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## Screening of Antimicrobial Agents for *In Vitro* Radiation Protection and Mitigation Capacity, Including those Used in Supportive Care Regimens for Bone Marrow Transplant Recipients\*

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### Abstract

Antibiotic and antifungal agents used in supportive care regimens for bone marrow transplantation recipients contribute to a significant dose-modifying effect of otherwise lethal total body irradiation. To determine whether drugs used in supportive care and other commonly used antibiotics such as tetracycline function as radiation protectors or damage mitigators in vitro, 13 drugs were tested for radiation protection and radiation damage mitigation of 32D cl 3 hematopoietic progenitor cells in clonogenic survival curves in vitro. Antibiotic/Antifungal agents including cilastatin, amikacin, ceftazidime, vancomycin, tetracycline, doxycycline, ciprofloxacin, metronidazole, methacycline, minocycline, meclocycline, oxytetracycline and rolitetracycline were added in 1, 10, or 100 micromolar concentrations to murine interleukin-3-dependent hematopoietic progenitor cell line 32D cl 3 cells either before or after irradiation of 0 to 8 Gy. Control irradiated 32D cl 3 cells showed radiosensitivity comparable to freshly explanted mouse marrow hematopoietic progenitor cells ( $D_0$   $1.1 \pm 0.1$  Gy,  $\tilde{N}$   $1.5 \pm 0.4$ ). Positive control GS-nitroxide JP4-039 (known radiation mitigator) treated 32D cl 3 cells were radioresistant ( $D_0$   $1.2 \pm 0.1$ ,  $\tilde{N}$   $5.8 \pm 2.4$  ( $p=0.009$ )). Of the 13 drugs tested, tetracycline was found to be a significant radiation mitigator ( $D_0$   $0.9 \pm 0.1$ ,  $\tilde{N}$   $13.9 \pm 0.4$  ( $p=0.0027$ )). Thus, the radiation dose-modifying effect of some antibiotics, but not those currently used in the supportive care (antibiotic/antifungal regimens) for marrow transplant patients, may act as radiation damage mitigators for hematopoietic cells as well as decreasing the growth and inflammatory response to microbial pathogens.

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## Keywords

Antibiotic; antifungal agent; radiation protection; radiation mitigators; bone marrow transplant; supportive care

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In experimental animals as well as clinical protocols of bone marrow transplantation, preparation of the host for infusion of donor hematopoietic stem cells often utilizes total-body irradiation or cytotoxic chemotherapy (1–5). In canine models (2–4,6–12), the LD 50/30 (dose which produces bone marrow death in 50% of irradiated dogs at 30 days after irradiation) has revealed a significant dose modifying effect (DME) of the antibiotic and antifungal supportive care regimen. The DME has been shown in some canine model systems to increase from 4 to 7 Gy (1–3,13–17). Supportive care regimens have been further modified in recent years, with the availability of new, more potent antibiotic and antifungal agents (1–3,12). A similar irradiation DME of supportive care regimens has been demonstrated in canine (1–4), and non-human primate models that include marrow transplantation (18).

Antibiotics and antifungal agents used in current clinical bone marrow transplant supportive care regimens have been combined to prevent opportunistic infections facilitated by the leukopenia of the bone marrow-toxic agents, which include total-body irradiation (18–31). A question which has arisen during discussion of the potential mechanism of supportive care-mediated irradiation DME is that one or more of the antimicrobial agents could be acting as radiation damage mitigators, outside of their antimicrobial actions. Radiation mitigators are defined as those agents which can improve survival when added after exposure to irradiation, but before the appearance of clinical pathological or pathophysiological symptoms or effects (32). Known radiation mitigators used in clinical radiotherapy include WR2721 (amifostine) (33), nitroxides (Tempol) (34), and in experimental model systems, a series of new small molecule radiation damage mitigators (33).

There is recent evidence from chemical library screening experiments that analogs of some known antibiotics may have DNA-intercalating capacity and may function to stimulate DNA repair. These data have suggested that some antimicrobial agents, including those used in supportive care regimens, may have radiation damage mitigative properties. We tested the effect of each of 13 antimicrobial agents, including those used in current bone marrow transplantation clinical protocols, in a radiation sensitivity assay utilizing the interleukin-3(IL-3)-dependent murine hematopoietic progenitor cell line 32D cl 3 (34–35) as a marker cell line for human bone marrow stem cells.

## Materials and Methods

### Cells and cell culture

The murine hematopoietic progenitor cell line 32D cl 3 (34,35), dependent for growth *in vitro* upon IL-3 was grown in McCoys modified medium contained at 37° C in a high humidity incubator according to published methods (34–35). This cell line has been shown to be a sensitive indicator of the radiation protective and radiation mitigative properties of new candidate drugs (33).

### Antimicrobial agents and analogs

Each of 13 drugs (Table I) was tested in triplicate experiments. Stock solutions of all drugs obtained from Sigma Chemical Co., St. Louis, MO, USA, were made by dissolving the drugs in sterile water at a concentration of 10 mM. Drugs were assayed for their ability to modify the radiosensitivity of 32D cl 3 murine hematopoietic progenitor cell line. The

antibiotics were used at a concentration of 1, 10, or 100  $\mu\text{M}$  by adding them to 32D cl 3 cells 1 hour before irradiation or immediately after irradiation. Cells were irradiated to doses ranging from 0 to 8 Gy, plated in methycellulose, and incubated at 37°C for 7 days, at which time colonies of greater than 50 cells were counted (34–35). The data show the mean $\pm$ SEM of three separate experiments for  $D_0$  (final slope of irradiation survival curve expressed in Gy) and  $\tilde{N}$  (shoulder on the survival curve) of each drug. The antibiotics were evaluated for their effect on  $D_0$  and  $\tilde{N}$ . Some drugs when administered at 100  $\mu\text{M}$  after irradiation showed no colonies, suggesting toxicity. The positive control radiation mitigator JP4-039 was added at 10  $\mu\text{M}$  to cultures before or after irradiation, as published elsewhere (36,37).

### Irradiation survival curves

Cells were irradiated using a Cesium 137 Gamma cell irradiator, dose rate 100 cGy/min over a total dose range of 0 to 8 Gy. For evaluation of radiation protective capacity, cells were incubated for 24 hours at each concentration of antimicrobial before irradiation. For irradiation mitigation experiments, irradiated cells were centrifuged to a cell pellet and resuspended in medium containing 1, 10, or 100 micromolar concentrations of each antimicrobial, then plated in 0.8% methylcellulose containing medium for clonogenic survival curve assay. Cells were plated at 500, 1000, or 2000 cells per plate in triplicate at each dose according to published methods (34,35). All experiments were carried out in triplicate.

At 7 days after plating, colonies of 50 or more cells per colony were scored using an inverted microscope according to published methods (34,35). Data are presented using each of two computer program plotting linear, quadratic, and the alpha/beta model as appropriate (35).

## Results

### Radiobiologic effects on 32D cl 3 cell clonogenic survival

We first tested the effect of each of 13 antibiotic and antifungal agents including those used in supportive care regimens in clinical and experimental (canine) bone marrow transplantation models. The results in Tables I and Figures 1 through 13 demonstrate some toxicity of drugs at high concentrations (for example, doxycycline at 100  $\mu\text{M}$  after irradiation). The antibiotic/antinocardial agents, minocycline and rolitetracycline was toxic at all doses when added after irradiation (Table I). Irradiation survival curves with 32D cl 3 demonstrated a significant increase in the shoulder reflected as ( $\tilde{N}$ ) by the addition of the positive control GS-nitroxide, JP4-039 (37) before or after irradiation (Table I). Tetracycline was also a radiation mitigator by this assay at 100  $\mu\text{M}$  (Table I) (Figure 8).

### Radiation protection and damage mitigating properties of tetracycline analogs

Each of 6 tetracycline analogs evaluated by chemical library screening were tested for radiation protection or damage mitigating properties against 32D cl 3 cells at each of the three concentrations described in the Materials and Methods. These agents were not significant radiation damage mitigators or protectors in the 32D cl 3 assay (Table I) (Figures 9–13).

