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Advances in Brief

Abrogation of the G_2 Checkpoint Results in Differential Radiosensitization of G_1 Checkpoint-deficient and G_1 Checkpoint-competent Cells[1]

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Abstract

We have examined the effect of abrogation of the G_2 checkpoint on the radiosensitivity of G_1 checkpoint-proficient and G_1 checkpoint-deficient cells. A549 human lung adenocarcinoma cells were transduced with the *E6* oncogene of the human papillomavirus type 16 to eliminate their radiation-induced G_1 arrest. These E6+ cells exhibited a dose-dependent increase in radiation resistance compared to control A549 cells transduced with the vector alone. Treatment (96 h) with 2 mM caffeine resulted in an abrogation of the cellular G_2 checkpoint in both E6+ and control cells and a differential radiosensitizing effect on the two cell lines such that the E6+ clones and the vector controls became equally radiosensitive. These data show that human tumors which are radioresistant due to the loss of the p53-mediated G_1 checkpoint can be made radiosensitive by abrogation of the G_2 checkpoint. The implications of these results for cancer therapy are discussed.

Introduction

The cellular response to ionizing radiation involves cell cycle regulatory genes which control progression of the cell through the cell cycle. The prototype for these regulatory genes is the *rad9* "checkpoint" gene of yeast, which delays cells in G_2 following radiation exposure (1). In mammalian cells, treatment with radiation results in delays in cell cycle progression, both in G_1 and in G_2. The G_1 delay has been shown to be under the control of the *p53* gene (2). Less is known about the genes controlling the radiation-induced G_2 delay in mammalian cells, because the homologue of the yeast *rad9* gene has not been identified. It has, however, been hypothesized that the magnitude of the G_2 delay in response to radiation may be a critical determinant of cellular radiosensitivity (3, 4). Abrogation of the G_2 delay with methylxanthines such as caffeine and pentoxifylline results in increased cellular radiation and chemosensitivity (5, 6), and the *rad9* mutation which abrogates the G_2 arrest in yeast results in marked radiosensitivity (1) .

Although wild-type p53 is believed to be present in most normal human tissues, p53 mutations are common in human malignancies. Consequently, cancer treatment strategies may be able to capitalize on this cell cycle regulatory deficiency which appears to be largely limited to tumors. Treatments which abrogate the G_2 arrest in p53-deficient cancer cells will result in cells lacking both G_1 and G_2 checkpoint functions. The same treatment administered to p53-proficient normal cells will eliminate only the G_2 checkpoint. This difference in checkpoint competence might result in a greater sensitization of the p53-deficient cells to DNA-damaging agents.

We have examined this hypothesis using the A549 human lung cancer cell line, which is stable in culture, expresses wild-type p53 (7), and demonstrates G_1 and G_2 arrests in response to ionizing radiation. By inactivating p53 activity in A549 cells through expression of the *E6* oncogene of the HPV[3] type 16, we have been able to eliminate the G_1 delay in response to radiation. This has permitted us to compare the effects of caffeine-mediated abrogation of the G_2 checkpoint on radiosensitivity in both the parental and G_1 checkpoint-deficient cells.

Materials and Methods

Supply Sources. Unless otherwise stated, all tissue culture media, serum, drugs, and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Lines: Production and Maintenance. A549 is a human adenocarcinoma cell line derived from a primary lung cancer. The cells were obtained from the American Type Culture Collection cell line repository (Rockville, MD) and were grown as attached monolayers in 25 cm^2 tissue culture flasks using "Sigma 1:1" media (a 1:1 mix of DMEM and Ham's F-12) supplemented with 10% heat-inactivated FCS, 100 units/liter penicillin, and 0.1 mg/ml streptomycin. Cells were maintained in exponential growth in humidified incubators at 37°C and 5% CO_2. Transduction of A549 with the HPV type 16 *E6* oncogene by the retrovirus vector LXSN and isolation of *E6* expressing polyclones was performed as described previously (8). In all experiments, cells transduced with the vector alone (LXSN+) were used as controls. Cell harvesting was performed by 0.5% trypsin and 0.2% EDTA detachment.

