

REPORT

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In vitro effects of tetraiodothyroacetic acid combined with X-irradiation on basal cell carcinoma cells

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ABSTRACT

We investigated radiosensitization in an untreated basal cell carcinoma (TE.354.T) cell line and post-pretreatment with tetraiodothyroacetic acid (tetrac) X 1 h at 37°C, 0.2 and 2.0 μM tetrac. Radioresistant TE.354.T cells were grown in modified medium containing fibroblast growth factor-2, stem cell factor-1 and a reduced calcium level. We also added reproductively inactivated (30 Gy) “feeder cells” to the medium. The *in vitro* doubling time was 34.1 h, and the colony forming efficiency was 5.09 percent. These results were therefore suitable for clonogenic radiation survival assessment. The 250 kVp X-ray survival curve of control TE.354.T cells showed linear-quadratic survival parameters of $\alpha_{X\text{-ray}} = 0.201 \text{ Gy}^{-1}$ and $\beta_{X\text{-ray}} = 0.125 \text{ Gy}^{-2}$. Tetrac concentrations of either 0.2 or 2.0 μM produced $\alpha_{X\text{-ray}}$ and $\beta_{X\text{-ray}}$ parameters of 2.010 and 0.282 Gy^{-1} and 2.050 and 0.837 Gy^{-2} , respectively. The surviving fraction at 2 Gy (SF_2) for control cells was 0.581, while values for 0.2 and 2.0 μM tetrac were 0.281 and 0.024. The SF_2 data show that tetrac concentrations of 0.2 and 2.0 μM sensitize otherwise radioresistant TE.354.T cells by factors of 2.1 and 24.0, respectively. Thus, radioresistant basal cell carcinoma cells may be radiosensitized pharmacologically by exposure to tetrac.

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Introduction

Basal cell carcinoma (BCC) is the most common clinical skin malignancy and neoplasm,^{1–7} amounting to approximately 500 cases per 100,000 population. The lifetime risk of contracting BCC is approximately 30%. Exposure to ultraviolet radiation (UV) is the primary causative factor in development of BCC. de Gruijl et al. showed a maximum effectiveness of UV at 293 nm (UVB; 280 to 320 nm in wavelength) in skin cancer induction in mice.⁸


Treatment of BCC is both surgical (including curettage, cryosurgery, excision and micrographic surgery) and non-surgical. The 5 y cure rate is approximately 95%. Non-surgical techniques include radiotherapy, photodynamic therapy, and application of topical fluorouracil,⁹ cisplatin or doxorubicin,¹⁰ or the immunomodulator imiquimod.¹¹

Lovett et al. found that tumor control was achieved in 91% of patients with BCC and treated with superficial x-irradiation (50–200 kVp), electrons, or megavoltage irradiation (e.g., 10 MeV¹²), or some combination of these modalities.¹³ The control rate was related to lesion size. For lesions < 1 cm in diameter, control was 97%, while for lesions of 1–5 cm or for lesions > 5 cm diameter, BCC control values were 87 and 76%, respectively. For all lesions treated with superficial X-rays, tumor control was 97.4%. For electrons, control was 77.2%, and for megavoltage irradiation control was 66.7%. For combined modality treatments, the control rate was 75.8%. However, it is difficult to obtain the physical parameters for these irradiations.

As summarized by Veness et al., the incomplete understanding of susceptibility of BCC to radiation is a function of marked variations in methodology used, the dose fractionation schedule, the extent of field margins, and dose prescription.¹⁴

These differences in control rates raise the issue of linear energy transfer (LET, the number of ionizations within a given distance) for BCC responses. For superficial X-rays, the LET is about 3 keV/micron, while for orthovoltage (e.g., 250 kVp), 10 MeV electron irradiations,¹² and ⁶⁰Co irradiations, LET values are 1.9, 2.0, and 0.2 keV/micron,¹⁵ respectively. Such LET differences raise the issue of the energy needed to produce an ion pair. This is approximately 34 eV.^{16,17} Per micron of distance traveled through the cell, there are approximately 88 ion pairs produced for superficial x-irradiation, while for ⁶⁰Co there are 6. While these LET differences are small, they suggest that decreasing LET (i.e., fewer ionizations per unit distance) leads to poorer results (i.e., superficial x-irradiation was 97.4% tumor control, while for ⁶⁰Co irradiations tumor control was 66.7%).