## Discussion

The development of protocols for clinical bone marrow transplantation in patients with leukemia, lymphoma, multiple myeloma, and other non-hematopoietic system malignancies has taken into account the significant problem of opportunistic infection during the interval between transplant of donor hematopoietic stem cells and recovery of peripheral white blood

cell counts (7–8,34). This interval of leucopenia which can last 14–30 days, subjects the transplant recipient to a significant risk of opportunistic infections (19,20). The use of laminar-flow rooms and meticulous sterile technique in the Marrow Transplant Unit has greatly minimized the incidence and severity of these opportunistic infections (38). Antibiotic and antifungal regimens used in the prophylactic care of transplant recipients prior to marrow ablative therapy (total-body irradiation or alkalinating agent therapy) as well as after donor marrow infusion have been continuously modified with the availability of newer, less toxic broad-spectrum antimicrobial agents (8–9,12).

Bone marrow transplantation recipients are prepared using techniques of single fraction or fractionated total-body irradiation at a dose of radiation that produces the hematopoietic syndrome. The hematopoietic syndrome is by definition one that can be corrected by replacement of hematopoietic stem cells (32). Gastrointestinal, pulmonary, or other non-hematopoietic organs reversibly damaged by radiation are not rescued by bone marrow transplantation (33). Total body irradiation doses, fractionation schemes, and efforts to protect other non-hematopoietic tissues such as the lung, using a pulmonary transmission blocks have established guidelines for appropriate radiation doses and preparative regimens (26).

It is also assumed that these drugs work secondarily to decrease any inflammatory cytokine response to infection. Supportive care regimens may also have utility in the management of those victims of irradiation accidents who may not be candidates for marrow transplantation (32).

In the present studies, we sought to determine whether the common antibiotic tetracycline as well as one or more of the agents used in the modern supportive care regimen also act as radiation damage mitigators. To determine whether small molecules including antibiotic or antifungal agents are acting as radiation damage mitigators at this basic cellular level, an *in vitro* clonogenic survival assay system was used (39). Rapid assays for immediate irradiation induced apoptosis, or cell death after the first cell division are of value as well (36). The clonogenic survival curve measures cell death within the first 7 cell doublings after irradiation, and allows detection of lethal events which may be delayed past the first cell division (36). The results showed that tetracycline, like the positive control drug JP4-039 (37), was a radiation mitigator *in vitro*. Another study using siRNA screening of mRNA targets identified doxycycline as a potential radioprotector, but the drug was not effective in that study, as in the present study in clonogenic survival curve assays using 32D cl 3 cells, nor was it effective *in vivo* (39).

The present study supports efforts to design future generations of antibiotics and antifungal agents with a goal of also using them as radiation damage mitigators. Antibacterial/antifungal agents could be modified to have normal tissue radiation mitigation capacity to aid recovery of the total-body irradiated transplant recipient or radiation terrorist victim. Concerns for clinical application would include confirmation that the agents not alter the tumor cell killing effects of total-body irradiation. Screening new antimicrobials as well as those used in clinical supportive care regimens for radiation damage mitigation capacity should be of value in the development of treatments for victims of radiation accidents and radiation terrorism.

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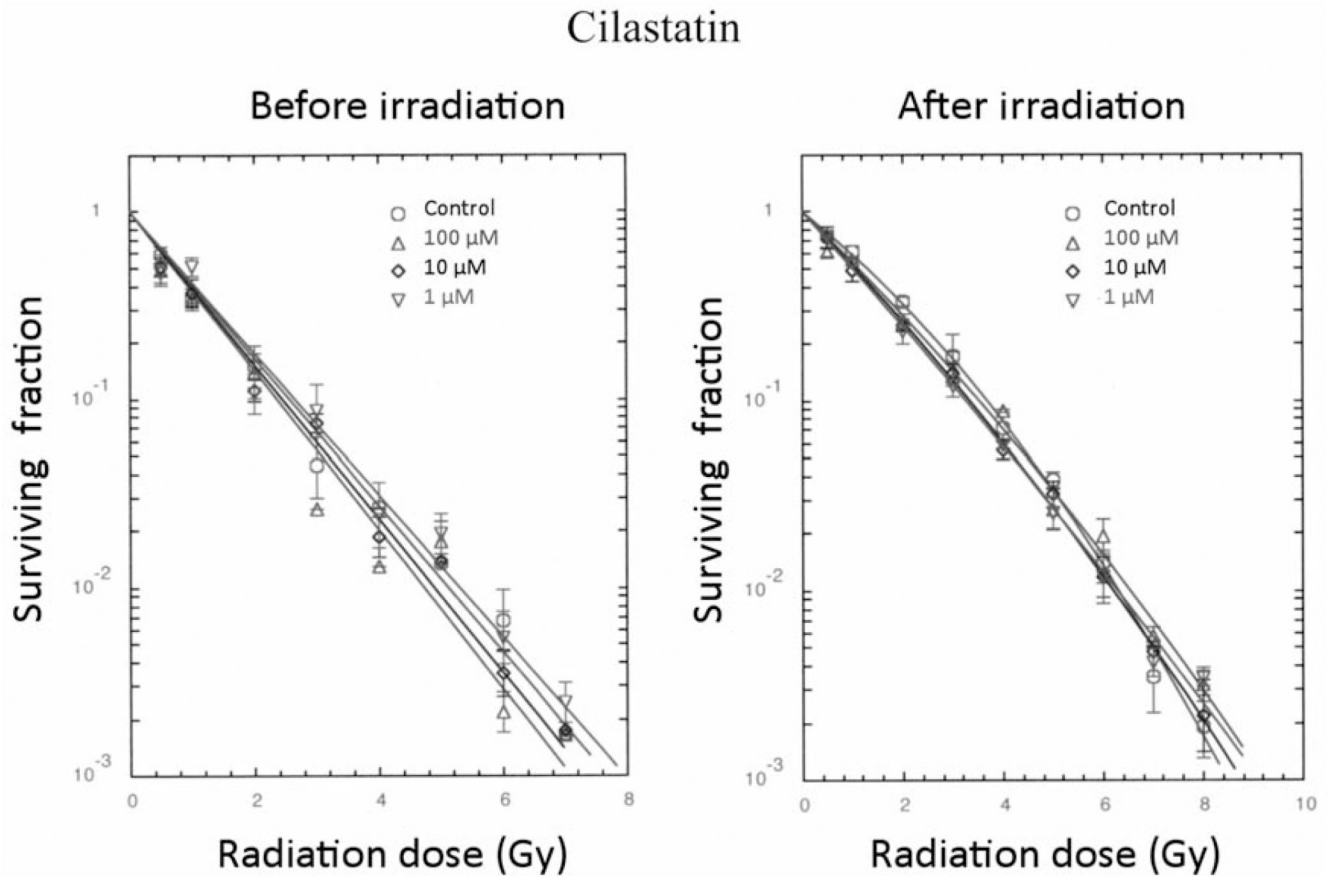
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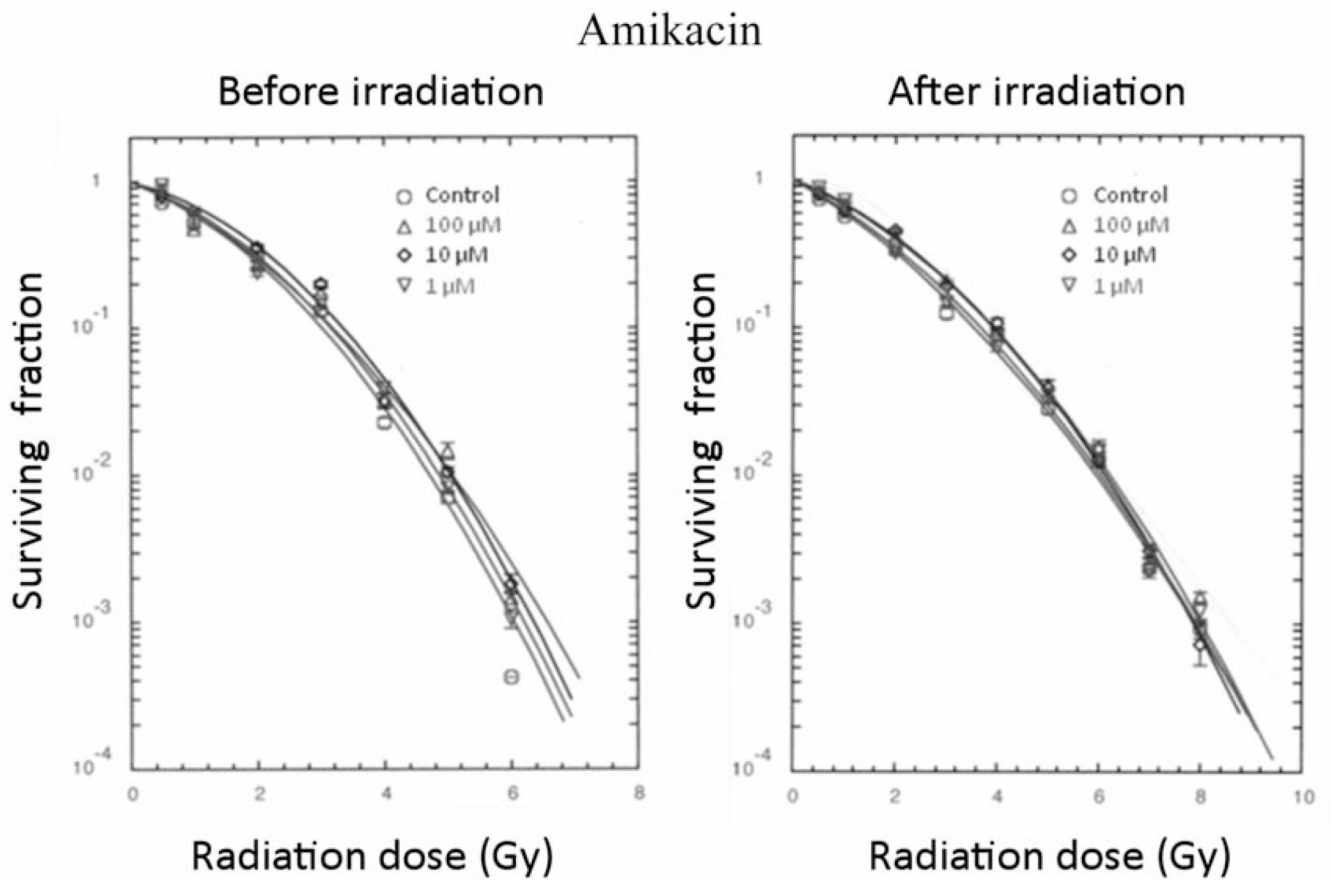


