Enhanced Tumor Cell Radiosensitivity and Abrogation of G₂ and S Phase Arrest by the Hsp90 Inhibitor 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin

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ABSTRACT

Purpose: Because of the potential for affecting multiple signaling pathways, inhibition of Hsp90 may provide a strategy for enhancing tumor cell radiosensitivity. Therefore, we have investigated the effects of the orally bioavailable Hsp90 inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) on the radiosensitivity of human tumor cells in vitro and grown as tumor xenografts.

Experimental Design: The effect of 17-DMAG on the levels of three proteins (Raf-1, ErbB2, and Akt) previously implicated in the regulation of radiosensitivity was determined in three human solid tumor cell lines. A clonogenic assay was then used to evaluate cell survival after exposure to 17-DMAG followed by irradiation. For mechanistic insight, the G₂- and S-phase checkpoints were evaluated in 17-DMAG-treated cells. Finally, the effect of in vivo administration of 17-DMAG in combination with radiation on the growth rate of xenograft tumors was determined.

Results: 17-DMAG exposure reduced the levels of the three radiosensitivity-associated proteins in a cell line-specific manner with ErbB2 being the most susceptible. Corresponding concentrations of 17-DMAG enhanced the radiosensitivity of each of the tumor cell lines. This sensitization seemed to be the result of a 17-DMAG-mediated abrogation of the G₂- and S-phase cell cycle checkpoints. The oral administration of 17-DMAG to mice bearing tumor xenografts followed by irradiation resulted in a greater than additive increase in tumor growth delay.

Conclusions: These data indicate that 17-DMAG enhances the in vitro and in vivo radiosensitivity of human tumor cells. The mechanism responsible seems to involve the abrogation of radiation-induced G₂- and S-phase arrest.

INTRODUCTION

Although the molecular events determining cell survival after exposure to ionizing radiation have not been completely defined, it is clear that critical processes in determining radioresponse include DNA repair, cell cycle checkpoint activation, and apoptosis. Because these events are under the control of a variety of signaling pathways, it is apparent that multiple independent and interacting processes can regulate radiation-induced cell death. In an evaluation of malignant and normal cells, Slupianek et al. (1) showed that, in response to DNA-damaging drugs, leukemic cells had enhanced DNA repair capability, prolonged checkpoint activation, and increased resistance to apoptosis as compared with their normal cell counterpart. These data are consistent with the existence of multiple drug resistance mechanisms in leukemic cells (2). Extrapolation of these results to solid tumor cells would suggest that multiple and possibly redundant processes contribute to the resistance of tumor cells to radiation.

Current efforts to develop strategies for enhancing tumor radiosensitivity have focused on the use of agents that target a single molecule putatively involved in regulating radiation-induced cell death. However, assuming that a combinatorial process determines radiosensitivity, a more effective approach would be to target multiple radiosresponse regulatory molecules. Moreover, the ability of a specific molecule to affect radiosresponse often depends on the genetic background of the tumor cell. For example, p53 (3, 4), Chk1 (5), and nuclear factor κB (6) have been shown to influence the radiosensitivity of some but not all tumor cells. Thus, the ability to affect more than one radiosresponse regulatory molecule should offer advantages in terms of the probability of enhancing radiosensitivity.

Toward the development of a multitarget approach to radiosensitization, we have focused on the molecular chaperone Hsp90. Hsp90 is unique among chaperones in that its clients are primarily proteins involved in signal transduction (7), a process critical to the regulation of radiosensitivity. In addition to signaling proteins such as steroid hormone receptors, Src family kinases, and certain cyclin-dependent kinases, the client proteins of HSP90 also include proteins that have been associated with radiosresponse (Akt, Raf-1, and ErbB2; ref. 8–11). We have shown recently that the Hsp90 inhibitor 17-allylaminogeldanamycin (17-AAG) enhances the in vitro radiosensitivity of four human tumor cell lines yet does not affect the radiation-induced cell killing of nonimmortalized normal human fibroblasts (12).
17-AAG is currently undergoing clinical trials as a single agent. However, the delivery of this Hsp90 inhibitor to patients requires the use of a relatively complex vehicle that includes egg phospholipids, which can have its own toxicities (13). Moreover, 17-AAG seems to undergo extensive metabolism that can result in the generation of toxic species (14). Alternatively, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), an analog of 17-AAG, is water soluble, orally bioavailable, and does not seem to undergo extensive metabolism (15).