Bergh et al.¹⁸ and Cody et al.¹⁹ showed that tetraiodothyroacetic acid (tetrac) binds to the thyroid hormone receptor on the extracellular domain of the plasma membrane integrin $\alpha v \beta 3$ protein and modulates multiple intracellular activities regulated by the receptor. These include inhibition of DNA repair processes after irradiation. Using the neutral Comet assay, we have shown that tetrac radiosensitizes GL261 and U87MG brain tumor cells through inhibition of repair of damaged DNA.^{20,21} In the present study, we have examined DNA

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double-strand break repair post-radiation in radioresistant BCC cells using the γ -H2AX assay;²²⁻²⁶ the effects of 2 concentrations of tetrac on DNA repair were studied.

Results

In vitro growth rates and colony forming efficiencies (CFEs) of TE.354.T BCC cells

TE.354.T BCC cells were initially slow-growing in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, sodium pyruvate, HEPES and fetal bovine serum (FBS) (10%) (see Materials and Methods). This was termed "standard medium" (SM).

To shorten doubling times and increase the CFE of BCC cells, we increased FBS concentration from 10% to 15%²⁷ and added fibroblast growth factor-2 (FGF-2)^{28,29} and stem cell factor-1 (SCF-1)³⁰ (Materials and Methods) and also reduced the medium calcium content to 0.3 mM. Finally, we added heavily irradiated (30 Gy) and reproductively inactivated TE.354.T "feeder cells" (FCs) to all dishes to make the total cell number constant over all radiation doses. In control TE.354.T cells, the doubling time in new medium of TE.354.T growth was decreased to 34.1 h and CFE increased from 0.26% to 10.10%.

Use of the linear-quadratic equation to determine radiation results for control and tetrac-treated cells

The 250 kVp X-ray survival curve for control and tetrac-treated cells is shown in Fig. 1. The linear-quadratic equation is an equation,^{31,32} in which fractional survival (FxS) is defined by the parameters ($\alpha_{X\text{-ray}}$ and $\beta_{X\text{-ray}}$). A 10 point survival response of the TE.354.T cell line was generated by exposure to increasing doses of 250 kVp X-rays. We used a 0.5 Gy dose to decrease the error estimate on the $\alpha_{X\text{-ray}}$ coefficient. Experiments were replicated 4-6 times. The $\alpha_{X\text{-ray}}$ coefficient (Gy^{-1}) describes the responses of cells at low doses while the $\beta_{X\text{-ray}}$ coefficient (Gy^{-2}) describes the responses at higher doses. We also estimated the surviving fraction at 2 Gy (SF_2) because this is the dose used per fraction in multifraction patient treatments.

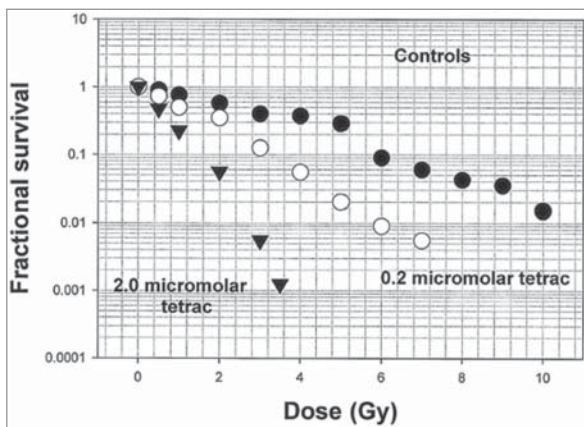


Figure 1. Survival of TE.354.T basal cell carcinoma cells *in vitro* after a 1 h exposure at 37°C to 2 different concentrations of tetraiodothyroacetic acid (0.2 and 2.0 μM tetrac) followed 1 h later by graded doses of 250 kVp x-irradiation.

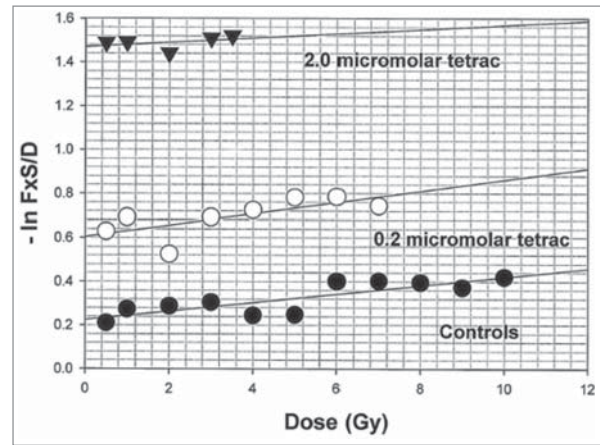


Figure 2. A plot of the transformed data shown in Fig. 1, using the relationship $-\ln FxS/D$ (FxS is the fractional survival) versus radiation dose. Tetrac administration primarily affects the $\alpha_{X\text{-ray}}$ parameter (intercept at 0 dose).