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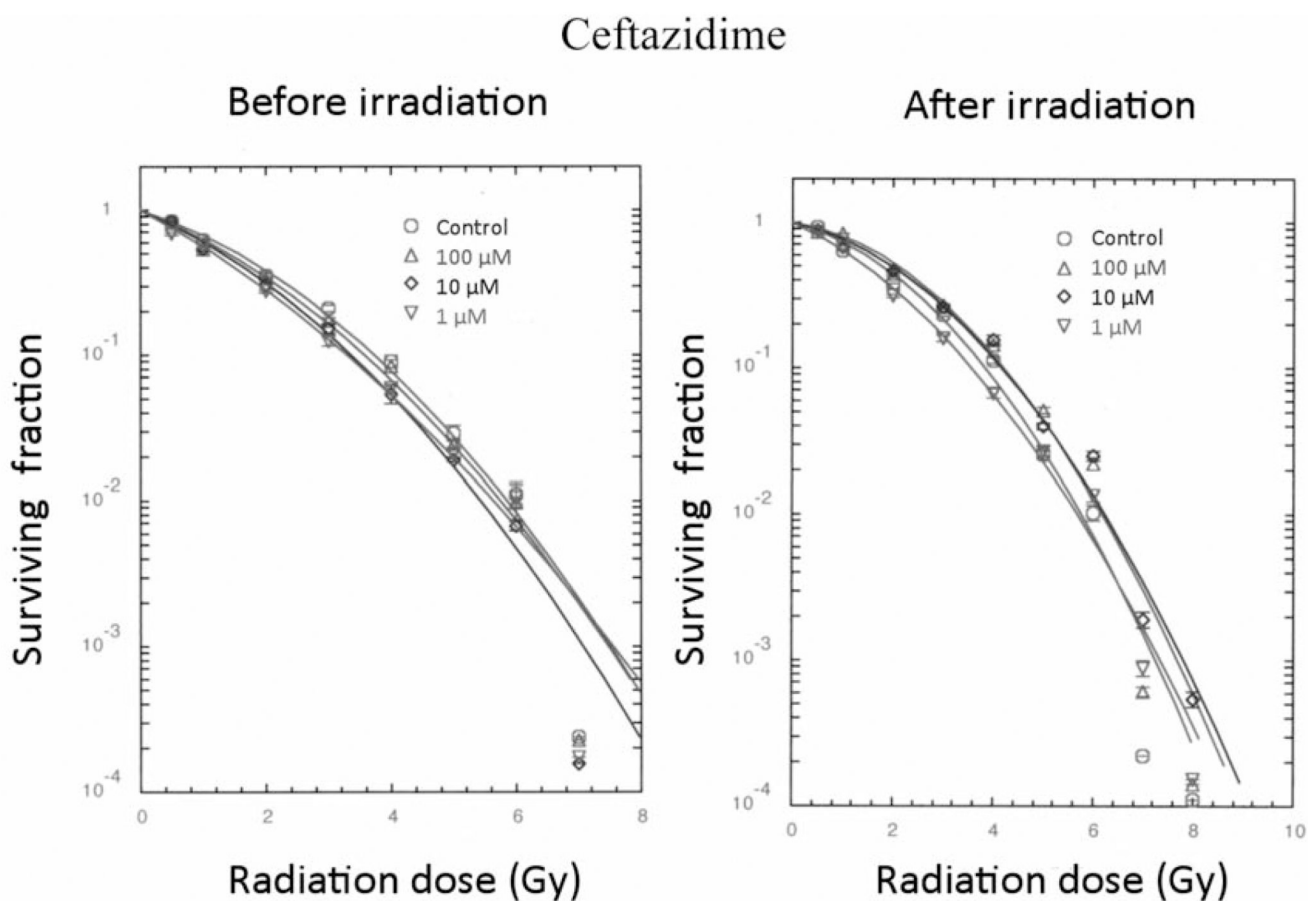
**Figure 1.** Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.