Cell Lines: Growth Conditions for Experiments. Cells were used under two different conditions of growth: (*a*) plateau, partially synchronized growth; and (*b*) exponential, asynchronous growth. Plateau phase cultures were achieved through nutrient depletion. Cells (1×10^5 cells/flask) in 5 cc of media were incubated for 5 days without media changes. This resulted in an equilibrium number of approximately 3×10^6 cells/flask with 80% in G_1, 10–15% in S, and 5–10% in G_2. After treatment, cells were subdivided and permitted to resume exponential growth for clonogenic assay. Asynchronous cultures were obtained by incubating 2×10^5 cells/flask 48 h prior to experimental use. Typical yields were 1×10^6 cells/flask, with 50% in G_1, 40% in S, and 10% in G_2. Cells in asynchronous culture were treated as attached monolayers in the growth flasks, and individual flasks were harvested serially after treatment for FACS cell cycle analysis or for clonogenic assay. The plating efficiency for both the cells transduced with *E6* (E6+) and the LXSN vector alone (LXSN+) was approximately 90%.

Radiation. Radiation was delivered by a Shepard #81–14 cesium irradiator (Glendale, CA) using a source-to-flask distance of 46 cm and a dose rate of 100 cGy/min. Unirradiated controls were sham irradiated.

Drug Preparation. Caffeine was dissolved in serum-free media, sterile filtered through a 0.22 μ filter, and diluted in full media. In experiments involving radiation, caffeine was added to the media immediately prior to radiation. The plating efficiency for both E6+ and LXSN+ cells incubated in caffeine was approximately 65%. Nocodazole was dissolved in warm sterile DMSO at a concentration of 1 mg/ml and diluted in DMSO to a stock concentration of 0.1 mg/ml. The working concentration was 0.4 μg/ml.

Flow Cytometry. Cells harvested for FACS were suspended in 10 mg/ml 4′,6-diamidino-2-phenylindole with 10% DMSO and cryopreserved at −70°C. Samples were run on an ICP-22 (Ortho Diagnostic Systems) flow cytometer

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[3] The abbreviation used is: HPV, human papillomavirus.

with UV excitation from a mercury arc lamp source, UG_1 excitation filter, and 400-nm long pass emission filter. A minimum of 20,000 cells were analyzed. The data were analyzed using the FACS software "Multicycle" (Phoenix Flow Systems, San Diego, CA).

Clonogenic Assay. Cell counting was performed on a Coulter ZM analyzer (Luton, United Kingdom) Cells were triplicate plated into 60-mm^2 tissue culture dishes and incubated in an undisturbed state for 12 days. Cells treated with caffeine underwent a media change 1–4 days after seeding in order to remove the caffeine. For these experiments, control cells underwent the same rinse procedures. After incubation, cells were fixed and stained with 0.25% crystal violet in formalin, and colony counts were performed by visual inspection. A colony was defined as ≥50 cells. All colony counts were adjusted for plating efficiency to yield corrected survivals of 100% for untreated controls. Similarly, colony counts for caffeine-treated cells were adjusted for drug toxicity to yield corrected survivals of 100% for unirradiated caffeine treated controls.

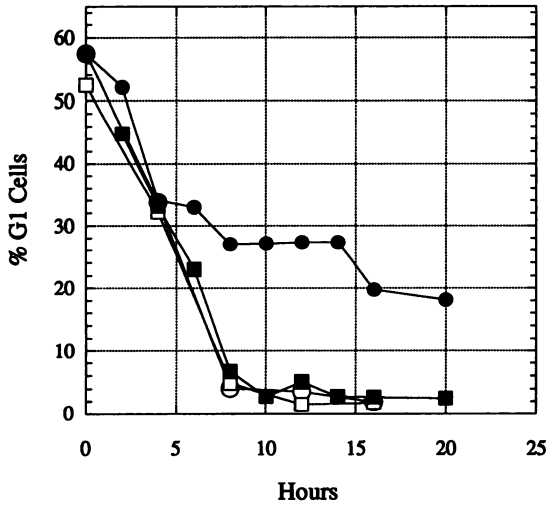

Fig. 1. FACS analysis of G_1 checkpoint abrogation in $E6^+$ cells. Asynchronous cultures of $LXSN^+$ and $E6^+$ cells were treated with 10 or 0 Gy irradiation at 0 h and incubated in nocodazole (0.4 μg/ml) to prevent cells from entering G_1 from G_2. Cells were reharvested for FACS at the indicated intervals. ●, LXSN+ (10 Gy); ○, LXSN+ (0 Gy); ■, E6+ (10 Gy); □, E6+ (0 Gy).