The $\alpha_{X\text{-ray}}$ (10^{-1} Gy) and $\beta_{X\text{-ray}}$ (10^{-2} Gy) values (and 95% confidence limits) for control cells were $0.225 (\pm 0.058)$ and $0.0195 (\pm 0.0097)$, respectively, and the SF_2 value was 0.60. For cells treated with the 0.2 μM tetrac concentration, $\alpha_{X\text{-ray}}$ and $\beta_{X\text{-ray}}$ values were $0.623 (\pm 0.301)$ and $0.108 (\pm 0.698)$, respectively. For treatment with 2.0 μM tetrac, $\alpha_{X\text{-ray}}$ and $\beta_{X\text{-ray}}$ values were $1.438 (\pm 0.162)$ and $0.073 (\pm 0.220)$, respectively. The use of 0.2 or 2.0 μM tetrac statistically significantly increased the $\alpha_{X\text{-ray}}$ value. $\beta_{X\text{-ray}}$ values were not statistically different. Transformed data are shown in Fig. 2. The SF_2 for control cells was 0.581, while values for 0.2 and 2.0 μM tetrac treatments were 0.281 and 0.024, respectively. The SF_2 data show that tetrac concentrations of 0.2 and 2.0 μM sensitize TE.354.T cells by factors of 2.1 and 24.0, respectively.

Investigation of the cellular effects of tetrac on repair of radiation injury

An early response to double-strand break (DSB) induction is the phosphorylation of histone H2A, which is then termed H2AX. This change can be visualized as discrete foci within cells using specific antibodies (EMD Millipore, Billerica, MA). H2AX foci co-localize with other proteins.²³ We found that the baseline level of such foci in TE.354.T cells was 1.92%.

The dose response for induction of γ -H2AX in control TE.354.T cells is shown in Fig. 3A. The equation for the control cells is $1.96 \text{ foci} (\pm 0.94) + 8.52 (\pm 0.27) \text{ foci/Gy}$ (errors are 95% confidence limits). In Fig. 3B, the γ -H2AX dose response curve is shown for treatment with 0.2 or 2.0 μM tetrac. The 0.2 μM tetrac curve equation is $1.92 (\pm 1.92) + 8.52 (\pm 0.81)$, and the curve for 2.0 μM tetrac is $1.91 (\pm 1.20) + 8.51 (\pm 0.48)$. There was no statistically significant difference between the induction of γ -H2AX foci as a function of dose between tetrac-treated cells and control cells; therefore, tetrac does not affect the initial induction of DSBs. In Fig. 4, the repair of DNA breaks is shown for control cells and for cells treated with the 0.2 or the 2.0 μM tetrac concentrations. We chose a dose of 2.5 Gy for the irradiation of control, 0.2, and 2.0 μM tetrac concentrations. The numbers of foci per cell seen at 1 h post-irradiation were: controls = 23.2; 0.2 μM tetrac = 22.8; and 2.0 μM tetrac = 23.0 (Fig. 3A, 3B).

