in vivo *26*: 341-354 (2012)

Ionizing Irradiation Protection and Mitigation of Murine Cells by Carbamazepine is p53 and Autophagy Independent

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Abstract. *Background: Carbamazepine, a sodium channel blocker and pro-autophagy agent used in the treatment of epilepsy and trigeminal neuralgia, is also an ionizing radiation mitigator and protector. Materials and Methods: We measured the effect of carbamazepine, compared to other pro-autophagy drugs (i.e. lithium and valproic acid), on irradiation of autophagy incompetent ($Atg5^{-/-}$) and competent ($Atg5^{+/+}$) mouse embryonic fibroblasts, $p53^{-/-}$ and $p53^{+/+}$ bone marrow stromal cells, and human IB3, KM101, HeLa, and umbilical cord blood cell and in total body-irradiated or orthotopic tumor-bearing mice. Results: Carbamazepine, but not other pro-autophagy drugs, was a radiation protector and mitigator for mouse cell lines, independent of apoptosis, autophagy, p53, antioxidant store depletion, and class I phosphatidylinositol 3-kinase, but was ineffective with human cells. Carbamazepine was effective when delivered 24 hours before or 12 hours after total body irradiation of C57BL/6HNsd mice and did not protect orthotopic Lewis lung tumors. Conclusion: Carbamazepine is a murine radiation protector and mitigator.*

The recent discovery that drugs which promote autophagy, including carbamazepine, can clear misfolded proteins from the liver (1) led to the investigation of other functions of carbamazepine, one of which was recently reported to be its effect as an ionizing irradiation protector and mitigator *in vitro* and *in vivo* (2). Carbamazepine is utilized clinically for the treatment of bipolar disorder, trigeminal neuralgia, and epilepsy (3, 5-6). The relatively safe history of administration of carbamazepine to patients with a variety of medical conditions, despite rare complications (7-8) led us to consider its use for radiation protection in humans. We therefore investigated its radiobiologic mechanism of action. We reasoned that identifying the specific molecular target of carbamazepine in radioprotection might facilitate its development for use in normal tissue protection during clinical radiotherapy, as well as for irradiation counter-terrorism.

The most frequently discussed mechanism of action of carbamazepine is in its amelioration of neurologic pathology by inactivation of voltage-gated sodium channels (3). How this action would affect cellular radiobiology is not known. Secondly, by up-regulating autophagy, carbamazepine promotes clearance of misfolded protein aggregates in α-anti-trypsin-deficient mice (1). Carbamazepine and other mood stabilizing drugs, including lithium and valproic acid (VPA), may therefore promote autophagy by depletion of intracellular inositol (4-7). Phosphoinositide 3-kinase (PI3K) is an enzyme involved in the inositol cycle and the production of inositol triphosphate (IP_3), an important second messenger phospholipid that binds to IP_3 receptors in the endoplasmic reticulum, releasing intracellular calcium stores, regulating both cell proliferation, and autophagy (9-11). Through a calcium surge regulated by IP_3, apoptosis might be induced directly or indirectly (12) and therefore, by promoting autophagy, carbamazepine might reduce irradiation-induced apoptosis (13). Thirdly, since carbamazepine can deplete antioxidant levels (14) and may increase levels of radical oxygen species (ROS) (15), neither of which facilitate radioprotection (16), a rebound increase in antioxidants might be the explanation for its radiobiologic action.

We evaluated the effects of carbamazepine on radiation-induced cell death pathways that are associated with autophagy by utilizing autophagy incompetent $Atg5^{-/-}$ and

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Key Words: Radioprotection, radiation mitigation, autophagy, p53, carbamazepine, murine hematopoietic progenitor cells, bone marrow stromal cells, Lewis lung carcinoma cells.

control Atg5$^{+/+}$ mouse embryonic fibroblast (MEF) cell lines (generously provided by Dr. Noboro Mizushima of Tokyo Medical and Dental University) (25). Other autophagy-promoting agents, including VPA and lithium chloride, were compared with carbamazepine. Since sodium channel inhibition by carbamazepine might alter intracellular p53, an important molecule in the DNA damage response to irradiation (17), we tested the effect of carbamazepine on the radiobiology of p53$^{-/-}$ compared to p53$^{+/+}$ cell lines. Inhibitory complexes of p53 with B-cell lymphoma extra large (BclXL) and B-cell lymphoma 2 (Bcl2) may alter the mitochondria permeability, inducing cytochrome c release and apoptosis (18). Since p53 induces autophagy in response to DNA damage in a Damage-Regulated Autophagy Modulator (DRAM)-dependent manner (19), this action may be protective against radiation damage (20), and p53$^{-/-}$ cells would not exhibit the carbamazepine effects.

We also tested the effects of carbamazepine as a radiation protector in mice with orthotopic tumors to determine if therapeutic irradiation was also mitigated by the drug. Finally, to be assured of translation of the findings to human cells, we tested carbamazepine as a radioprotector or mitigator in human cell lines and fresh umbilical cord blood hematopoietic progenitors.