Results

Abrogation of the G_1 Checkpoint by HPV 16 *E6*. The HPV *E6* gene targets p53 for early degradation through ubiquination (9). As demonstrated by Western analyses, polyclones of A549 lung cancer cells infected with an amphotropic retrovirus expressing HPV 16 *E6* (E6+) had very low levels of p53 protein compared to cells infected with the LXSN vector (LXSN+) alone. E6+ cells failed to induce p53 and p21 following irradiation, whereas p53 and p21 were induced in irradiated LXSN+ cells (data not shown).

To study the effect of *E6* on the G_1 checkpoint in these cells, exponential cultures of E6+ and LXSN+ A549 cells received either 10 or 0 Gy irradiation and were sequentially harvested for FACS analysis at time points up to 24 h following irradiation. At the onset of the experiments, all cells were incubated in nocodazole to prevent cells in G_2 from reentering G_1 (10) and complicating the interpretation of radiation effects on G_1 cells. Expression of the *E6* oncogene resulted in the loss of the radiation-induced delay in G_1, as seen in Fig. 1, in which the percentage of G_1 cells are plotted against time. By 12 h, less than 5% of both unirradiated cell lines remain in G_1. This is also the case for the irradiated E6+ cells, whose progression out of G_1 occurs at the same rate as the unirradiated controls. In contrast, 28% of the irradiated LXSN+ cells remain in G_1 at this time. Thus, the expression of *E6* abrogates the G_1 checkpoint in A549 cells.

Caffeine Abrogation of the Radiation-induced G_2 Arrest. In asynchronous cultures, the radiation-induced G_2 arrest peaked at 16–18 h following radiation. Fig. 2*A* shows the FACS data for unirradiated LXSN+ and E6+ cells, as well as for irradiated cells 16