To further pursue Hsp90 inhibition as an approach to radiosensitization, we have investigated the effects of 17-DMAG on the radiosensitivity of three human tumor cell lines of different histologic origins. The data presented indicate that 17-DMAG enhanced the in vitro radiosensitivity of each cell line, which correlated best with the loss of the Hsp90 client protein ErbB2. In addition, with respect to potential mechanisms involved, the sensitization was accompanied by the abrogation of G2- and S-phase checkpoints. Finally, xenograft studies showed that 17-DMAG administration results in a greater than additive increase in radiation-induced tumor growth delay.

MATERIALS AND METHODS

Cell Lines and Treatment. Three human tumor cell lines were evaluated: a glioma (U251), a pancreatic carcinoma (MiaPaCa), and a prostate carcinoma (DU145), each was obtained from American Type Culture Collection (Gaithersburg, MD). The cell lines were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) containing glutamate (5 mmol/L) and 5% fetal bovine serum and maintained at 37°C in an atmosphere of 5% CO2 and 95% room air. 17-DMAG, provided by the Therapeutics Program of the National Cancer Institute, was dissolved to a stock concentration of 1 mmol/L and stored at −20°C. We irradiated cultures using a Pantak (Solon, OH) X-ray source at a dose rate of 1.55 Gy/minute.

Clonogenic Assay. Cultures were trypsinized to generate a single cell suspension and a specified number of cells were seeded into each well of 6-well tissue culture plates. After allowing cells time to attach (6 hours), 17-DMAG or the vehicle control was added at specified concentrations, and the plates were irradiated either 6 or 16 hours later. Immediately after irradiation, the growth media was aspirated, and fresh media was added. Ten to twelve days after seeding, colonies were stained with crystal violet, the number of colonies containing at least 50 cells was determined and the surviving fractions were calculated. Survival curves were generated after normalizing for the cytotoxicity generated by 17-DMAG alone. Data presented are the mean ± SEM from at least three independent experiments.

Immunoblot Analysis. Cells were scraped into PBS, centrifuged, and the cell pellet resuspended in three volumes of lysis buffer [20 mmol/L HEPES (pH 7.9), 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP40, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 250 mg/mL benzamidine, 50 mmol/L NaF, 1 mmol/L Na3VO4]. For in vivo tumor xenograft samples, tumors were minced and homogenized in lysis buffer and centrifuged to remove debris. Immunoblot analysis was then done as described previously (12). The antibodies to c-Raf, Akt, and ErbB2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and to actin from Chemicon (Temecula, CA). The Typhoon scanner (Molecular Dynamics, Sunnyvale, CA) or enhanced chemiluminescence (Santa Cruz, CA) were used to do visualization.

Cell Cycle Phase Analysis. We evaluated cell cycle phase distribution using flow cytometry. The treatment protocols were essentially the same as in the clonogenic survival experiments, except the cells were initially seeded into 10-cm dishes. All cultures were subconfluent at the time of collection. We collected cultures for fixation, stained them with propidium iodide, and analyzed them using flow cytometry as described previously by the Clinical Services Program at the National Cancer Institute-Frederick (16). To evaluate the activation of the G2 cell cycle checkpoint, mitotic cells were distinguished from G2 cells, and the mitotic index was determined according to the expression of phosphorylated histone H3 (Upstate Biotechnology, Charlottesville, VA) as detected in the 4N DNA content population by the flow cytometric method of Xu et al. (17). In this assay, loss of mitotic cells (reduced mitotic index) reflects the onset of G2 arrest.