Materials and Methods

Cell culture. Murine hematopoietic progenitor cells (32Dcl3) (21, 22), murine p53$^{+/+}$ and p53$^{-/-}$ bone marrow stromal cells (23), 3LL Lewis Lung Carcinoma cells (24), and Atg5$^{+/+}$ Atg5$^{-/-}$ MEF cells (25) were cultured according to published methods. Briefly, 32Dcl3 cells were passaged in Iscove's modified medium supplemented with 15% conditioned medium from Walter and Elizabeth Hall Institue-3 cells (WEHI-3) as a source of interleukin 3 (IL-3), 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA), 1% L-glutamine (GIBCO, Gaithersburg, MD, USA) and 1% penicillin-streptomycin (P/S) (GIBCO). Murine bone marrow stromal cell lines (p53$^{+/+}$ and p53$^{-/-}$), 3LL cells, and MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD, USA) supplemented with 10% FBS, 1% L-glutamine and 1% P/S. Culture conditions for the human cell lines HeLa, IB3 (26) and KM101 (27) have been reported and were grown in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% P/S. Human umbilical cord blood cells were cultured and analyzed for CFU-GEMM multilineage colonies as published elsewhere (28).

In vitro irradiation experiments. Carbamazepine (Sigma Chemical Company, St. Louis, MO, USA) was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). Lithium chloride and VPA (Sigma Chemical Company) were prepared as 1 mM stock solutions in water. Cells were suspended at 1x10^6 cells/ml and irradiated with 0 to 8 Gy using a Shepherd Mark 1 irradiator with a cesium source (J.L. Shepherd, San Fernando, CA, USA). Carbamazepine was added at a final concentration of 10 μM (2) for one hour before or immediately after irradiation to murine Atg5-proficient and -deficient, murine p53-proficient and -deficient, and 3LL cells. With human IB3, HeLa, and KM101cells, 50 μM of carbamazepine was used. Lithium chloride or VPA was added to 32Dcl3 cell cultures at a final concentration of 0, 1 or 10 μM for one hour before or immediately after irradiation. 32Dcl3 cells were plated in triplicate in methylcellulose as previously described and incubated at 37˚C with 5% CO_2 for 7-14 days then colonies of >50 cells were counted (29). Adherent cells were plated in quadruplicate in 4-well Linbro plates (MP Biomedicals, LLC, Salon, OH, USA), incubated for 7 to 14 days at 37˚C with 5% CO_2, stained with crystal violet and colonies greater than 50 cells were counted with a colony counter (Oxford Optronix, Oxford, UK). Irradiated human umbilical cord blood mononuclear cells (MNC) were plated in triplicate in methylcellulose supplemented with recombinant human stem cell factor (rh SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL 3 and erythropoietin (Stemcell Technologies, Vancouver, Canada). Colony-forming unit-granulocyte macrophage (CFU-GM), burst-forming unit erythroid (BFU-E), and colony-forming unit-granulocyte-erythroid-megakaryocyte-monocytes (CFU-GEMM) were scored on day 14. The radiosensitivity of human cord blood progenitor cells was measured according to published methods (30).

Immunoblot. Autophagy was assayed by immunoblot for microtubule-associated protein light chain 3 (LC3) as described previously (2). Briefly, Atg5$^{+/+}$ and Atg5$^{-/-}$ MEF cells were harvested and lysed in NP-40 buffer [50 mM Tris, pH 7.8, 10 mM ethylenediaminetetaacetic acid (EDTA), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP-40 and a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA)]. Protein samples were separated in 15% polyacrylamide gels by electrophoresis. Primary LC3 (Novus Biologicals, Littleton, CO, USA) or α-tubulin (Sigma Aldrich, St. Louis, MO, USA) antibody were used. Horseradish peroxidase anti-rabbit or anti-mouse secondary antibody (Promega, Pittsburgh, PA, USA) was applied and membranes developed with Super Signal West Dura ECL (Thermo Scientific, Rockford, IL, USA).

Immunofluorescent staining of autophagic vacuoles in Atg5 $^{-/-}$ and Atg5$^{+/+}$ cell lines. Atg5$^{+/+}$ and Atg5$^{-/-}$ cells were grown on glass coverslips in the presence or absence of 50 μM carbamazepine or 50 μM chloroquine (InvivoGen, San Diego, CA, USA) for 16 or 24 hours. Paraformaldehyde-fixed cells were stained with a rabbit polyclonal primary antibody against LC3II, AlexaFluor 488 secondary antibody and AlexaFluor 568 phalloidin (Invitrogen, Gaithersburg, MD, USA).

Lewis lung carcinoma (3LL) orthotopic tumor model. A total of 1×10^6 3LL cells were injected subcutaneously into the left hind limbs of C57BL/6NTac 6 week old female mice (20-22 grams in weight) (Taconic Farms, Inc., Hudson, NY, USA). One week after injection, mice received an intra-peritoneal (*i.p.*) injection of 10 mg/kg carbamazepine in Cremphor-EL (29) prior to or immediately after 20 Gy irradiation to the tumor-containing leg using a LINAC (Varian Medical Systems, Palo Alto, CA, USA). Tumor diameter was monitored with caliper measurement.

Apoptosis and mitochondria permeability. Cells from the IL3-dependent hematopoietic progenitor cell line 32Dcl3 (29) were incubated with 10 μM carbamazepine for one hour before or after irradiation with 5 or 10 Gy. Cells were harvested 48 hours after irradiation and apoptosis and mitochondrial membrane depolarization

were quantified by commercial TUNEL stain (Promega, Madison, WI, USA) and JC1 (Immunochemistry Technologies, Bloomington, MN, USA) kits, respectively. Cell viability was calculated using an automated cell counter (Oxford Optronix, Milton Park, Oxford, UK). As a positive control, 32Dcl3 cells were grown in the absence of IL3, the deprivation of which induces apoptosis.