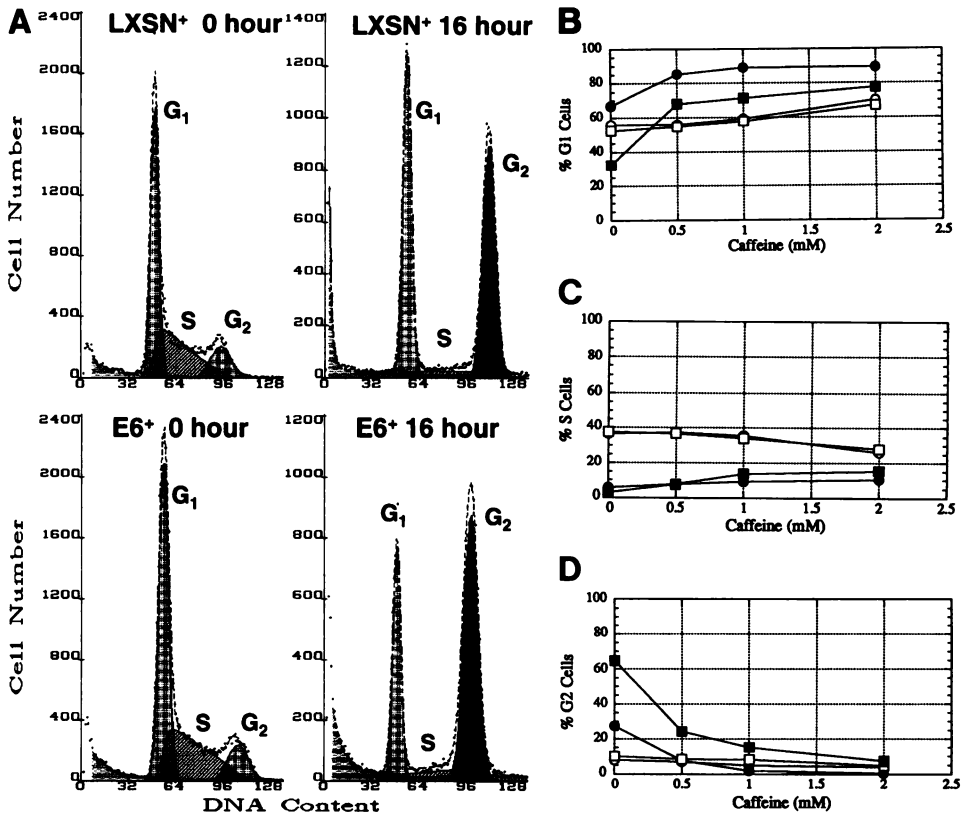

Fig. 2. *A*, FACS distributions of exponentially growing LXSN+ and E6+ cells treated with 10 Gy radiation alone. Time after irradiation was 0 and 16 h. *B-D*, cell cycle changes in exponentially growing LXSN+ and E6+ cells 18 h after 10 or 0 Gy radiation ± incubation with 0.5, 1.0, or 2.0 mM caffeine. *B*, $\%G_1$; *C*, %S; *D*, $\%G_2$. ●, LXSN+ (10 Gy); ○, LXSN+ (0 Gy); ■, E6+ (10 Gy); □, E6+ (0 Gy).

h following 10 Gy. The irradiated E6+ cells have a relative paucity of G_1 cells compared to the LXSN+ cells and have a correspondingly increased percentage of G_2-arrested cells relative to LXSN+ cells. In contrast to the irradiated LXSN+ cells, where the majority of the G_1 population is due to a G_1 checkpoint, the G_1 population in the irradiated E6+ cells is due to the contribution of cells from G_2 reentering G_1, as demonstrated by preventing the egress of cells from G_2 with nocodazole (see Fig. 1).

Fig. 2, *B-D*, shows the effects on the cell cycle of 10 Gy irradiation; incubation with 0.5, 1.0, and 2.0 mM caffeine; and the two treatments in combination. Asynchronous cultures were harvested 18 h after irradiation and/or 18 h caffeine incubation, and the three figures display the data for G_1, S, and G_2 phases respectively. In the absence of caffeine, the low percentage of S-phase cells in the irradiated cells is due to the radiation-induced arrest of cells in G_2 in the E6+ population and in G_1 and G_2 in the LXSN+ cells. Increasing concentrations of caffeine progressively abrogated the radiation-induced G_2 arrest in both cell lines. For both cell lines, caffeine treatment (± radiation) also resulted in an increased percentage of cells in G_1 (Fig. 2*B*). Nocodazole experiments confirmed that these cells were due to an increased influx from G_2, rather than a decrease in egress from G_1 (data not shown).

Preferential Caffeine Radiosensitization of G_1 Checkpoint-deficient Cells. Table 1 shows the radiation survival of asynchronous cultures of the two cell lines treated with 10 Gy and incubated for 4 days in the concentrations of caffeine used in the previous experiments. We chose 4 days of caffeine treatment because prior results demonstrated that LXSN+ radiation survival did not differ over a range of 1–4 days of caffeine incubation, but E6+ radiation survival diminished with increasing caffeine incubation time and was most pronounced at 3–4 days treatment (data not shown; see "Discussion").

E6+ cells were approximately 7-fold more radioresistant than LXSN+ cells. Radiation sensitivity was increased in the E6+ cells with caffeine concentrations of 0.5 mM. Radiosensitization of the LXSN+ cells only occurred at 1.0 mM concentration. At 2 mM caffeine, both cell lines appeared to be equally radiosensitive.

Fig. 3 expands upon the data of Table 1 and shows the radiation survival of LXSN+ and E6+ cells treated with a range of doses of radiation ± a 4-day incubation with 2 mM caffeine. We elected to use 2 mM caffeine in order to obtain equal radiation cytotoxicity. This permitted us to compare differences in cell cycle effects between LXSN+ and E6+ lines under conditions of equitoxic injury. In Fig. 3*A*, the cells were irradiated under conditions of exponential growth. In addition, cells were also irradiated under plateau growth conditions (Fig. 3*B*) in which a higher percentage of cells were in G_1 (~80%; data not shown) and under the control of the G_1 checkpoint.

Confirming the prior experiments, the E6+ line was more radioresistant than the LXSN+ cells and in a dose-dependent manner. At a dose of 10 Gy, E6+ cells had approximately a 7-fold greater radiation survival than LXSN+ cells. Caffeine cotreatment resulted in increased radiation sensitivity of both cell lines, but with a more profound effect on the E6+ line, such that both cell lines became equally radiosensitive. Overall, caffeine radiosensitization of the E6+ line was 17-fold, compared to 3-fold for the LXSN+ cell line. These results were the same for both asynchronous and plateau cultures.