**Figure 2.**

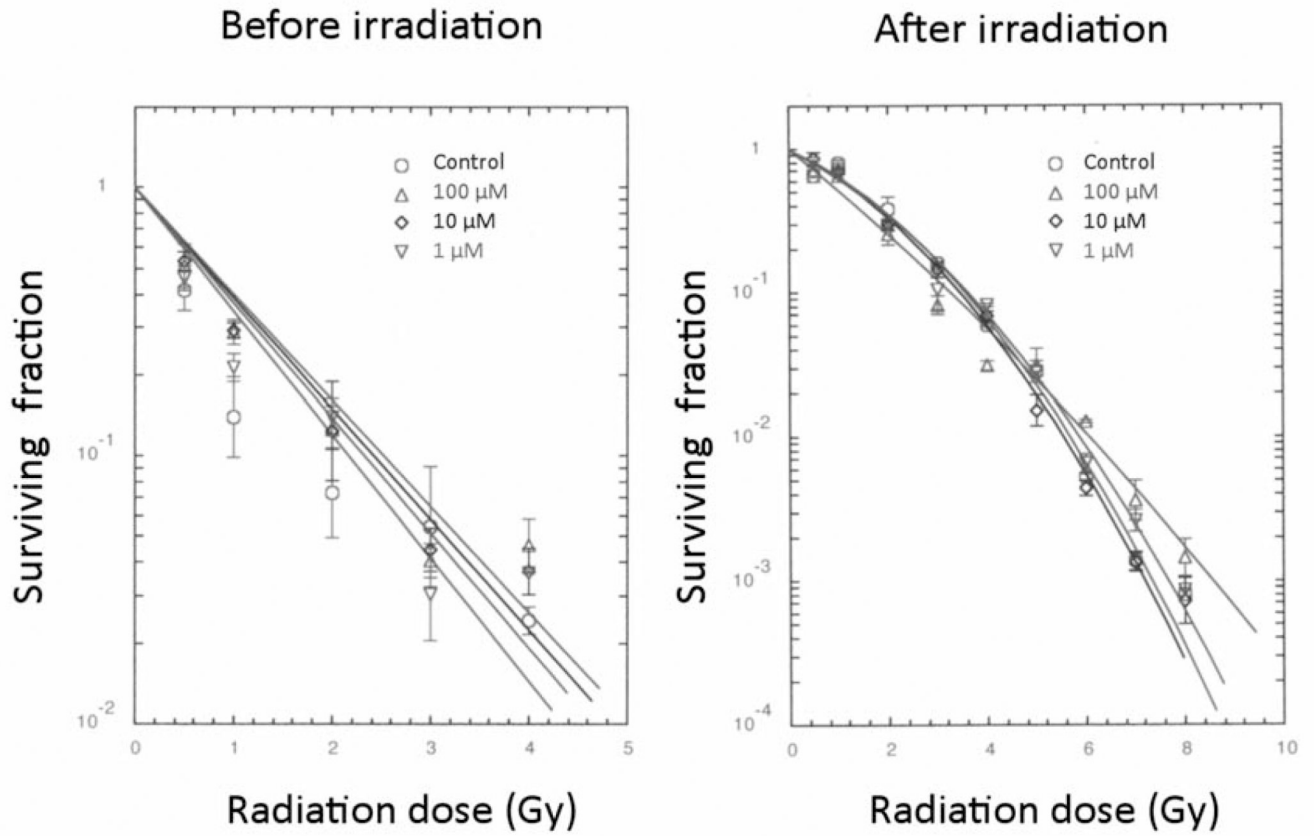
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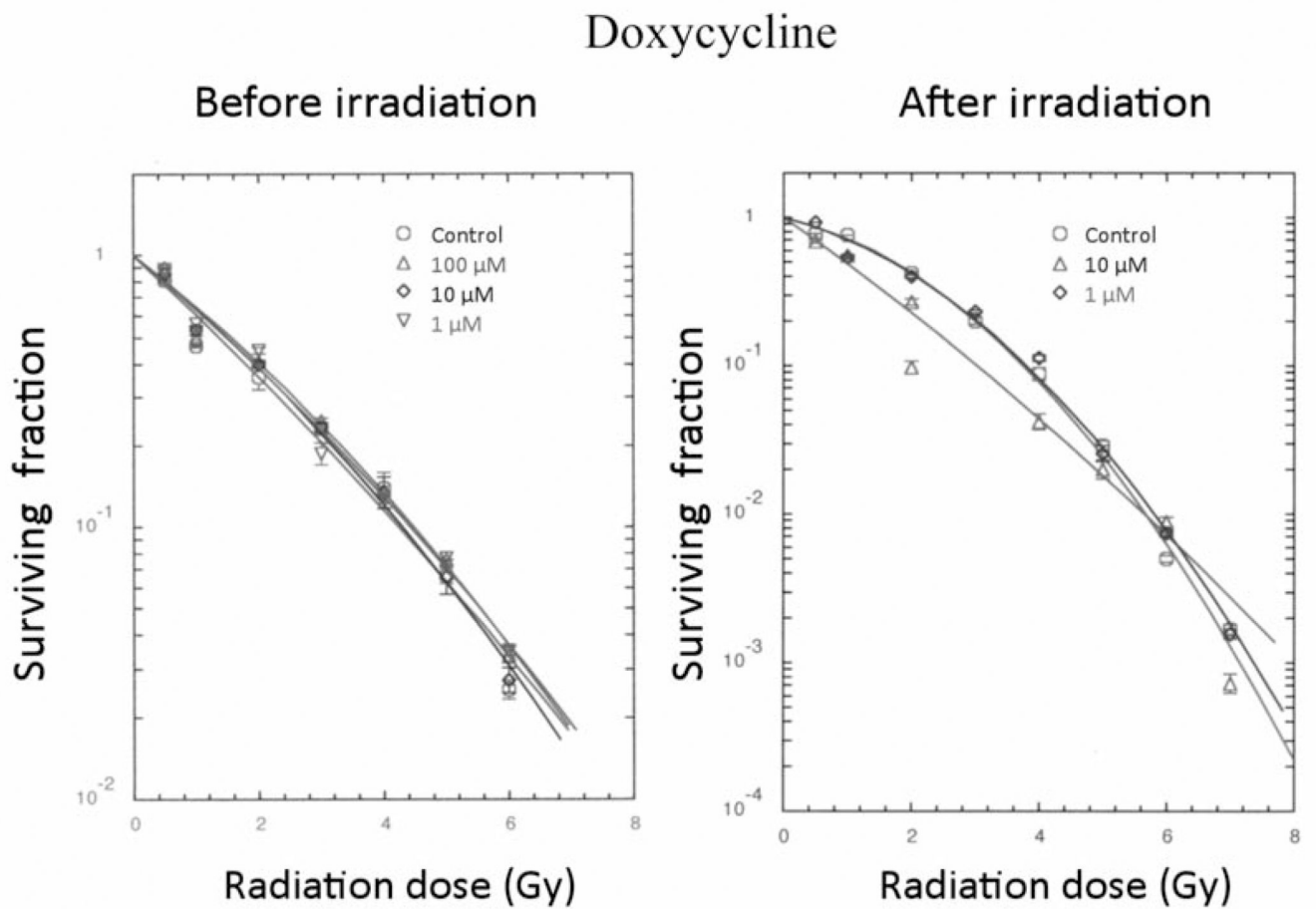
**Figure 3.**

Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.

## Vancomycin

**Figure 4.**

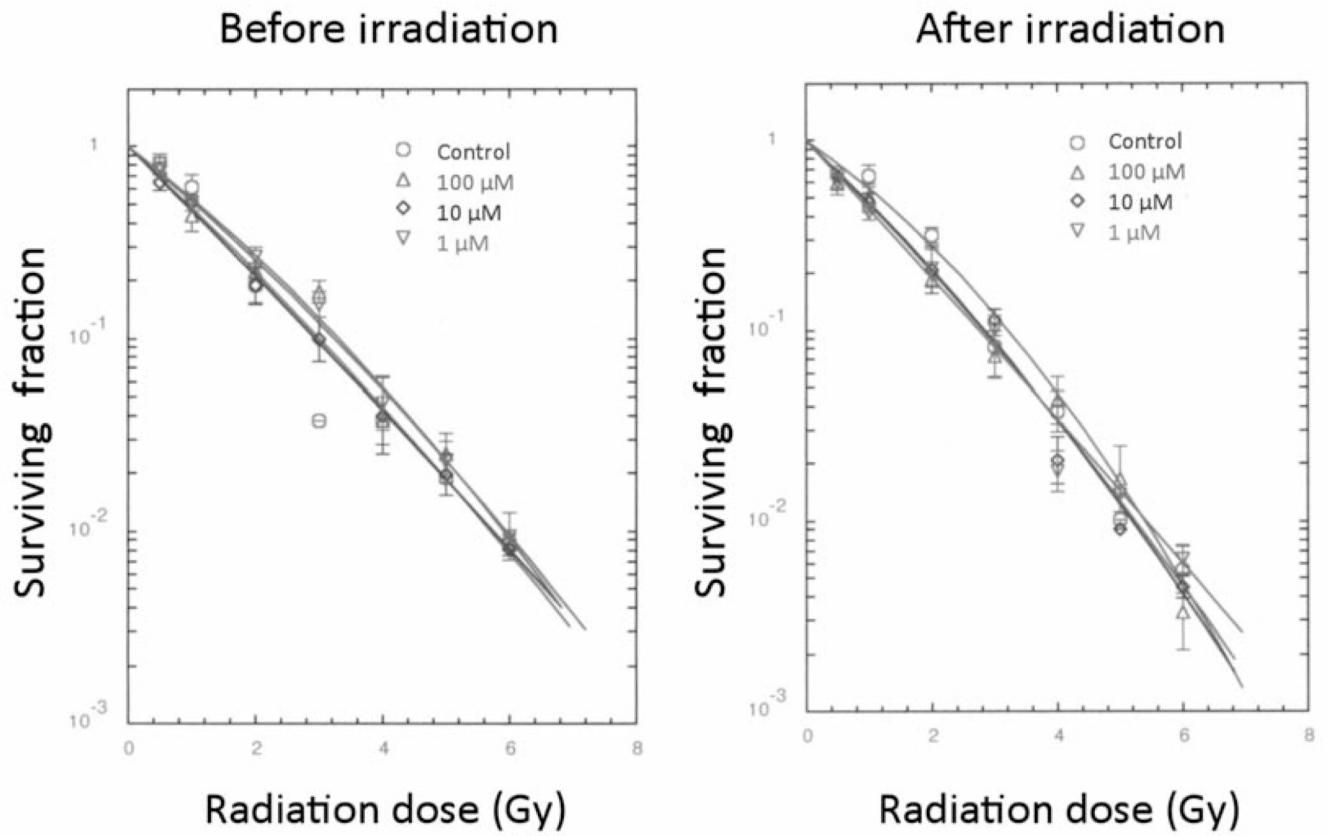
Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.