Antioxidant assay. Cells from the MEF cell lines $Atg5^{+/+}$ and $Atg5^{-/-}$ were incubated with 10 μM carbamazepine for one hour prior to 6 Gy ionizing radiation. Cells were harvested after 10, 30, 60, 90 and 120 minutes and snap-frozen in liquid nitrogen. Cell pellets were then thawed and mechanically homogenized in cold phosphate buffer solution. Protein concentrations were standardized by Bradford assay and antioxidant levels measured using a commercial kit (Northwest Life Science Specialties, Vancouver, WA, USA).

Class I PI3K assay. The *in vitro* effect of carbamazepine on class I PI3K activity was measured by use of a commercial ELISA kit (Echelon Biosciences Inc., Salt Lake City, UT, USA). Each reaction mixture contained 0.025 ng/μl class I PI3K enzyme (Echelon Biosciences Inc.) and was incubated at 37˚C for 1.5 hours in the presence of different concentrations (6.3-200 μM) of the control inhibitor LY-294,002 (Enzo Life Sciences Inc., Farmingdale, NY, USA) or carbamazepine. PI3K activity was quantified by phosphatidylinositol 3,4,5-trisphosphate (PIP_3) production and the resultant absorbance change at 450 nm.

Statistics. The *in vitro* radiation survival curves were analyzed with the linear-quadratic model and the single-hit multi-target model, and were compared using the final slope representing multiple-event killing (D_0) and the extrapolation number measuring the width of the shoulder on the radiation survival curve (ñ) (29, 31). Results for D_0 and ñ are presented as the mean±standard error of the mean (SEM) from multiple measurements. The two-sided two sample *t*-test was used to compare means of different groups.

The tumor volume data are summarized as the mean±standard deviation for each of the four treatment groups (namely, 0 Gy, 20 Gy, carbamazepine before 20 Gy irradiation and carbamazepine after 20 Gy irradiation) at each day of measurement. Linear mixed models were built for the log-transformed tumor volume where group and day of measurement, as well as their interaction, were used as fixed explanatory variables, and day of measurement, the within subject variable, was used as a repeated measure. The F-test was used to examine the significance of interaction between group and day of measurement. A significant result indicates a significant difference in tumor growth rate between groups. For the *in vivo* mouse survival data, the two-sided log-rank test was used to compare each treatment group with the radiation only control group. For all these analyses, *p*-values less than 0.05 were interpreted as being significant.

Results

The autophagy-promoting drugs lithium chloride and VPA are not radiation dose modifiers in vitro. To test whether other autophagy-promoting drugs with similar clinical uses and effects on the inositol pathway were radiation protectors/mitigators, we tested lithium chloride and VPA in radiation clonogenic assays using 32Dcl3 cells. Lithium chloride or VPA added at 1 mM or 10 mM before or after irradiation did not change the ñ or D_0 (Table I). Thus, unlike carbamazepine, neither lithium chloride nor VPA were radiation protectors or mitigators for 32Dcl3 cells (2). We next tested the autophagy dependence of CBZ radioprotection and mitigation.

Table I. *Effects of lithium chloride and valproic acid (VPA) on 32Dcl3 cell clonogenic radiation survival curves.*

Lithium (mM)	Pre-irradiation		Post-irradiation	
	Do (Gy)	ñ	Do (Gy)	ñ
0	1.3±0.1	1.3±0.3	1.3±0.1	1.3±0.3
1	1.3±0.1 (p=0.8340)	1.0±0.1 (p=0.3465)	1.5±0.1 (p=0.7415)	1.0±0.1 (p=1.000)
10	1.5±0.1 (p=0.1841)	1.1±0.1 (p=0.2508)	1.7±0.1 (p=0.1145)	1.0±0.1 (p=0.7888)
VPA (mM)				
0	1.5±0.1	1.0±0.1	1.5±0.1	1.0±0.1
1	1.6±0.1 (p=0.6037)	1.5±0.5 (p=0.2529)	1.2±0.1 (p=0.0602)	1.5±0.5 (p=0.3122)
10	1.3±0.1 (p=0.4431)	1.1±0.1 (p=0.3801)	1.4±0.1 (p=0.7159)	1.3±0.3 (p=0.1527)

Four-well plates with 500 or 1000 cells per plate were scored at day 7 for colonies of ≥50 cells as described in the Materials and Methods. The *p*-values compare cell survival with lithium and VPA to that of non-drug treated irradiated cells using the single-hit, multi-target model.

Radiation protection and mitigation by carbamazepine is autophagy independent. Western blot analysis of LC3 was first performed to confirm that $Atg5^{-/-}$ MEF cells were autophagy deficient. The absence of the LC3II band in control or carbamazepine-treated $Atg5^{-/-}$ cells and the absence of autophagosomes, the vacuoles necessary for autophagy (25), in control as well as in irradiation-, chloroquine-, or in carbamazepine-treated cells, confirmed that $Atg5^{-/-}$ cells were autophagy deficient (Figures 1 and 2).

To determine whether carbamazepine protected and mitigated ionizing radiation damage in autophagy-deficient cells, $Atg5^{-/-}$ MEF cells were incubated with 50 μM CBZ for one hour before or immediately after irradiation. Autophagy-deficient MEF cells supplemented with 50 μM CBZ before irradiation had an increase in D_0 from 1.53±0.05 to 1.99±0.05 (p=0.0028). Wild-type MEF cells had a similar increase in D_0 from 1.67±0.09 to 2.90±0.35 (p=0.0260) (Table II, Figure 3A and B).