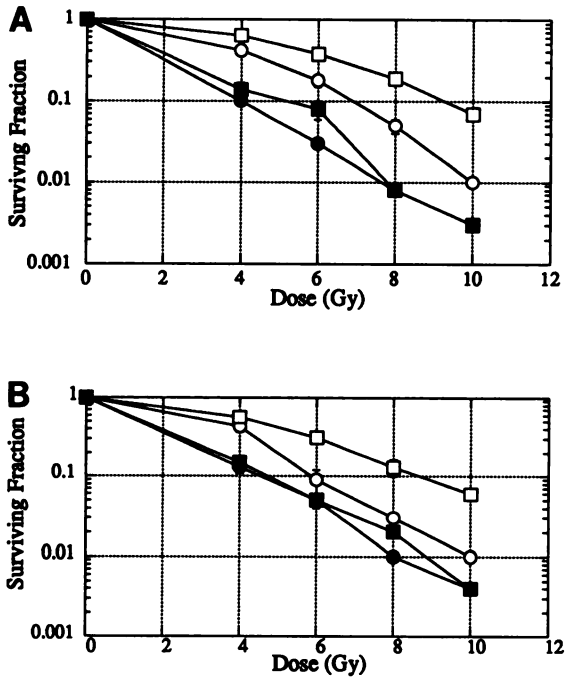

Fig. 3. Clonogenic survival of $LXSN^+$ and $E6^+$ cells treated with graded doses of radiation ± 96 h incubation of 2 mM caffeine. *Bars*, ±1 SD. *A*, cells irradiated under conditions of exponential growth. *B*, cells irradiated under conditions of plateau phase growth. ●, LXSN+ (2 mM caffeine); ○, LXSN+ (no caffeine); ■, E6+ (2 mM caffeine); □ E6+ (no caffeine).

Table I *Clonogenic surviving fraction (± 1 SD) of exponentially growing LXSN+ and E6+ cells treated with 10 Gy radiation ± 96 h incubation with 0.5, 1.0, or 2.0 mM caffeine*

The plating efficiency for untreated E6+ and LXSN+ cells is 90% and declines to 65% when both cell lines are incubated in 2 mM caffeine. All colony counts are adjusted for plating efficiency to yield corrected survivals of 100% for untreated controls. Similarly, colony counts for caffeine-treated cells are adjusted for drug toxicity to yield corrected survivals of 100% for unirradiated, caffeine-treated controls.

	LXSN+		E6+	
Caffeine Dose	0 Gy	10 Gy	0 Gy	10 Gy
No caffeine	1.00 ± 0.05	0.01 ± 0.0009	1.00 ± 0.10	0.07 ± 0.001
0.5 mM	1.00 ± 0.07	0.01 ± 0.001	1.00 ± 0.08	0.02 ± 0.001
1.0 mM	1.00 ± 0.12	0.004 ± 0.0001	1.00 ± 0.12	0.01 ± 0.0004
2.0 mM	1.00 ± 0.10	0.003 ± 0.0005	1.00 ± 0.11	0.004 ± 0.0003

Discussion

These results indicate, within the limitations of the single malignant cell line studied, that the radiosensitivity of cells can be manipulated *in vitro* by selective inactivation of the G_1 and G_2 checkpoints. Expression of the *E6* oncogene resulted in a p53-deficient phenotype with a loss of the radiation-induced G_1 checkpoint function. Abrogation of this G_1 checkpoint resulted in an increase in radiation resistance compared to the parental cell line. Concurrent abrogation of the radiation-induced G_2 arrest in both cell lines by treatment with caffeine enhanced the radiation sensitivity of both cell lines. A greater effect was seen in the E6+ cells which were G_1 checkpoint deficient.

The implications of these data for the treatment of human cancers are provocative. It is becoming clear that p53 mutations are commonplace in human malignancies. If the resulting loss of the G_1 checkpoint leads to radiation resistance, it suggests that radiation treatment would be less cytotoxic to these malignant cells than to surrounding normal tissues. Our results suggest that a therapeutic gain might be achieved in the radiation treatment of selected human cancers by a strategy which inactivates the G_2 checkpoint. Such a strategy would overcome the relative radiation resistance of the p53-deficient phenotype and would provide a degree of selective radiosensitization if the cancer under treatment were p53 deficient and if the surrounding normal human tissues were p53 proficient.