Radioreistant DNA Synthesis. Detection of DNA synthesis after irradiation was done essentially as described by Xu et al. (18) with slight modifications. Briefly, cells were incubated with [3H]thymidine (10 nCi/mL; Perkin-Elmer, Wellesley, MA) for 24 hours; the cells were then fed fresh growth media with or without 17-DMAG (50 mmol/L) and irradiated 16 hours later. Immediately after irradiation, [3H]thymidine (2.5 μCi/μL; Perkin-Elmer, Wellesley, MA) was added for 15 minutes. After a 2 hour chase period in complete growth media, the cells were collected onto filter paper and washed in 70 and 90% methanol. Measurement of radioactivity was done on an LS 6500 Multipurpose Scintillation Counter (Beckman, Fullerton, CA). The ratio of [3H] to [14C], reflecting the rate of DNA synthesis, was then calculated.

In Vivo Tumor Growth Delay Assay. Male 6-week-old athymic nude mice (NCr nu/nu) were used in these studies. Mice, housed in filter-topped cages, were provided autoclaved feed and hyperchlorinated water ad libitum. DU145 tumor cells were implanted subcutaneously at the base of the tail. Irradiation was done with a Pantak (Solon, OH) irradiator with animals restrained in a custom lead jig, which allowed for the localized irradiation of tumors at the base of the tail. When tumors reached 177 mm3 (7 × 7 mm), 17-DMAG (50 mg/kg) was delivered by oral gavage twice at 12 hours intervals, the next day (12 hours after the last 17-DMAG administration) tumors were irradiated with 5 Gy. To obtain growth curves, perpendicular diameter measurements of each tumor were collected every 3 days with calipers, and we calculated the volumes using the formula (L × W × W)/2. Tumors were followed until the group’s tumors were >800 mm3. Absolute tumor growth delay was calculated as the number of days for the treated tumors to grow to 800 mm3 — the number of days for the control group to reach the same size. Each experimental group contained 10 mice, and the untreated group contained 20 mice; data were expressed as the mean volume ± SE of each group. We did statistical analysis using Student’s t test with a 2-tail distribution and 2-sample unequal variation. All animal studies were con-
ducted in accordance with the principles and procedures outlined in the USPHS Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility under an approved animal protocol.

RESULTS

Although Hsp90 has numerous client proteins (7), c-Raf-1, Akt, and ErbB2 were of particular interest because they have also been associated with radioresistance (8–11). Moreover, reduced levels of these proteins have been suggested to serve as general markers of Hsp90 inhibition (19). Therefore, as an indicator of Hsp90 inhibition, the effects of 17-DMAG exposure on the levels of Raf-1, Akt, and ErbB2 proteins were initially determined in DU145 cells. As shown in Fig. 1A, a 16 hour exposure to 17-DMAG resulted in concentration-dependent decreases in ErbB2 with c-Raf-1 decreased significantly only at the highest concentration tested, 50 nmol/L. Akt levels were not affected in DU145 cells over this concentration range. The 17-DMAG–mediated decreases in the levels of ErbB2 and c-Raf-1 were next determined as a function of exposure time to 50 nmol/L (Fig. 1B). The decrease in ErbB2 was detectable by 3 hours reaching a maximum by approximately 6 hours of 17-DMAG exposure, whereas the decrease in c-Raf-1 was not reached until approximately 16 hours. Consistent with the results in Fig. 1A, Akt levels remained at untreated levels out to at least 24 h. These results are indicative of a 17-DMAG–mediated inhibition of Hsp90 activity. The lack of a decrease in Akt levels is consistent with previous reports in which this signaling protein was the most resistant to the Hsp90 inhibitor 17-AAG (19, 20).