We next evaluated the effects of carbamazepine on class I PI3K, an enzyme which inhibits autophagy and participates in the cellular inositol cycle (32, 33). We reasoned that if carbamazepine inhibited class I PI3K then autophagy would be up-regulated. Inhibition of PI3K might prevent production of IP_3, calcium release, and cell death (34). To

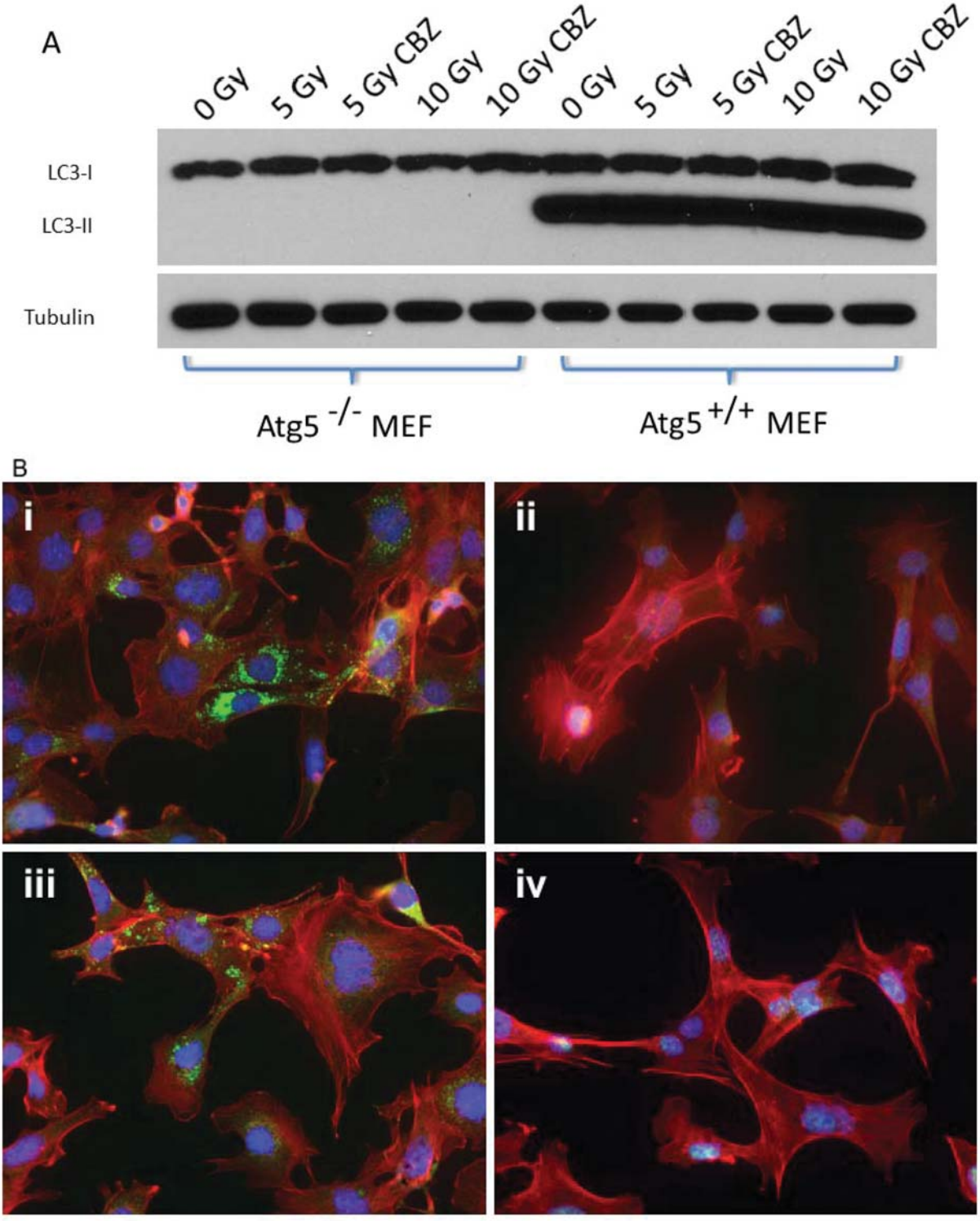

Figure 1. *Atg5$^{-/-}$ mouse embryonic fibroblast (MEF) cells exhibit markers of autophagy deficiency. A: Immunoblot for microtubule associated light chain-3 type II (LC3II). Cells were treated with 5 or 10 μM carbamazepine (CBZ) and/or irradiated to 5 or 10 Gy to induce autophagy. There was a lack of LC3II in Atg5$^{-/-}$ cells B: LC3II staining of Atg5$^{+/+}$ and Atg5$^{-/-}$ cells. Atg5$^{+/+}$ and Atg5$^{-/-}$ cells were incubated with 50 mM CBZ 1 h prior to or post 7 Gy irradiation, then stained for LC3II to identify the induction of autophagic vacuoles by irradiation. CBZ prior to 7 Gy induced autophagic vacuoles in Atg5$^{+/+}$ cells (i) but not Atg5$^{-/-}$ cells (ii). Similar induction was evident in Atg5$^{+/+}$ cells treated with 50 mM CBZ post 7 Gy (iii), but not with Atg5$^{-/-}$ cells (iv). Original magnification ×40.*