Some elements of this hypothesis are established and others remain conjectural. It is known that p53 deficiency results in a loss of the radiation-induced G_1 arrest (2, 11) whether the p53 deficiency is on the basis of mutation or through expression of the *E6* oncogene which

targets p53 for early ubiquination (9). Our results with *E6* transduction into the A549 cells confirm these prior results. However, the dependence of radiosensitivity or chemosensitivity on p53 status is unresolved (12–14). In some cell lines where radiation or chemoresistance has been associated with loss of p53 function, it appears to be due to an inability of the cells to undergo p53-dependent apoptosis (14, 15). We are currently investigating radiation-induced apoptosis in the LXSN+ and E6+ cell lines. While we have emphasized the effect of E6 on p53, we cannot rule out the possibility that some of the effects of *E6* on radiation sensitivity may be independent of p53.

The effects of caffeine we have observed on LXSN+ and E6+ cell cycle kinetics are in agreement with prior reports. Caffeine clearly abrogated the G_2 arrest that normally occurred in response to ionizing radiation. This effect is well known (4). In contrast to other reports, we did not observe that caffeine abrogated the G_1 arrest following irradiation (16). The radiosensitizing effects of caffeine on our cell lines is in agreement with the radiobiology literature on methylxanthines (4, 5). There are thought to be multiple mechanisms that account for the increased radiosensitivity (17).

The differential effect of methylxanthines on the radiosensitivity of the G_1 checkpoint-proficient and G_1 checkpoint-deficient cells is a new finding. Similar observations have been made by Powell *et al.* (18) and Fan *et al.* (19). Presumably, the G_1 checkpoint-deficient E6+ cells enter G_2 with more damaged DNA than the G_1 checkpoint-proficient cells. Thus, these cells would be more dependent on an intact G_2 checkpoint to repair damage compared to the G_1 checkpoint-proficient LXSN+ cells. Accordingly, the abrogation of the G_2 checkpoint by caffeine would result in the greater radiosensitization of the E6+ cells. Although radiosensitization occurred for both cell lines with 24-h caffeine treatment, the maximum differential radiosensitization was observed after 4 days of caffeine incubation. This suggests that multiple cell cycles in caffeine may be necessary for maximal abrogation of G_2-associated damage repair. We note, however, that there are conflicting reports regarding the relationship between the cell cycle effects and the radiosensitizing properties of caffeine (20, 21).

At present, it is not possible to achieve human serum levels of caffeine approaching the concentrations necessary to achieve the levels of radiosensitization observed in our experiments. We are also exploring the use of other methylxanthines such as pentoxifylline, a radiosensitizer which is tolerated in humans at serum levels which have been reported to exceed those required for tumor sensitization in experimental animals (6, 22). Our preliminary radiation survival experiments using pentoxifylline have yielded both qualitatively and quantitatively comparable results to the caffeine data shown and are similar to the data reported by Fan *et al.* (19). Regardless of whether caffeine or pentoxifylline prove to be clinically useful radiosensitizers, our results clearly suggest that targeting of the G_2 checkpoint in the presence of DNA damage may be an important strategy for cancer therapy.

In order to extend our results to clinical radiation oncology, we have also carried out preliminary experiments substituting the single large fraction of radiation used with multiple daily fractions of 2 Gy, which are the doses routinely used in clinical practice. The relationships in radiation sensitivity between the untreated and caffeine-treated cells are reproduced under these experimental conditions, showing that large radiation fractions are not required to achieve the results we have shown above.

In conclusion, *E6* oncogene inactivation of p53 leads to a dose-dependent increase in radioresistance in A549 human lung adenocarcinoma cells relative to vector-transduced control A549 cells. Caffeine abrogation of the G_2 arrest differentially radiosensitizes these cell lines such that both cell lines become equally radiation sensitive at a caffeine concentration of 2 mM. The differential effect of G_2 abrogation on these two cell lines suggests a cancer treatment strategy where the therapeutic selectivity of treatment is based on the prevalence of p53 or other mutations that lead to abrogation of the G_1 checkpoint in cancer cells relative to normal tissues. Success of such a strategy in humans will require that therapeutic serum levels of inhibitors of the G_2 checkpoint be achievable and sustainable over a course of daily fractionated radiation and/or a course of DNA-damaging chemotherapeutics.

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