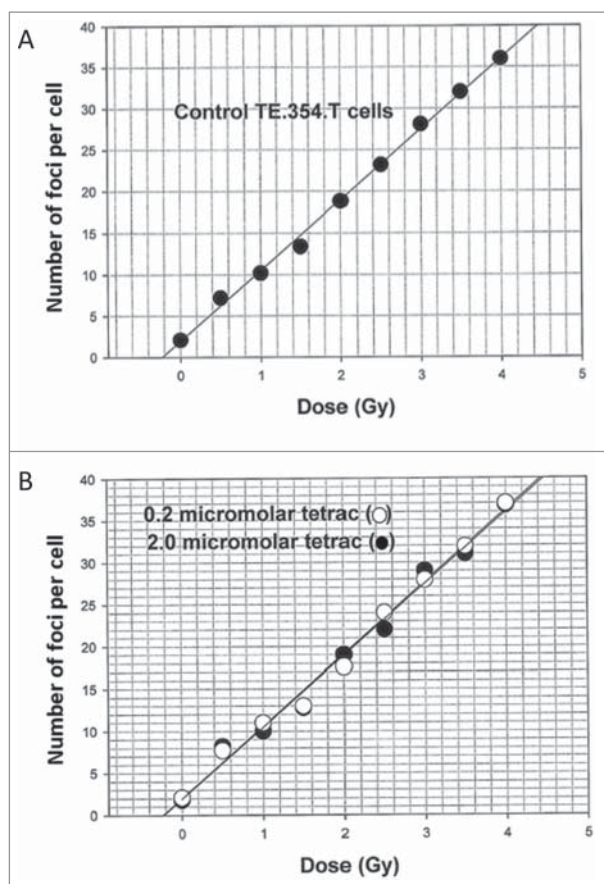


Figure 3. (A) Dose response dependence of the number of foci per cell for control cells for the fast repair portion of the H2AX response curve (0 to 1 h post-irradiation). (B) Dependence of the number of foci per cell for cells for the fast repair portion of the H2AX response curve (0 to 1 h post-irradiation) for cells at 37°C treated for 1 h with 0.2 or 2.0 μM tetrac before irradiation.

To calculate the percentage of DNA DSBs repaired, we took data from 10 to 40 h post-irradiation, converted the information to log values, and calculated the zero time intercept value³³ by back-extrapolation (Fig. 4). The value for control cells at zero time was 3.74 ($\pm 2.96\%$). The percentage of “slow repair” was 16.1% (3.4 to 28.9%) for control TE.354.T cells and fast repair was (100 – 16.1%) or 83.9%.

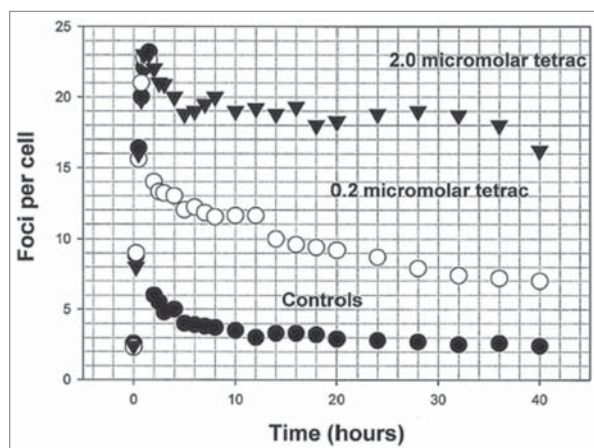


Figure 4. DNA double-strand break repair as indicated by the γ -H2AX assay (foci per cell) for control TE.354.T basal cell carcinoma cells and for cells pretreated for 1 h before graded dose irradiation with 2.5 Gy of 250 kVp x-irradiation.

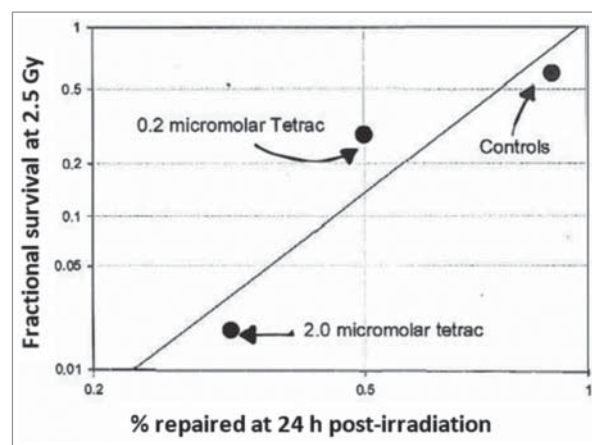


Figure 5. Probability plot of the survival at 2.5 Gy vs. the percent of DNA damage repaired at 24 h post-irradiation.

For cells treated with 0.2 μM tetrac, the zero time intercept was 13.40 ($\pm 2.93\%$). This value for the percentage of slow repair yielded a value of 58.8%, while the percentage of fast repair decreased to (100 – 58.8%) or 41.2%.