Given the previous associations between Hsp90 inhibition and radiosensitization (12, 21–23), we determined the effect of 17-DMAG on the radiosensitivity of DU145 cells using a clonogenic assay (Fig. 2). Exposure to the 17-DMAG for 6 hours before irradiation resulted in a concentration-dependent increase in radiosensitivity with DEFs (dose enhancement factors at a surviving fraction of 0.1) of 1.2, 1.3, and 1.5 for 10, 25, and 50 nmol/L, respectively. 17-DMAG treatment alone resulted in surviving fractions of 0.91 ± 0.02, 0.72 ± 0.04, and 0.60 ± 0.09 for 10, 25, and 50 nmol/L, respectively. When DU145 cells were exposed to the same concentrations for 16 hours before irradiation (Fig. 2B), DEFs of 1.8 to 1.9 were obtained for all three concentrations (10, 25, and 50 nmol/L). In this protocol, the surviving fractions for 17-DMAG exposure...
alone were $0.53 \pm 0.08$, $0.29 \pm 0.05$, and $0.31 \pm 0.02$ for 10, 25, and 50 nmol/L, respectively. These data indicate that 17-DMAG increases the radiosensitivity of DU145 in a time-dependent fashion; at the early time point, the increase is concentration dependent.

To determine whether these effects of 17-DMAG were cell line dependent, we did similar studies using two additional human cell lines of different histologic origins: MiaPaCa (pancreatic carcinoma) and U251 (glioma). Exposure of MiaPaCa cells to 17-DMAG for 16 hours resulted in reduced levels of ErbB2 beginning at 10 nmol/L, c-Raf-1 at 25 nmol/L, and in contrast to DU145 cells, Akt levels were reduced after exposure to 25 nmol/L (Fig. 3A). The effects of 17-DMAG on MiaPaCa cell radiosensitivity were then determined (Fig. 3B). Irradiation after 16 hours of exposure to 10 nmol/L 17-DMAG enhanced radiosensitivity with a DEF of 1.4; preirradiation exposure to 25 nmol/L resulted in a greater enhancement with a DEF of 1.8. The surviving fractions for 17-DMAG exposure alone were $0.73 \pm 0.02$ and $0.35 \pm 0.03$ for 10 and 25 nmol/L, respectively. No further increase in radiosensitivity was achieved with a 17-DMAG concentration of 50 nmol/L (data not shown). Similar analyses were then done on U251 cells (Fig. 4). Exposure of U251 cells to 17-DMAG for 16 hours resulted in reduced levels of ErbB2 beginning at 10 nmol/L, c-Raf-1 at 50 nmol/L and Akt at 25 nmol/L (Fig. 4A). Exposure to 17-DMAG also increased U251 cell radiosensitivity, however, to a lesser degree than for the other two cell lines (Fig. 4B). The preirradiation exposure (16 hours) to 10 or 25 nmol/L 17-DMAG each enhanced radiation-induced cell killing to similar degrees with DEFs of 1.3. The surviving fractions for 17-DMAG exposure alone were $0.79 \pm 0.08$ and $0.36 \pm 0.02$ for 10 and 25 nmol/L, respectively. We achieved no additional radiosensitization using a concentration of 50 nmol/L (data not shown). These data indicate that the 17-DMAG-mediated enhancement in radiosensitivity is not limited to one cell line and occurs in human tumor cell lines of different histologic origins.

Initial attempts to identify the fundamental cell processes responsible for 17-DMAG-induced radiosensitization focused on...
the activation of cell cycle checkpoints in DU145 cells. The activation of the G2 checkpoint allows for DNA repair before progression into mitosis and is considered to protect against radiation-induced cell death (24). To evaluate the effects of 17-DMAG on radiation-induced G2 arrest, the method of Xu et al. (17) was used, which distinguishes between G2 and mitotic cells. This assay determines the percentage of mitotic cells in the 4N population according to the flow cytometric analysis of phosphorylated histone H3, which is specifically expressed in mitotic cells. Done as a function of time after irradiation, this analysis provides a measure of the progression of G2 cells into M phase and thus the activation of the G2 checkpoint. As shown in Fig. 5, irradiation of DU145 results in a decrease in mitotic cells by 1 hour reaching a maximum reduction by 3 hours, consistent with the rapid onset of G2 arrest and with previously published results (17, 25). Exposure to 17-DMAG alone, which was rinsed off and the cells were fed fresh growth media at the 0 time point, corresponding to the time of irradiation, resulted in an increase in the percentage of mitotic cells over the 6-hour observation period. The mitotic index for cells exposed to the 17-DMAG/radiation combination did not decrease as it did for radiation alone, but increased in a manner similar to 17-DMAG-treated cells. Thus, these data indicate that 17-DMAG abrogates radiation-induced G2 arrest.