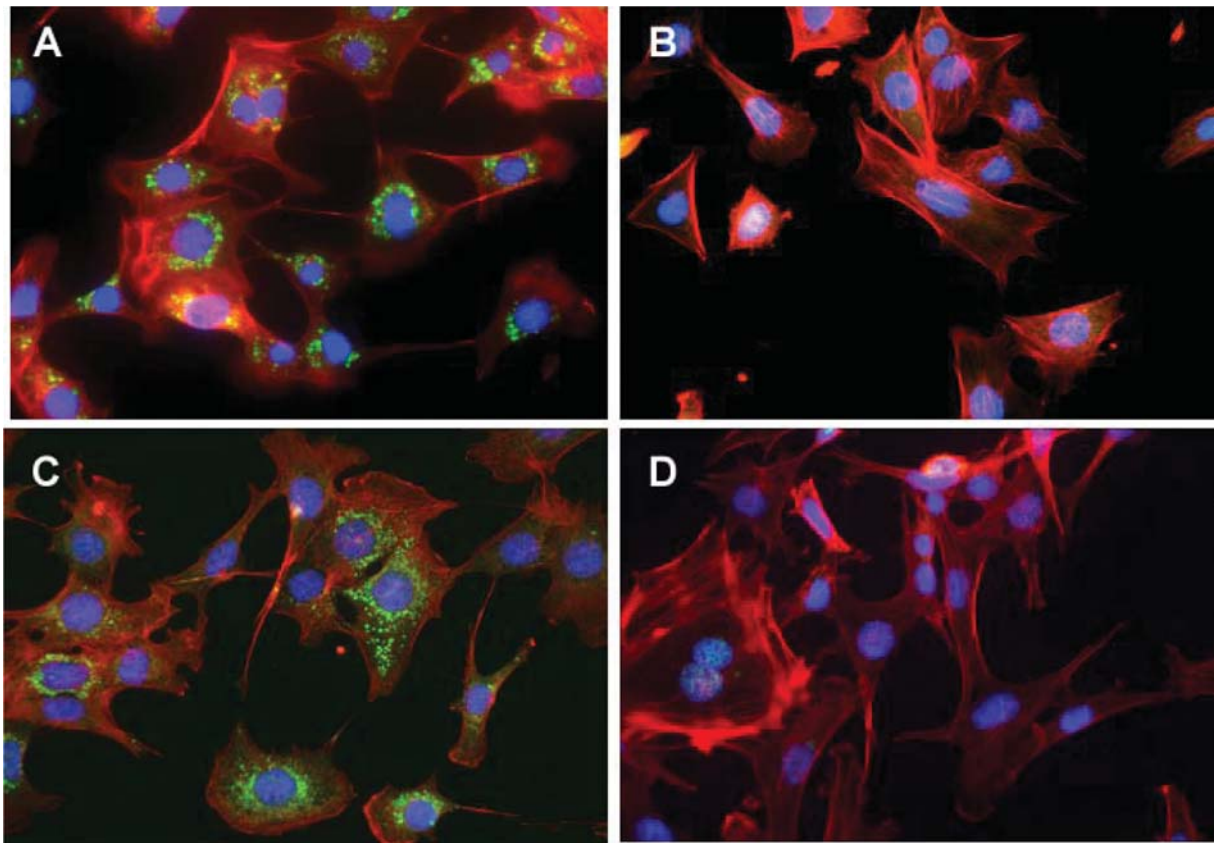

Figure 2. *Effect of chloroquine diphosphate and carbamazepine (CBZ) on induction of autophagic vacuoles in Atg5$^{+/+}$ and Atg5$^{-/-}$ cell lines. Atg5$^{+/+}$ and Atg5$^{-/-}$ cells were incubated with 50 μM chloroquine for 16 hours or 50 μM CBZ for 24 hours, then stained for LC3II to identify the presence of autophagic vacuoles. Chloroquine induced autophagic vacuoles in Atg5$^{+/+}$ cells (A) but not in Atg5$^{-/-}$ cells (B). Induction of autophagic vacuoles was evident in Atg5$^{+/+}$ cells treated with 50 mM CBZ for 24 hours (C) but not in Atg5$^{-/-}$ cells (D). Original magnification ×40.*

Table II. *Effect of carbamazepine (CBZ) on the radiosensitivity of Atg5$^{+/+}$ and Atg5$^{-/-}$ mouse embryonic fibroblast cell lines.*

Experimental conditions	Pre or post irradiation	Cell type			
		ATG5$^{+/+}$		ATG5$^{-/-}$	
		D_0	ñ	D_0	ñ
Cells only		1.67±0.09 (n=3)	9.76±0.84 (n=3)	1.53±0.05 (n=3)	7.43±2.70 (n=3)
DMSO	Pre	1.80±0.31 (n=3)	7.0±0.30 (n=3)	1.72±0.02 (n=3)	7.39±3.48 (n=3)
		p1=0.6965	p1=0.0964	p1=0.0622	p1=0.9916
	Post	2.01±0.25 (n=3)	9.20±3.53 (n=3)	1.69±0.01 (n=3)	7.61±4.32 (n=3)
		p1=0.2794	p1=0.8673	p1=0.0932	p1=0.9723
CBZ	Pre	2.90±0.35 (n=3)	5.00±2.15 (n=3)	1.99±0.05 (n=3)	4.02±1.07 (n=3)
		p1=0.0260	p1=0.0687	**p1=0.0028**	p1=0.3089
		p2=0.0759	p2=0.5245	**p2=0.0246**	p2=0.3342
	Post	1.49±0.08 (n=3)	15.15±1.71 (n=3)	1.64±0.19 (n=3)	13.23±7.74 (n=3)
		p1=0.2794	**p1=0.0299**	p1=0.6404	p1=0.2622
		p3=0.1386	p3=0.5245	p3=0.8533	p3=0.4125

Clonogenic survival curves were determined as described in the Materials and Methods. Data are summarized as the mean±SEM. *P*-values were calculated with the two-sided two-sample *t*-test, where p1 is the *p*-value for comparison with the control cells only, p2 is for the comparison with the pre-irradiation dimethyl sulfoxide (DMSO) group, and p3 is for the comparison with the post-irradiation DMSO group. Significant *p*-values are written in bold.