For TE.354.T cells treated with 2.0 μM tetrac, the zero time intercept was 19.5 (± 0.59) percent. The percentage of slow repair was 84.8% and the percentage of fast repair was (100 – 84.8%) or 15.2%. The rate of slow repair in control cells over the period of 10 to 40 h post-irradiation was 0.74 ($\pm 0.46\%$). For cells treated with 0.2 μM tetrac the repair rate was 0.15 (± 0.04), and with 2.0 μM tetrac, the repair rate was 0.03 ($\pm 0.01\%$). Both the rate and the extent of slow repair are affected by tetrac treatment. The rate of slow repair decreases by approximately 80% with 0.2 μM tetrac and by 96% with 2.0 μM tetrac.

Correlation between cell survival at 2.5 Gy and slow repair

In Fig. 5 we show the correlation between the survival seen in control cells and in cells treated with 0.2 and 2.0 μM tetrac. The slope of this fit is 0.964. However, as the sample number (n) is only 3, the t value on this fit is 0.113. This is not significant at the $P = 0.05$ level. The fit is nonetheless instructive because it strengthens the concept that an increasing concentration of tetrac results in increased cell killing because of inhibition of DNA repair.

Absence of cytotoxicity with exposure of cells to tetrac

We have previously noted that tetrac in μM concentrations lacks cytotoxic action on immortalized nonmalignant human and kidney cells.³⁴ In the current studies, we exposed cultured TE.354.T cells *in vitro* to 0.2 and 2 μM tetrac for up to 8 h or to cisplatin (2 μM) for 30 min. Tetrac-treated cells uniformly excluded trypan blue, whereas all cisplatin-exposed cells contained trypan blue (H-Y Tang, P.J. Davis: results not shown).

Discussion

The radiation survival parameters given here for these TE.354.T BCC cells is the first time that these values have been reported (Fig. 1). These cells are radioresistant, as shown by the dose

surviving fraction at 2 Gy of 0.575. This places BCC cells in the mid-range of radiosensitivity of cancer cells.^{35–39} The current studies were facilitated by altering the doubling times and colony-forming efficiencies of BCC by changing several factors in the medium in which cells were cultured.^{28,40–42}

We have previously reported that tetrac may radiosensitize certain tumor cells^{20,21} and does so by inhibiting repair of radiation-induced double-strand DNA breaks. The effects of the agent on radioresistant cells or skin cancer cells have not previously been studied. Tetrac acts at a thyroid hormone-tetrac receptor identified on the extracellular domain of integrin $\alpha v \beta 3$;^{18,43–45} this plasma membrane protein is essential to cell-cell and cell-extracellular matrix protein interactions and is generously expressed by cancer cells, but not by normal cells.⁴³ At this receptor, tetrac has anticancer and anti-angiogenic—as well as radiosensitizing—properties and blocks the proliferative and pro-angiogenic activities of agonist thyroid hormone (L-thyroxine, T₄; 3,5,3'-triiodo-L-thyronine, T₃).⁴³ An index of DNA double-strand break repair, H2AX phosphorylation studied in the current paper is due to mitogen-activated protein kinase (MAPK) activity.⁴⁶ One of the actions of tetrac and its formulations is to downregulate MAPK activity in cancer cells⁴³ and we propose that this may be one of the mechanisms by which tetrac achieves radiosensitization.

The present results are valid for 250 kVp X-rays. Given the difference in patient survival seen when BCC is treated with superficial X-ray (⁶⁰Co gamma rays), and given the concomitant LET differences, pre-treatment of BCC with tetrac before irradiation should yield beneficial results. Tetrac has been shown to increase cancer cell radiosensitivity by inhibiting DNA repair.²¹ In particular, the slow repair of DNA DSBs appears to be the drug target as indicated by γ -H2AX results. These results argue for a more detailed inclusion of radiation therapy parameters in the treatment of BCC, both from estimation of physics (e.g., LET) and from estimation of DNA repair inhibition. These effects of tetrac are concentration-dependent *in vitro* and they would likely be concentration-dependent in the patient. It may be feasible to apply tetrac topically to BCC for the purpose of radiosensitization.