**Figure 5.**

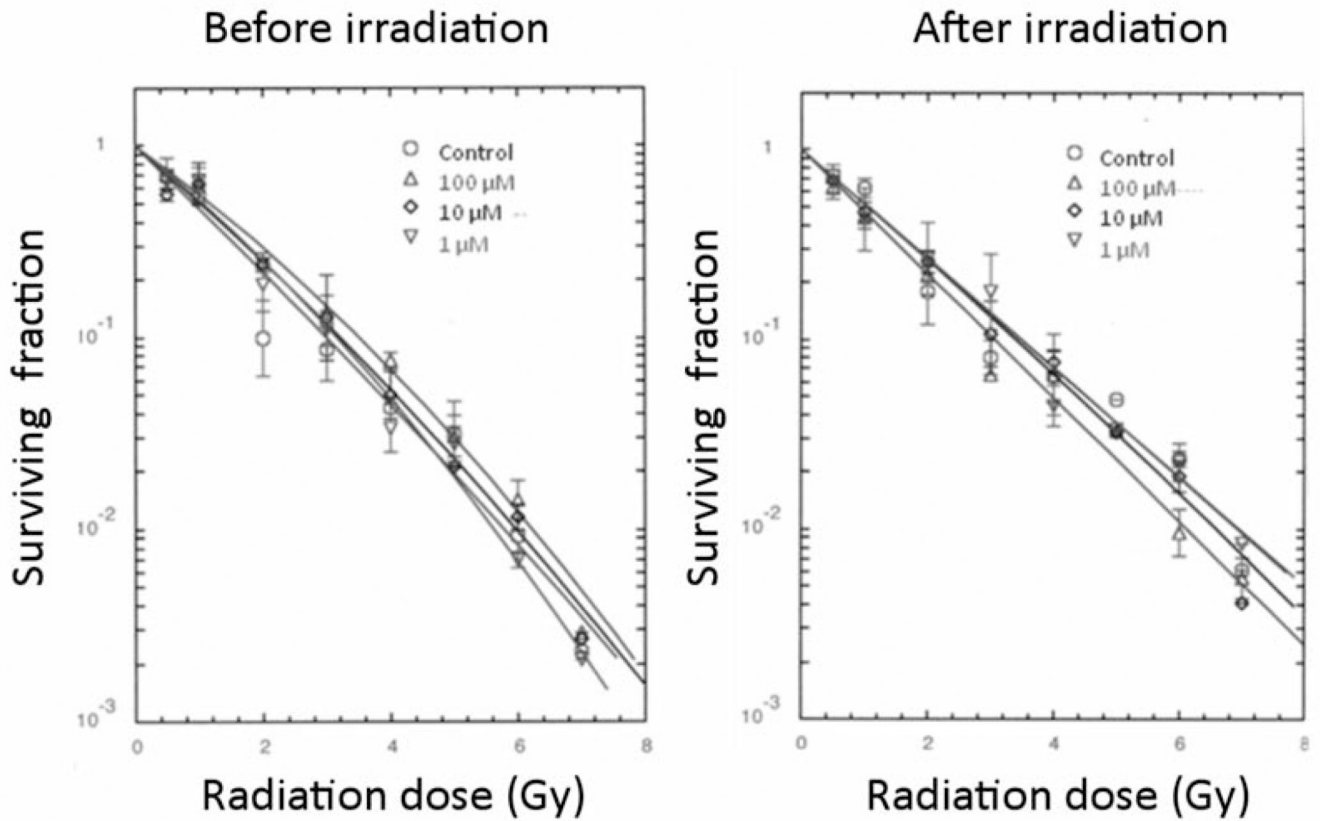
Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean  $\pm$  SEM of each agent at each concentration.

## Ciprofloxacin

**Figure 6.**

Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.

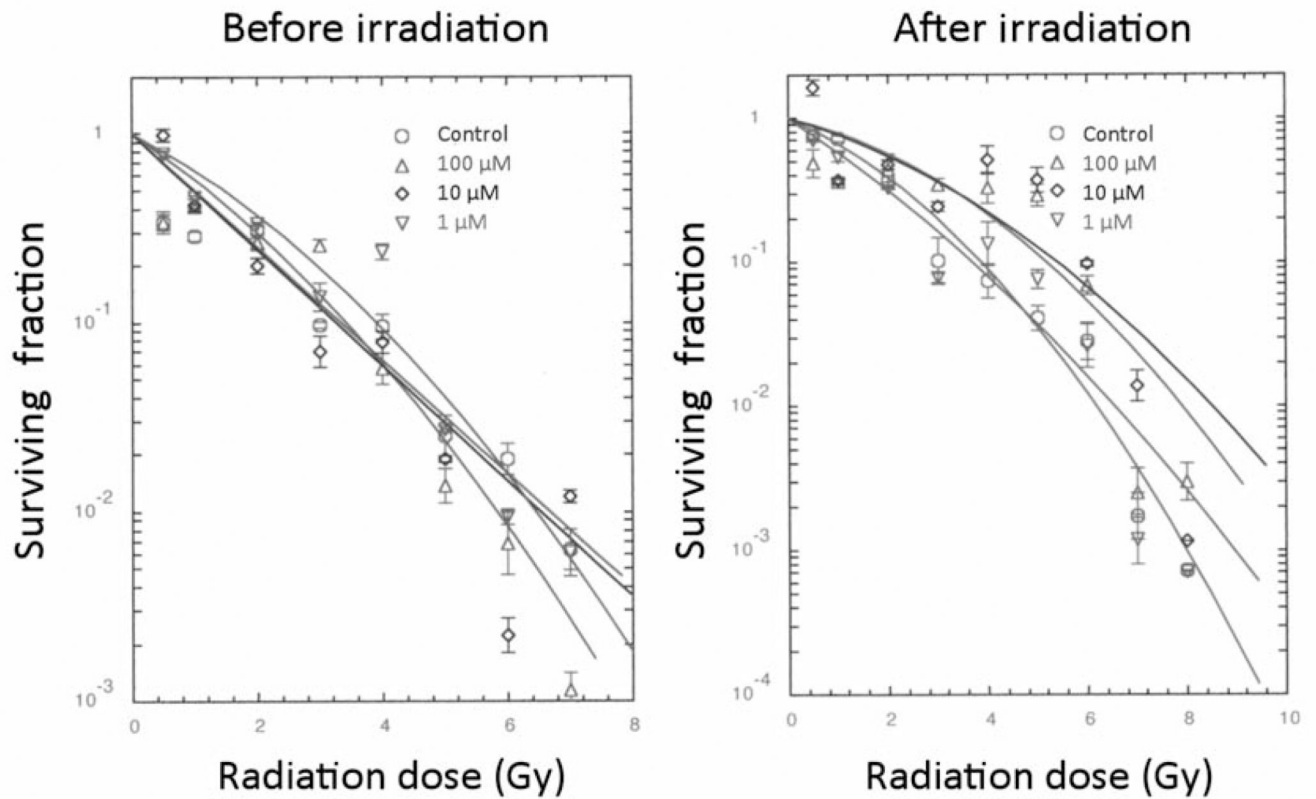
## Metronidazole (Flagyl)



**Figure 7.**

Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean  $\pm$  SEM of each agent at each concentration.