During the studies evaluating mitotic index, we noted that radiation resulted in an accumulation of cells in S phase (Fig. 6A), consistent with the rapid onset of an S-phase arrest (25). However, in cells treated with 17-DMAG or 17-DMAG and radiation there was no such increase in the percentage of S-phase cells after irradiation. To more specifically address the activation of the S-phase checkpoint, radioresistant DNA synthesis (25) was evaluated in irradiated cells with and without prior 17-DMAG exposure (Fig. 6B). Radiation alone resulted in a decrease in DNA synthesis corresponding to the activation of the S-phase checkpoint. However, in cells exposed to 17-DMAG for 16 hours before irradiation, DNA synthesis was essentially unchanged as compared with unirradiated cells. These data indicate that in addition to the abrogation of G2 arrest, 17-DMAG inhibits radiation-induced S-phase arrest.

To determine whether the radiosensitizing effects of 17-DMAG can be extended to an in vivo model, DU145 cells were grown as tumor xenografts at the base of the tail of nude mice. Animals bearing 177 mm3 (7 7 mm) tumors were given 17-DMAG (50 mg/kg delivered by oral gavage at 12 hour intervals) the day before tumor irradiation (5 Gy). Because of its susceptibility to 17-DMAG-mediated reduction in vitro (Fig. 1), ErbB2 was used as an indicator of drug activity in vivo. As shown in Fig. 6A, DU145 cells were exposed to 50 nmol/L 17-DMAG for 16 hours before irradiation with 6 Gy. Cells were collected at 1 to 6 hours after irradiation. The percentage of S-phase cells was determined by flow cytometry according to analysis of propidium iodide-stained cells. The values represent the mean ± SE of three to four independent experiments. Comparison of radiation versus 17-DMAG + radiation resulted in P values of <0.04 at 6 Gy and <0.08 at 12 Gy.
in Fig. 7A, 12 hours after the final drug treatment (corresponding to the time of irradiation), ErbB2 levels were reduced in the DU145 xenografts isolated from mice that received 17-DMAG as compared with tumors isolated from vehicle-treated mice. These data suggest that the in vivo 17-DMAG–treatment protocol used in this study affected a similar target as that of the in vitro treatment, which enhanced tumor cell radiosensitivity.

The growth rates of DU145 tumors in mice treated with vehicle (control), 17-DMAG, 5 Gy, and the 17-DMAG/5 Gy combination are shown in Fig. 7B. For each group, we calculated the time to grow from 177 mm$^3$ (volume at the time of treatment) to 800 mm$^3$ using the tumor volumes from the individual mice in each group (mean ± SE). 17-DMAG treatment significantly inhibited tumor growth ($P < 0.01$ versus vehicle): i.e., the time required for tumors to grow from 177 to 800 mm$^3$ increased from 21.1 ± 3.8 days for vehicle-treated mice to 31.5 ± 3.0 days for 17-DMAG–treated mice. Radiation treatment alone increased the time to reach 800 mm$^3$ to 29.2 ± 2.4 days ($P < 0.01$ versus vehicle). However, in mice that received the 17-DMAG/radiation combination, the time for tumors to grow to 800 mm$^3$ increased to 47.4 ± 7.7 days ($P < 0.01$ versus vehicle), which is also significantly greater than the individual treatment groups ($P < 0.001$ versus 17-DMAG and versus 5 Gy). The absolute growth delays (the time in days for tumors in treated mice to grow from 177 to 800 mm$^3$ — the time in days for tumors to reach the same size in vehicle-treated mice) were 10.4 for 17-DMAG only, 8.1 days for 5 Gy only, and 26.3 for the combination. Thus, the growth delay after the combined treatment was more than the sum of the growth delays caused by 17-DMAG alone and radiation alone. Furthermore, the combination treatment produced tumor regression in contrast to the single agent treatments that did not. To obtain a dose-enhancement factor comparing the tumor radioresponse in mice with and without 17-DMAG treatment, the normalized tumor growth delay was determined, which accounts for the contribution of 17-DMAG to tumor growth delay induced by the combination treatment. Normalized tumor growth delay is defined as the time in days for tumors to grow from 177 to 800 mm$^3$ in mice treated with the combination of 17-DMAG and radiation — the time in days for tumors to grow from 177 to 800 mm$^3$ in mice treated with 17-DMAG only, which was 15.9 days (26.3 days − 10.4 days). The dose enhancement factor, obtained by dividing the normalized tumor growth delay in mice treated with the 17-DMAG/radiation combination by the absolute growth delay in mice treated with radiation only (8.1 days), was 1.96. Thus, 17-DMAG alone slows
tumor growth and enhances the effect of radiation, which is similar to the results obtained in *in vitro*. This occurs in the absence of overt toxicity because there was no body weight loss in any of the treatment groups (data not shown).