Materials and methods

Cells and medium

We used TE.354.T BCC cells obtained from the American Type Culture Collection, Manassas, VA (ATCC; cat. No. CRL-7762). The cells were female human tumor cells that are adherent and fibroblastic in culture. Cells were initially grown in Dulbecco's modified Eagle's medium (DMEM) in T₇₅ cm² flasks, to which fetal bovine serum (FBS) was added at a concentration of 10%. The medium was further supplemented with L-glutamine (0.058 g/L), sodium pyruvate (0.11 g/L) and HEPES (5.96 g/L). In this 'standard medium' (SM), TE.354.T cells were slow growing, with a doubling time of 48.4 (± 3.3) h *in vitro* and a CFE of 0.26 (± 0.09) percent. The medium was modified to shorten doubling time and increase CFE. The modifications included: 1) increase in FBS concentration to 15%; 2) reduction in calcium content of medium from 0.5% to 0.3%; 3) addition of fibroblast growth factor (FGF-2) (50 ng/mL); 4) addition of

stromal derived growth factor-1 (SDF-1) (100 ng/mL); 5) addition of heavily irradiated (30 Gy) "feeder cells" to all dishes to keep the total cell concentration constant. Cells were pre-treated with tetraiodoacetic acid (tetrac) at 2 different concentrations (0.2 and 2.0 μ M) for 1 h at 37°C before graded dose x-irradiation.

Tetrac formulation

Tetrac powder (Sigma-Aldrich Co. St. Louis, MO) was solubilized in potassium hydroxide (KOH) and propylene glycol to a stock concentration of 200 μ M and was then stored at –20°C until use. For use, the tetrac solution was added to complete medium to make differing concentrations (e.g., 0.2 and 2.0 μ M).

Irradiation techniques

For survival experiments, cells were seeded 48 h before irradiation at 10⁴ cells/T₂₅ cm² flask. Cells were grown for 3 d and then flasks were irradiated using a Philips 250 kVp X-ray machine (Philips Corp., Fall River, MA). After irradiations, flasks were placed on ice for 30 min. Medium was then removed from the flasks and trypsin-EDTA was added. Flasks were placed at 37°C. After 5–7 min, cells detached and the trypsin-EDTA was poured into a centrifuge tube to which an equal volume of ice-cold medium was added. The suspension was centrifuged (1000 \times g) for 5 min, and the supernatant removed. We then added 1 mL of the new medium, and the cell pellet was retrituated, using a sterile glass pipette. Next, 4 mL of medium was added, and the suspension was mixed to provide a cell suspension for hemacytometry. Cells were counted (Olympus phase contrast microscope, Olympus Corp., Center Valley, PA) to determine the cell concentration. Determination was made of cell multiplicity and corrections for this were made for experiments. The cell multiplicity for both control and tetrac-treated cells was 1.06 (95% confidence limits 1.02–1.10). Cells were seeded into flasks for survival determinations. We added irradiated TE.354.T feeder cells using a ¹³⁷Cs source (dose 30 Gy; dose rate 10 Gy/min; J.L. Shepard Co., Glendale, CA). Feeder cells were added to cultures at 2.5 \times 10⁴ cells/T₂₅ cm² flask.

Use of the single-hit, multitarget equation to determine radiation results for control and tetrac-treated cells, and BCC survival

The single-hit, multitarget equation is a model that has been used to describe cell survival after exposure to ionizing radiation (e.g.,^{35,47,48}). FxS is the fractional survival after graded dose irradiations, x is the number of radiation "hits" per cell, D₀ is the dose that creates one-lethal hit per cell (the mean lethal dose), and n is the number of targets per cell (the extrapolation number). With regard to a multitarget model, the survival probability of a cell is represented by:

$$FxS = 1 - (1 - e^{-Dx/D_0})^n \quad (1)$$

where FxS = probability of survival, Dx is the experimental dose given, D₀ = a dose that causes an average of one-hit per

cell (mean lethal dose), and n = number of “targets” (required number of hits for cell death). This equation describes the cell-survival curve and the radiosensitivity of the cell line using ‘ D_Q (quasi-threshold dose)’, ‘ D_0 (slope)’, and n (extrapolation number)’. These parameters are found by first fitting the data lying below 0.368 fractional survival using a semilogarithmic approach. The slope yields the D_0 value. This linear fit is back-extrapolated to the Y-axis. For survival curves that are not exponential, this yields an intercept that is greater than 1.0, and is termed the extrapolation number (n), which represents the number of “targets” within each cell. The third parameter is the D_q (Gy) value, which is obtained from the D_0 fit. However, the intercept of this back-extrapolation with the survival at a value of 1.0 (100 percent survival) is obtained.