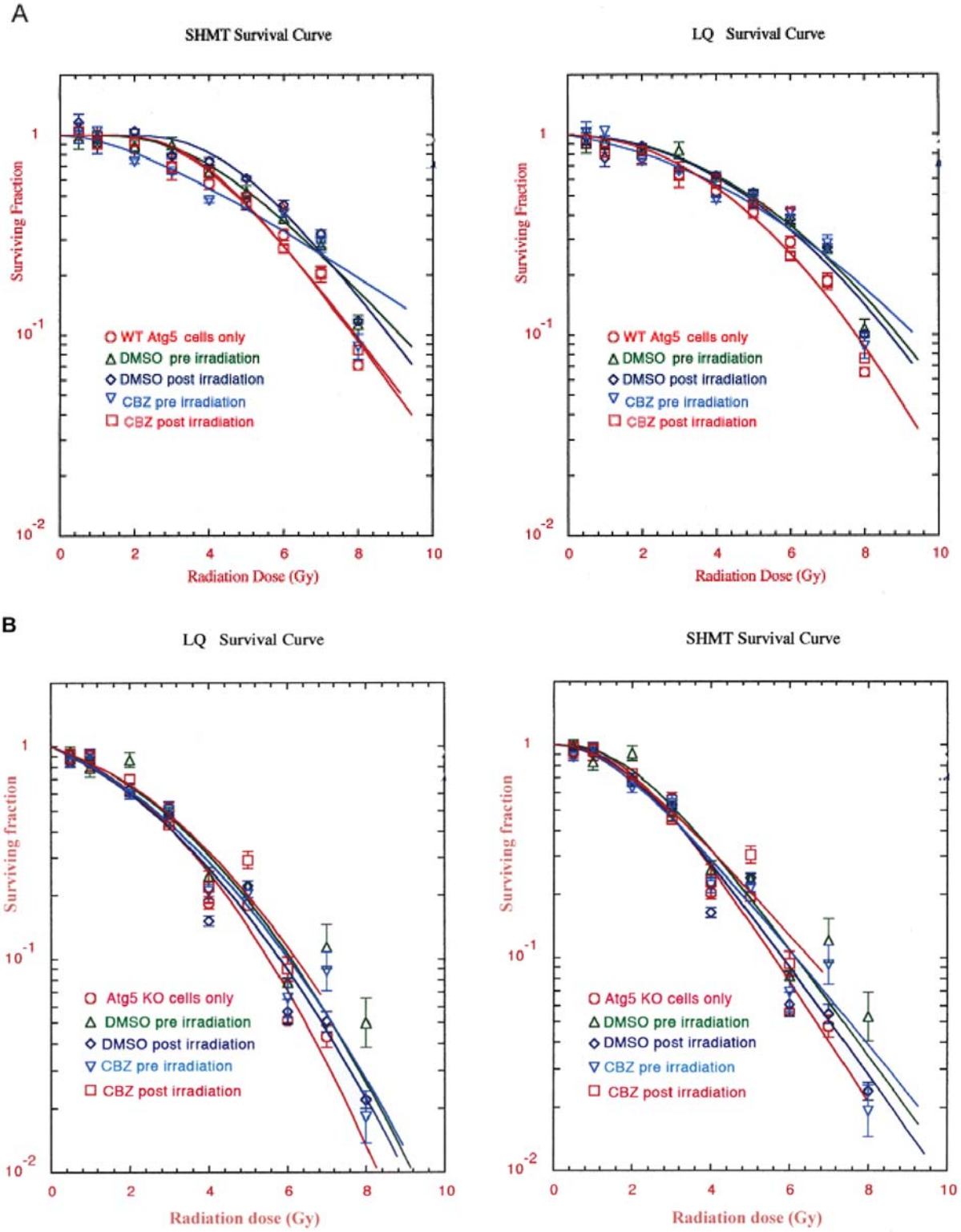

Figure 3. *Effect of (50 μM) carbamazepine (CBZ) treatment pre- and post-irradiation on Atg5$^{+/+}$ and Atg5$^{-/-}$ cells. Cells from cell lines Atg5$^{+/+}$ (WT)(A) and Atg5$^{-/-}$ (KO)(B) were tested. MEF cells were irradiated to doses from 0 to 8 Gy then plated in clonogenic assay and colonies greater than 50 cells were scored at 7 days. CBZ added before irradiation or after irradiation protected and mitigated against radiation damage in both the autophagy-proficient and -deficient lines. CBZ added pre or post-irradiation to Atg5$^{-/-}$ clone 4 (KO) cells protected and mitigated against irradiation damage. Data are presented in single-hit multi-target and linear quadratic format.*

Table III. *Effects of carbamazepine (CBZ) on class I PI3K enzyme activity in 32Dcl3 cells in vitro.*

LY-294,002	PIP_3	CBZ	PIP_3
200 μM	1.2	1000 μM	9.5
100 μM	1.8	250 μM	25
50 μM	1.5	62.5 μM	25
25 μM	2	15.6 μM	28
12.5 μM	4	3.9 μM	28
6.3 μM	6	1 μM	25
0 μM	22	0 μM	25

Class I PI3K activity was measured in the presence of serial fold dilutions of control inhibitor LY-294,002 and CBZ. The amount of phosphatidylinositol 3,4,5-triphosphate (PIP3) produced is proportionate to the enzyme activity.