**DISCUSSION**

Recent efforts to develop agents that enhance tumor cell radiosensitivity have focused on targeting a specific molecule involved in regulating cellular radioresponse. However, there are numerous examples in which targeting a selected radiore- sponse-associated molecule affects the radiosensitivity of some tumor cell lines but not others (9, 11, 26, 27). Such results suggest that the regulatory prowess of a single molecule regarding radioresponse is dependent on the genetic and/or epigenetic context of a tumor cell. Given this situation, it would seem that the effectiveness of target-based radiation sensitizers against solid neoplasms would be significantly constrained by inter- and intratumor heterogeneity. In an attempt to increase the probability of enhancing tumor radiosensitivity, we have explored a multitarget approach to radiosensitization focusing on the inhibition of Hsp90 and consequently its client proteins. The rationale for combining an Hsp90 inhibitor with radiation is essentially an extension of the motivation for investigating the use of these compounds as a single modality cancer treatment. That is, this work is based on the assumption that the simultaneous, combinatorial blockade of multiple signaling pathways will be more efficient that inhibiting a single protein (28).

For predicting the clinical performance of new chemotherapeutic agents, Voskoglou-Nomikos et al. (29) suggested that results obtained from a panel of human tumor cell lines evaluated *in vitro* or as xenograft tumors are predictive of efficacy in phase II clinical trials. It may be possible to extrapolate this analysis to the development of clinically effective radiation modifiers. Previous studies have shown that the Hsp90 inhibitors geldanamycin and 17-AAG enhance the *in vitro* radiosensitivities of cell lines initiated from a variety of human tumors (12, 21–23). Thus, the ability of Hsp90 inhibition to enhance radiosensitivity across a spectrum of human tumor cell lines *in vitro* suggests that this chaperone protein can serve as a general target for radiosensitization. The data presented here have extended these initial observations to a water-soluble, orally bioavailable Hsp90 inhibitor 17-DMAG, which enhanced the *in vitro* radiosensitivity of tumor cell lines originating from three different histologies. Moreover, this study also showed that 17-DMAG delivered *in vivo* resulted in a greater than additive increase in radiation-induced tumor growth delay with an *in vivo* enhancement factor of almost 2. Thus, these results suggest that 17-DMAG has potential clinical utility as a radiosensitizing agent.