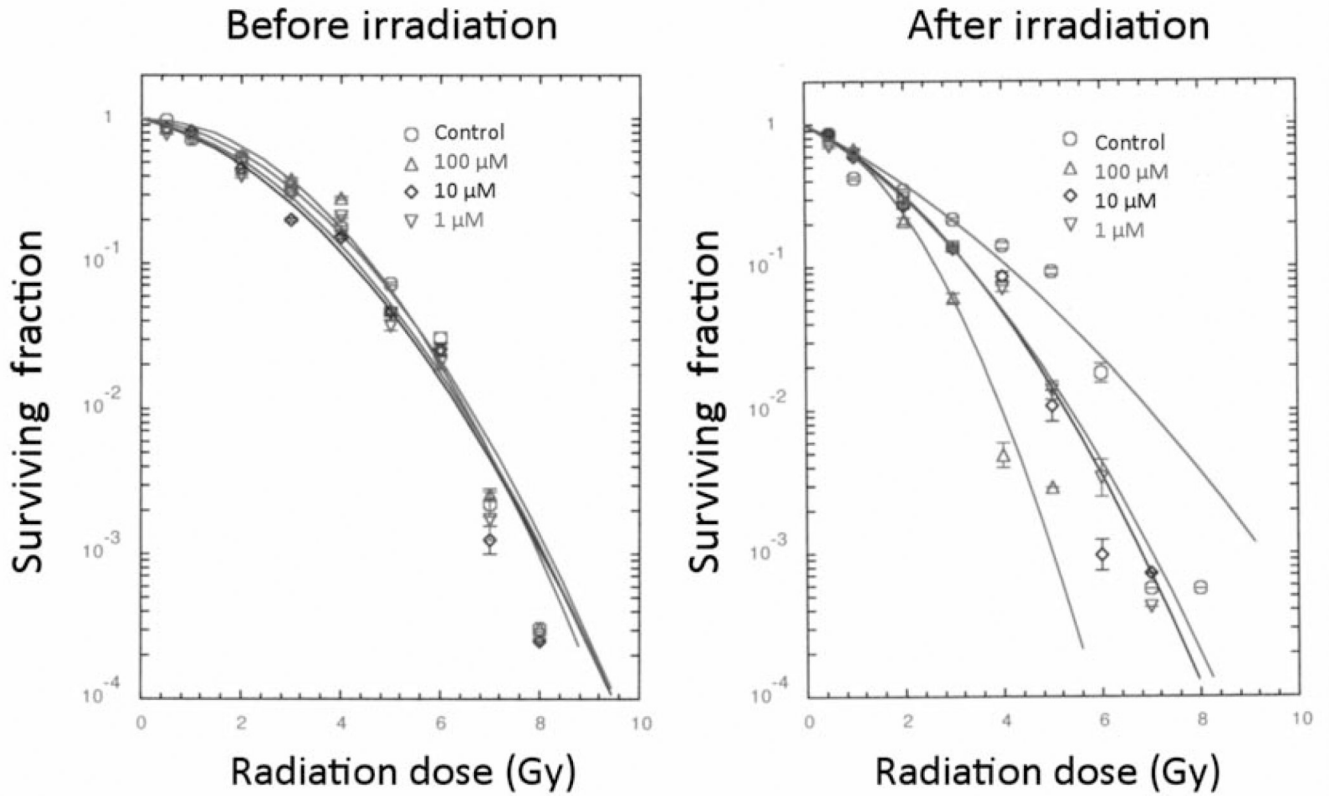
## Tetracycline



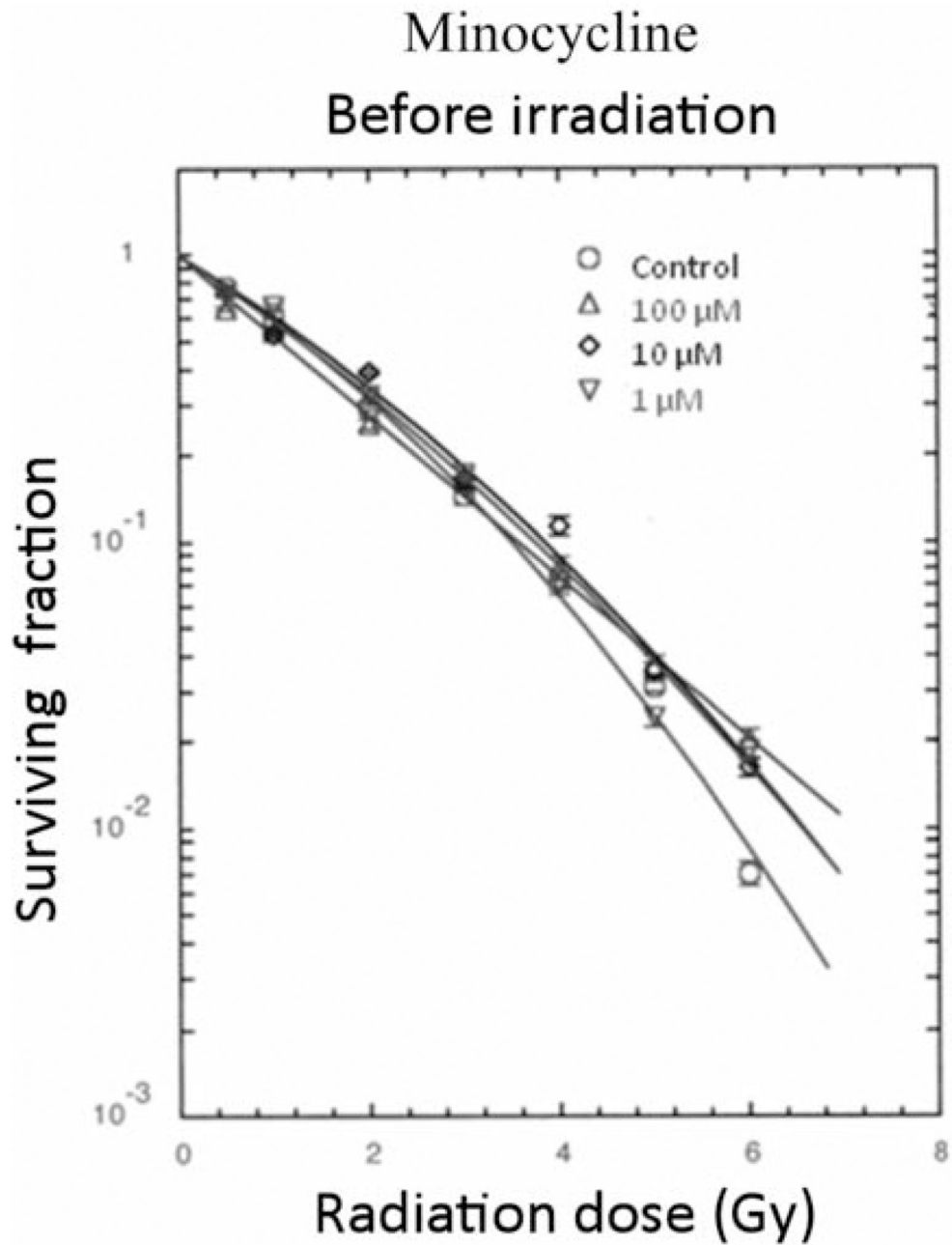
**Figure 8.** Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.