Table IV. *Effects of carbamazepine (CBZ) on 32Dcl3 cell line cell viability, mitochondrial membrane depolarization and apoptosis.*

Condition	Viability (%)	Mitochondrial membrane depolarization (%)	Apoptosis (%)
Control	95.5±1.7	11.5±2.1	3.2±0.7
Without IL3	35.0±7.2	55.2±6.5	76.1±6.6
5 Gy	74.9±2.0	43.6±6.7	23.2±3.0
CBZ + 5 Gy	78.3±2.5	38.7±5.4	24.7±3.7
	(p=0.3503$^{\#}$)	(p=0.6093$^{\#}$)	(p=0.8250$^{\#}$)
5 Gy + CBZ	76.7±2.7	45.5±3.7	25.4±3.1
	(p=0.6362$^{\#}$)	(p=0.8013$^{\#}$)	(p=0.7330$^{\#}$)
10 Gy	64.1±2.6	58.6±5.7	46.0±4.6
CBZ + 10 Gy	61.2±3.2	57.7±6.3	41.9±3.9
	(p=0.5233*)	(p=0.9208*)	(p=0.5274*)
10 Gy + CBZ	61.6±2.2	51.9±6.9	44.1±3.7
	(p=0.4899*)	(p=0.5333*)	(p=0.7597*)

$^{\#}p$-value *vs*. 5 Gy; *p-value *vs*. 10 Gy. Viability, mitochondrial depolarization and apoptosis were determined on 32Dcl3 cells treated with 10 μM CBZ either 1 hour before irradiation or added to the media after either 5 or 10 Gy irradiation. As a positive control, 32Dcl3 cells were grown in the absence of IL3, a condition which induces apoptosis. Cells were assayed 48 hours after either irradiation or removal of IL3. Viability was determined by trypan blue exclusion; mitochondrial membrane depolarization was determined using a MitoPT-JC1 Assay kit; and apoptosis using a TUNEL kit.

determine whether carbamazepine had an effect on IP_3 levels, class I PI3K enzyme was incubated with carbamazepine and combined with PIP3 substrate. As a positive control LY-294,002 was added. With 32Dcl3 cells, there was nonspecific inhibition of the PI3K enzyme at 1000 μM carbamazepine but no inhibition at 250 μM or lower concentrations (Table III). The positive control PI3K inhibitor, LY-294,002, did inhibit PI3K activity at concentrations as low as 6.3 μM (Table III). Thus, the data indicated that carbamazepine did not directly inhibit class I PI3K in 32Dcl3 cells. We cannot rule out possible carbamazepine interaction with a modulator of PI3K.

Carbamazepine induces the formation of autophagic vacuoles in Atg5$^{+/+}$ cells but not in Atg5$^{-/-}$ cells. To test whether carbamazepine induced the formation of autophagic vacuoles in Atg5$^{+/+}$ and autophagy-deficient Atg5$^{-/-}$ cells, we treated the cells with carbamazepine, pre and post 7 Gy irradiation, and used chloroquine diphosphate, which induces autophagosome production, as a positive control. Cells were stained for LC3II, which is a general marker for autophagic vacuoles. Atg5$^{+/+}$ and Atg5$^{-/-}$ cells were incubated with 50 mM CBZ for one hour prior to or after 7 Gy irradiation. Cells were stained for LC3II to identify the presence of autophagic vacuoles induced by irradiation compared to chloroquine diphosphate or carbamazepine. Carbamazepine at 50 mM for one hour before 7 Gy irradiation induced the formation of autophagic vacuoles in Atg5$^{+/+}$ cells but not in Atg5$^{-/-}$ cells (Figure 1B). Similar induction was detected in Atg5$^{+/+}$ cells treated with 50 mM carbamazepine after 7 Gy irradiation. Vacuoles were not detected in Atg5$^{-/-}$ cells (Figure 1B). When Atg5$^{+/+}$ and Atg5$^{-/-}$ cells were incubated with 50 μM chloroquine for 16 hours, and were then stained for LC3II, autophagic vacuoles were detected in Atg5$^{+/+}$ but in not Atg5$^{-/-}$ cells (Figure 2A and B). When cells were treated with 50 μM carbamazepine for 24 hours prior to staining for LC3II, autophagic vacuoles were detected with Atg5$^{+/+}$, but not with Atg5$^{-/-}$ cells (Figure 2C and D). The data support the studies with PI3K and Atg5$^{-/-}$ cells, and indicate that carbamazepine acts as a radiation protector and mitigator independent of autophagy.

Carbamazepine does not alter mitochondrial permeability or prevent apoptosis. We next used Mito PT-JC1 and TUNEL staining to determine whether carbamazepine altered mitochondrial permeability and/or prevented apoptosis in irradiated cells. The percentage of cells with depolarized mitochondrial membrane after 5 Gy or 10 Gy irradiation did not change significantly when carbamazepine was added before or after irradiation (Table IV). Furthermore, the percentage of apoptotic cells after 5 or 10 Gy irradiation did not change significantly if carbamazepine was added before or after irradiation (Table IV). The viability of cells at 24 hours after irradiation was unchanged between the drug-treated and the irradiation-treated control group. Thus, carbamazepine did not alter irradiation-induced apoptosis in 32Dcl3 cells at 24 hours after irradiation and did not significantly alter mitochondrial membrane permeability. These results establish that the effect of carbamazepine on irradiated cells *in vitro* was mediated by events occurring after the first cell division not measurable by assays for apoptosis.