Although there are a considerable number of other Hsp90 client proteins, the study presented herein addressed the effects of 17-DMAG on the levels of ErbB2, c-Raf-1, and Akt. These proteins are of interest because inhibition of each had been associated with enhanced radiosensitivity in some systems (8–11). In the three cell lines evaluated, ErbB2 was the most susceptible to 17-DMAG-mediated reduction. For each cell line, enhanced radiosensitivity was first detected when ErbB2 levels began to decrease, at concentrations that had no detectable effects on the levels of the other two proteins. For DU145 cells there was a correlation between enhanced radiosensitivity and loss of ErbB2 with respect to 17-DMAG concentration and time of exposure. Although a causal relationship cannot be established, the data presented here suggest that ErbB2 may serve as an indicator of 17-DMAG–induced enhancement in radiosensitivity. The contribution of a reduction in the other proteins evaluated (c-Raf-1 and Akt) to the enhanced radiosensitivity is unclear. In MiaPaca cells, a 17-DMAG concentration of 25 nmol/L resulted in loss of ErbB2, c-Raf-1, and Akt and resulted in a greater DEF as compared with 10 nmol/L, which induced a decrease in ErbB2 only. However, for DU145 cells, whereas 50 nmol/L 17-DMAG reduced ErbB2 and c-Raf-1, there was only a slightly further increase in radiosensitivity as compared with 10 nmol/L, which resulted in a reduction of ErbB2 only. Finally, in U251 cells, 10 nmol/L 17-DMAG resulted in a reduction of only ErbB2 and induced the same degree of radiosensitization as did 25 nmol/L, which reduced ErbB2, c-Raf-1, and Akt. These data suggest that the contribution of c-Raf-1 and Akt to radiosensitivity may be cell type dependent, as suggested in previous reports (9, 11, 26). Although indicative of a correlation between loss of ErbB2 and 17-DMAG–induced radiosensitization, clearly these results do not establish a causal role for ErbB2 nor do they eliminate a potential role for other Hsp90 client proteins in the regulation of radioresponse.

Identifying the specific molecules mediating 17-DMAG–induced radiosensitization is likely to be severely complicated by reductions in other Hsp90 client proteins that could contribute to radioresponse. In an attempt to develop some type of mechanistic insight into the effects of 17-DMAG on tumor cell radiosensitivity, we bypassed the individual protein level and focused on a general cellular process subject to a high degree of regulation by signal pathways, the activation of cell cycle checkpoints. With respect to the modulation of radiation-induced cell death, the G2 checkpoint has received considerable attention in that arrest in G2 is considered to allow for more DNA repair, reducing the probability that a cell will enter mitosis in the presence of genomic injury. Indeed, Kao et al. (24) showed that a significant amount of DNA repair takes place during G2 arrest. Agents that abrogate G2 arrest such as UCN-01 (30) and the Wee1 kinase inhibitor PD0166285 (27) have been shown to enhance radiosensitivity. However, these agents do not affect radiation-induced cell killing in all cell types. In addition, studies selectively deleting aspects of BRCA1 function, which plays a role in the activation of the G2 and S-phase checkpoints (18), revealed a disassociation between a defect in G2 arrest and the enhanced radiosensitivity of BRCA1 mutant cells (17). These results suggest that abrogation of G2 arrest alone is not always sufficient to enhance radiosensitivity. Because of its defect in ATM mutant cells (31), the S-phase checkpoint has also been implicated in the recovery response to radiation. However, Xu et al. (17) showed that selectively deleting the S-phase checkpoint function of BRCA1 function did not account for the enhanced radiosensitivity of a BRCA1 mutant cell line. Thus, studies to date suggest that loss of the G2 or S-phase checkpoints individually may not consistently affect cellular radiosensitivity and that additional defects in the recovery process must be present to affect radiosensitivity.

Such a combination of defects resulting in enhanced radiosensitivity may include deficiencies in more than one of the cell cycle checkpoints. For example, ATM is a critical regulator of cell
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radioresponse; cells containing an ATM mutation are extremely radiosensitive and, among other abnormalities, have defective G2- and S-phase checkpoints (31). The data presented here indicate that exposure to 17-DMAG abrogates the G2- and S-phase checkpoints, which is consistent with the hypothesis that 17-DMAG targets multiple cell signaling processes. Whereas a reduction in ErbB2 correlates with 17-DMAG–mediated enhancement in radiation-induced cell death, it is unclear at this time whether its loss specifically contributes to the radiosensitization process or merely serves as an indicator of 17-DMAG activity. The specific proteins and pathways mediating the 17-DMAG-induced effects on cell cycle regulation and radiosensitivity await additional study. However, the data presented here do indicate that 17-DMAG enhances radiosensitivity across a spectrum of human tumor cell lines and that its oral administration can increase the radiosensitivity to tumor xenografts. These results suggest that combining 17-DMAG and radiation may have clinical potential as a cancer treatment strategy.

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