## Methacycline



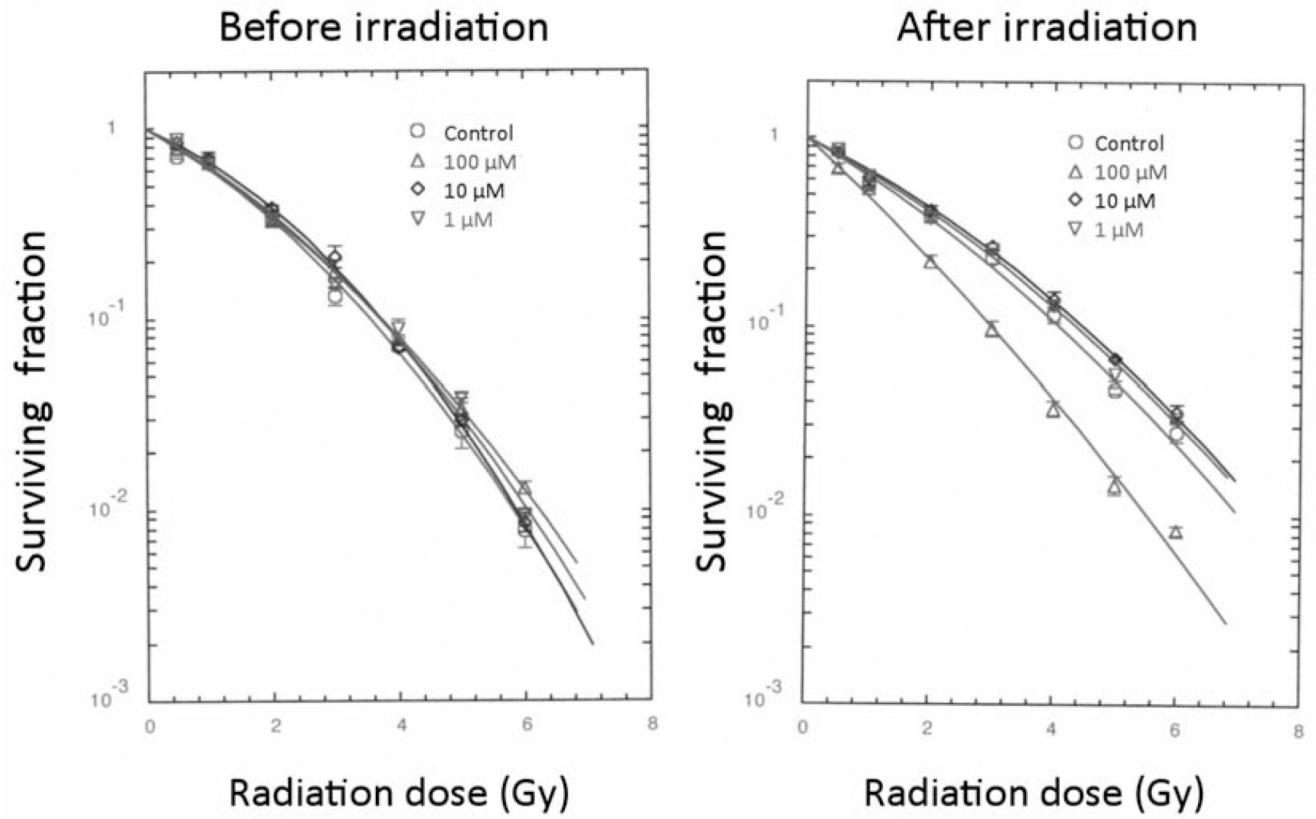
**Figure 9.** Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean  $\pm$  SEM of each agent at each concentration.



**Figure 10.**

Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.

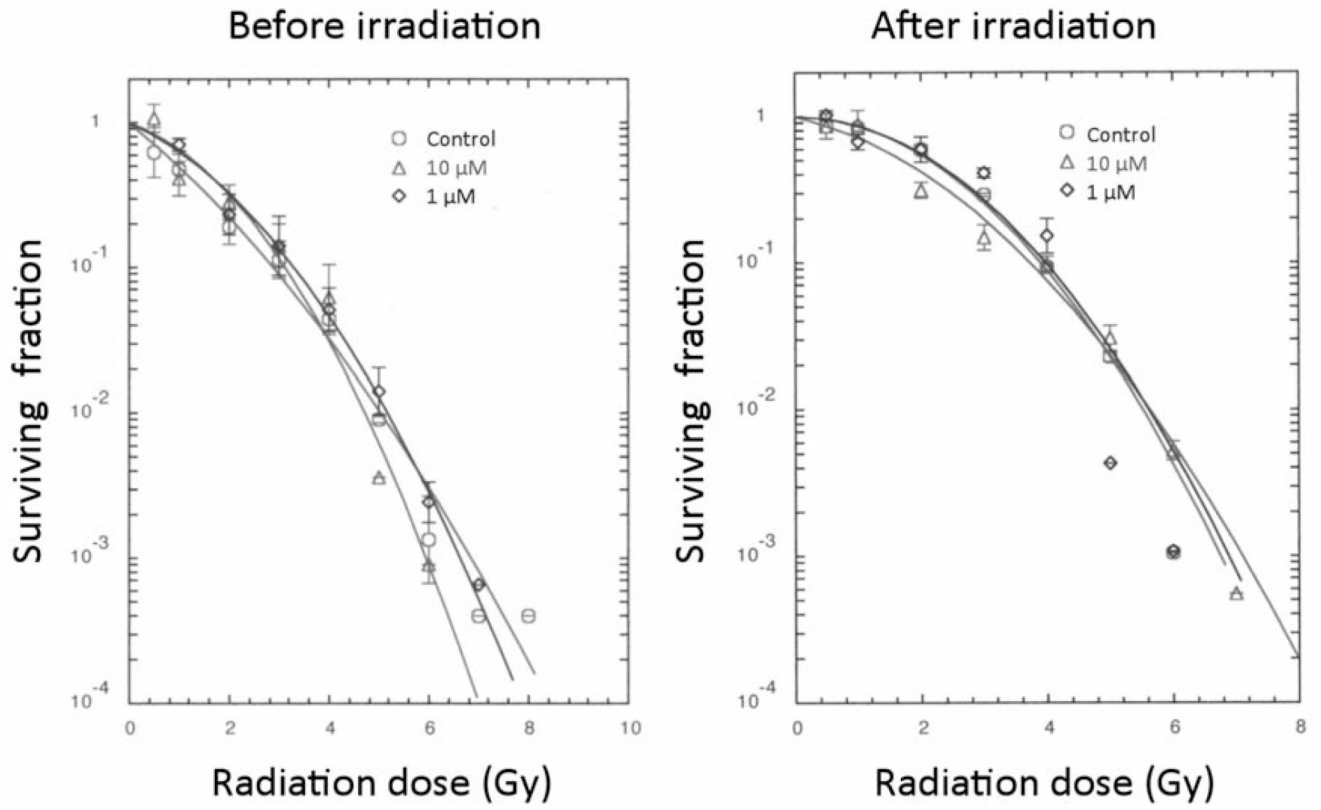
## Meclocycline



**Figure 11.**

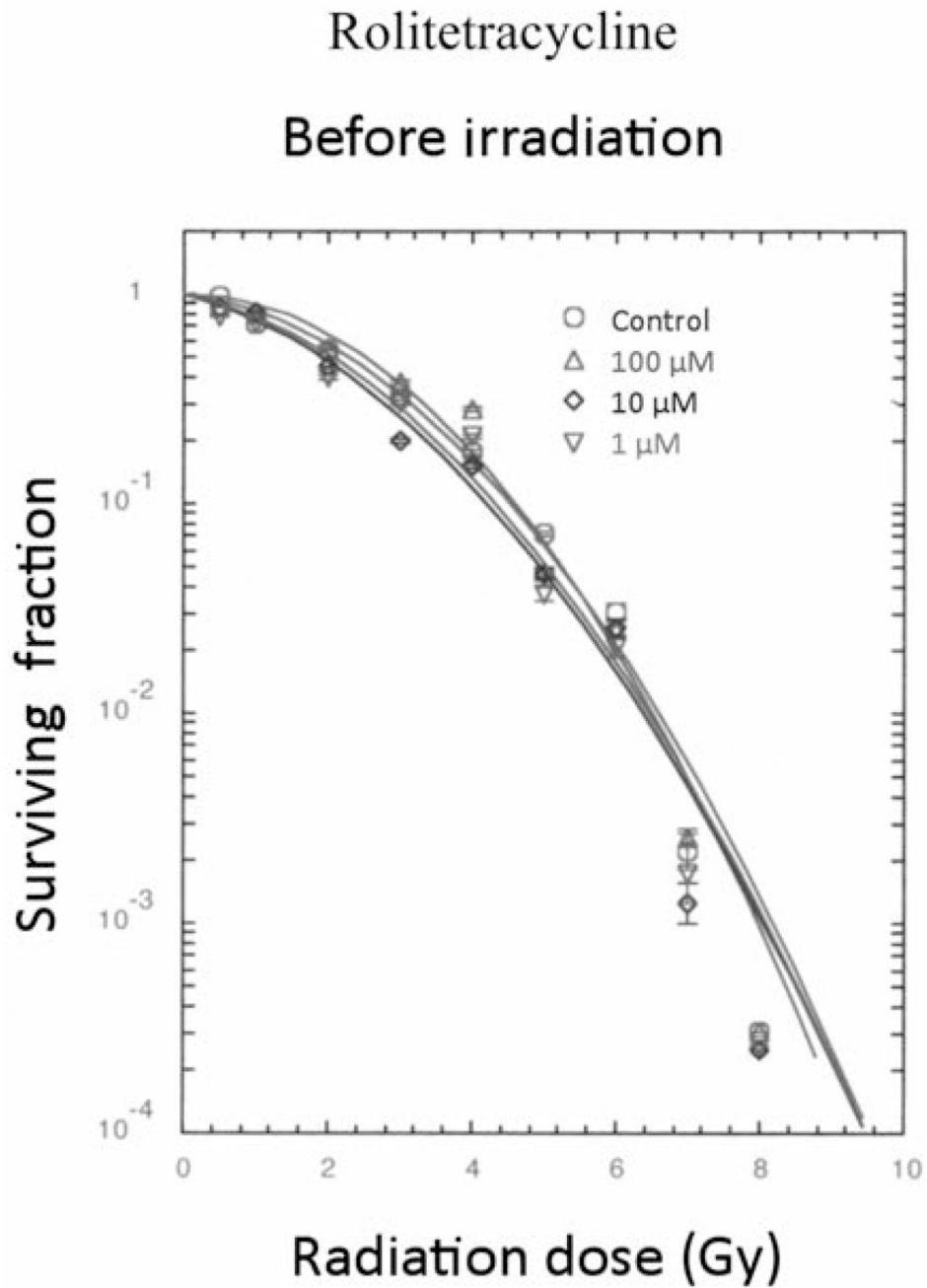
Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.