Linear-quadratic equation to calculate BCC survival

The equation for analysis of dose-response data is the linear-quadratic equation.^{31,49,50} This equation is:

$$FxS = \exp[-(\alpha_{x\text{-ray}}D + (\beta_{x\text{-ray}}D^2))] \quad (2)$$

FxS is fractional survival, and $\alpha_{x\text{-ray}}$ and $\beta_{x\text{-ray}}$ represent the inactivation coefficients of one-hit or 2-hit inactivation. D is the dose. This equation was transformed by dividing by dose (D), e.g.,

$$FxS / D = \exp(\alpha_{x\text{-ray}} + \beta_{x\text{-ray}}D) \quad (3)$$

Radiation produces DNA double-strand breaks (DSBs) with a yield proportional to dose. DSBs can be repaired. The ($\alpha_{x\text{-ray}}D$) term represents production of a DSB by a single radiation track, while the $\beta_{x\text{-ray}}D^2$ term represents DSBs produced by 2 adjacent events.⁵² DSBs can be produced either by direct ionization or by production of free radicals or other reactive species.⁵¹ Data were plotted using:

$$-\ln FxS / D = (\alpha + \beta D) \quad (4)$$

The transformed data were analyzed using SigmaPlot (v. 13, Point Richmond, CA) to obtain the $\alpha_{x\text{-ray}}$ and $\beta_{x\text{-ray}}$ values for each experiment and 95% confidence limits. In these plots, $\alpha_{x\text{-ray}}$ is represented by extrapolation of the curve to zero dose, while $\beta_{x\text{-ray}}$ is the slope of these curves.

Effects of tetrac on repair of radiation injury (γ -H2AX assay)

After definition of the survival curves for BCC cells receiving pre-irradiation tetrac concentrations of 0.2 and 2.0 μ M, we determined the ability of BCC cells to repair radiation injury using the γ -H2AX assay.^{52,53} Cells were spotted on glass Superfrost Plus slides (VWR International, Radnor, PA) at a concentration of 1×10^8 cells/mL, fixed with 2% formaldehyde/PBS for 10 min, washed once in PBS, permeabilized using 0.5% (v/v) Triton-X/PBS for 10 min, and washed again in PBS. Blocking was done using 1% (w/v) bovine serum albumin in PBS for 30 min. Cells were incubated with a combination of 1:500

mouse monoclonal γ -H2AX antibody (Abcam, Cambridge, MA) and 1:400 rabbit polyclonal anti-53BP1 antibody (Bethyl Laboratories, Montgomery, TX) in 1% BSA/PBS for 1 h at 20°C. Cells were washed in 1% BSA/PBS, incubated in 1:200 mouse AlexaFluor 488 conjugated antibody (Invitrogen, Carlsbad, CA), 1:200 (rabbit tetramethyl rodamine isocyanate conjugated antibody, Jackson ImmunoResearch, West Grove, PA), and 200 ng/mL of 4',6-diamidino-2-phenylindole (DAPI) in 1% BSA/PBS for 1 h at 20°C, washed in PBS, dried, mounted with a coverslip using Vectashield (Vector Laboratories, Burlingame, CA) and sealed using nail polish. γ -H2AX foci were scored using a Nikon (Melville, NY) epifluorescence microscope (100 \times objective with 1.3 NA). One hundred and fifty cells were scored for each dose point in each experiment.

To analyze data, points on the fast and the slow components of repair were converted to their log values. The slow component was analyzed with SigmaPlot, and extrapolation of these slow repair data to intercept the induction curve (0 to 2 h post-irradiation) yielded the percentage of the total repair performed by the slow repair process and the extent of slow repair at various times post-irradiation (e.g., at 3, 3.5, and 4 h). These slow repair estimates were then subtracted from fast repair and the fast repair data were fit using SigmaPlot.

Statistics

Use of SigmaPlot (v. 13, Point Richmond, CA) gave us the respective $\alpha_{x\text{-ray}}$ and $\beta_{x\text{-ray}}$ values and their 95% confidence limits. The 95% confidence limits on SF₂ were obtained by error propagation of the individual $\alpha_{x\text{-ray}}$ and $\beta_{x\text{-ray}}$ values.⁵⁴

Abbreviations

BCC	basal cell carcinoma
CFE	colony forming efficiency
DSB	double-strand break
FBS	fetal bovine serum
FC	feeder cells
FGF-2	fibroblast growth factor 2
LET	linear energy transfer
NM	new medium
SCF-1	stem cell factor-1
SF ₂	surviving fraction at 2 Gy
tetrac	tetraiodothyroacetic acid

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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