## Oxytetracycline



**Figure 12.**

Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean  $\pm$  SEM of each agent at each concentration.



**Figure 13.** Each drug was added before or after irradiation to 32D cl 3 cells as described in the Materials and Methods. Cells were plated and scored as described in the Materials and Methods. Results are the mean $\pm$ SEM of each agent at each concentration.

Radiation protection and/or damage mitigation by supportive care antimicrobials. 32D cl 3 cells were incubated in the presence of 1, 10 or 100  $\mu\text{M}$  of the antibiotics for 1 hour before irradiation or added to the cells after irradiation of 0 to 8 Gy. The cells were plated in methycellulose, incubated at 37°C for seven days at which time colonies of greater than 50 cells counted. Data was analyzed using linear quadratic and single-hit, multi-target models.-- Indicates that no colonies were detected at that concentration. All antibiotics were obtained from Sigma Chemical Company, St. Louis, MO.

**Table 1**

Antibiotic	Concentration ( $\mu\text{M}$ )	Before irradiation		After irradiation	
		$D_0$ (Gy)	$\bar{N}$	$D_0$ (Gy)	$\bar{N}$
None	0	1.0 $\pm$ 0.1	1.5 $\pm$ 0.4		
JP4-039	10	0.9 $\pm$ 0.1	9.4 $\pm$ 1.7	1.2 $\pm$ 0.1	5.8 $\pm$ 2.4
			( $p=0.0101$ )		( $p=0.009$ )
Cilastatin	100	1.0 $\pm$ 0.1	1.4 $\pm$ 0.3	1.5 $\pm$ 0.2	1.2 $\pm$ 0.1
	10	1.0 $\pm$ 0.1	1.3 $\pm$ 1.2	1.4 $\pm$ 0.2	1.6 $\pm$ 0.3
	1	0.9 $\pm$ 0.1	1.4 $\pm$ 0.3	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1
Amikacin	100	1.0 $\pm$ 0.1	2.0 $\pm$ 0.1	1.1 $\pm$ 0.1	4.1 $\pm$ 0.4
	10	0.8 $\pm$ 0.1	4.2 $\pm$ 1.6	1.2 $\pm$ 0.2	2.9 $\pm$ 1.9
	1	1.0 $\pm$ 0.1	2.5 $\pm$ 0.6	1.1 $\pm$ 0.1	3.4 $\pm$ 0.2
Ceftazidime	100	1.1 $\pm$ 0.1	2.5 $\pm$ 0.7	1.1 $\pm$ 0.1	5.6 $\pm$ 1.9
	10	1.2 $\pm$ 0.1	3.2 $\pm$ 1.1	1.1 $\pm$ 0.1	4.7 $\pm$ 1.6
	1	1.1 $\pm$ 0.2	4.7 $\pm$ 3.1	1.1 $\pm$ 0.1	3.5 $\pm$ 0.4
Vancomycin	100	1.2 $\pm$ 0.3	1.0 $\pm$ 0.1	1.7 $\pm$ 0.1	1.0 $\pm$ 0.1
	10	1.5 $\pm$ 0.3	1.0 $\pm$ 0.1	1.6 $\pm$ 0.2	1.0 $\pm$ 0.1
	1	1.3 $\pm$ 0.3	1.0 $\pm$ 0.1	2.1 $\pm$ 0.1	1.1 $\pm$ 0.1
Doxycycline	100	0.9 $\pm$ 0.1	2.3 $\pm$ 0.1	--	--
	10	0.9 $\pm$ 0.1	2.8 $\pm$ 0.1	1.0 $\pm$ 0.1	7.5 $\pm$ 3.7
	1	0.8 $\pm$ 0.1	2.9 $\pm$ 0.1	1.0 $\pm$ 0.1	4.4 $\pm$ 0.4
Ciprofloxacin	100	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1
	10	1.2 $\pm$ 0.1	1.0 $\pm$ 0.1	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1
	1	1.3 $\pm$ 0.1	1.1 $\pm$ 0.1	1.3 $\pm$ 0.1	1.5 $\pm$ 0.3
Metronidazole	100	1.1 $\pm$ 0.1	1.4 $\pm$ 0.3	1.3 $\pm$ 0.1	1.2 $\pm$ 0.2
	10	1.2 $\pm$ 0.1	1.3 $\pm$ 0.2	1.2 $\pm$ 0.1	1.1 $\pm$ 0.1
	1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.2

Antibiotic	Concentration ( $\mu$ M)	Before irradiation		After irradiation	
		$D_0$ (Gy)	$\tilde{N}$	$D_0$ (Gy)	$\tilde{N}$
Tetracycline	100	1.2 $\pm$ 0.3	1.2 $\pm$ 0.2	0.9 $\pm$ 0.1	13.9 $\pm$ 0.4
	10	1.2 $\pm$ 0.3	1.2 $\pm$ 1.1	0.9 $\pm$ 0.1	5.5 $\pm$ 4.5
Methacycline	1	1.3 $\pm$ 0.1	1.5 $\pm$ 0.5	1.1 $\pm$ 0.2	5.3 $\pm$ 2.9
	100	1.0 $\pm$ 0.1	2.9 $\pm$ 0.9	0.7 $\pm$ 0.1	4.4 $\pm$ 0.3
Minoocycline	10	1.0 $\pm$ 0.1	3.8 $\pm$ 0.2	1.2 $\pm$ 0.2	2.0 $\pm$ 0.5
	1	1.1 $\pm$ 0.1	3.5 $\pm$ 1.0	1.1 $\pm$ 0.1	2.6 $\pm$ 0.6
Meclocycline	100	1.2 $\pm$ 0.1	2.1 $\pm$ 0.9	--	--
	10	1.1 $\pm$ 0.1	5.5 $\pm$ 2.2	--	--
Oxytetracycline	1	1.1 $\pm$ 0.2	4.0 $\pm$ 2.1	--	--
	100	1.5 $\pm$ 0.2	1.7 $\pm$ 0.3	1.1 $\pm$ 0.1	1.3 $\pm$ 0.1
Rolitetracycline	10	1.3 $\pm$ 0.1	2.5 $\pm$ 0.4	1.4 $\pm$ 0.1	2.8 $\pm$ 1.0
	1	1.4 $\pm$ 0.1	1.8 $\pm$ 0.4	1.4 $\pm$ 0.1	3.1 $\pm$ 1.0
Meclocycline	100	--	--	--	--
	10	0.8 $\pm$ 0.1	7.0 $\pm$ 1.5	0.9 $\pm$ 0.1	8.3 $\pm$ 2.8
Meclocycline	1	0.9 $\pm$ 0.1	8.8 $\pm$ 2.7	1.0 $\pm$ 0.1	7.7 $\pm$ 4.2
	100	1.4 $\pm$ 0.1	1.1 $\pm$ 0.1	--	--
Meclocycline	10	1.3 $\pm$ 0.1	3.3 $\pm$ 1.1	--	--
	1	1.5 $\pm$ 0.2	2.4 $\pm$ 1.4	--	--

(p=0.0027)