Table V. *Effect of carbamazepine (CBZ) as a radiation protector in $p53^{-/-}$ compared to $p53^{+/+}$ cell lines.*

Cell line	CBZ concentration (μM)	D_0 (Gy)	ñ
$p53^{-/-}$	0	3.9±0.8	1.8±0.4
	1	2.0±0.1	6.0±0.6 (p=0.0018)
	10	3.3±0.6	3.7±1.9 (p=0.3286)
	100	3.1±0.6	3.6±1.5 (p=0.2433)
$p53^{+/+}$	0	1.9±0.5	3.0±0.7
	1	4.0±0.2 (p=0.0179)	1.5±0.1
	10	4.6±0.6 (p=0.0279)	3.7±0.7
	100	4.3±2.5	1.9±0.9

Cells from $p53^{+/+}$ and $p53^{-/-}$ bone marrow stromal cell lines were incubated in the presence of 0, 1, 10 or 100 μM CBZ for one hour and then were irradiated to doses ranging from 0 to 8 Gy, plated in 4-well plates, incubated for 7 days at 37˚C, stained with crystal violet and colonies of greater than 50 cells counted. The data was analysed using linear quadratic and single-hit, multi-target models. The p-values are in comparison to results using 0 μM. CBZ had different, but still protective effects on $p53^{+/+}$ (increased D_0) and $p53^{-/-}$ (increased ñ) cells.

Protection by carbamazepine is p53 independent. We next evaluated whether carbamazepine-mediated ionizing irradiation protection and mitigation was dependent on p53. The effects of treatment with carbamazepine before or after irradiation of $p53^{-/-}$ murine bone marrow stromal cells were assessed by clonogenic survival curve assay and results were compared to those with a $p53^{+/+}$ cell line. The $p53^{-/-}$ cells that were incubated with carbamazepine for one hour before or immediately after irradiation demonstrated both protection and mitigation, with an increase in ñ from 1.8±0.4 to 6.0±0.6 (p=0.0018) for protection (Table V, Figure 4), and by an increase in ñ from 1.9±0.5 to 4.5±0.8 (p=0.0318) for mitigation (data not shown). Thus, the mechanism by which carbamazepine modifies cellular irradiation damage was not dependent on p53.

Carbamazepine increases antioxidant levels in $Atg5^{+/+}$ MEF cells. The cell lines $Atg5^{+/+}$ and $Atg5^{-/-}$ were incubated with carbamazepine for one hour prior to 6 Gy irradiation and were then harvested at various time points. $Atg5^{+/+}$ cells that were supplemented with 10 μM carbamazepine demonstrated an increase in antioxidant levels 10 minutes after irradiation, compared to control irradiated cells (Figure 5). This result may reflect a rebound from the response of cells to carbamazepine-induced oxidative stress which was caused by adding carbamazepine one hour prior to irradiation. However, carbamazepine was also effective as a radiation mitigator with continual exposure to drug in the medium in the mitigation experiment. Continuous exposure would have been expected to deplete antioxidant levels through any such rebound. Increased antioxidant levels were not detected in autophagy-deficient $Atg5^{-/-}$ cells, which were also protected and mitigated from irradiation by carbamazepine. $Atg5^{-/-}$ cells demonstrated consistently low antioxidant (Figure 5) and glutathione (GSH) levels (Table VI). The data support the conclusion that radiation protection and mitigation of cells by CBZ was not mediated by alterations in cellular antioxidant levels.

Carbamazepine does not protect tumor cells in vitro. An effective radioprotector for use in clinical radiation therapy should protect normal tissues but not tumor cells. To determine if carbamazepine protected tumor cells from ionizing irradiation, Lewis lung carcinoma (3LL) cells were incubated in 10 μM carbamazepine before or after irradiation and plated for clonogenic survival assay. Cells that received carbamazepine before or after irradiation did not exhibit a statistically different survival curve from control irradiated cells (Table VII). The results establish that carbamazepine did not protect 3LL tumor cells *in vitro*.

Carbamazepine does not modulate the radiation response of 3LL orthotopic tumors in vivo. We next tested the effect of carbamazepine on irradiated 3LL tumors *in vivo*. 3LL tumor cells were injected into the leg of mice and allowed to grow to a measurable 5 mm diameter mass prior to irradiation with 20 Gy to the hind limb, a dose known to reduce tumor growth. The irradiated tumors in mice that received intraperitoneal injection of CBZ before or after irradiation did not show faster regrowth compared to tumors in mice that received irradiation only (p=0.2431 and 0.5439, respectively) (Figure 6). Thus carbamazepine did not reduce the irradiation response of 3LL tumors *in vivo*. These data confirm and extend prior studies, showing that CBZ was an effective mitigator against total body irradiation when delivered at 12 but not at 24 hours after irradiation (2).

Carbamazepine is not a radioprotector or mitigator for human cell lines or fresh human umbilical cord blood hematopoietic progenitor cells in vitro. We tested the effect of carbamazepine pre and post-irradiation on three human cell lines: i) IB3, bronchoalveolar cells, ii) KM101 human bone marrow stromal cells, and iii) cervical cancer derived HeLA cells (Table VIII). We evaluated the effect of carbamazepine on human umbilical cord blood MNCs that form multilineage hematopoietic colonies *in vitro* (Table IX). We also tested the effects on the sorted and purified cord blood $CD34^+$ progenitor cells (Table X). The results showed no detectable radioprotection or mitigation by carbamazepine of any of the human cell sources (Tables VIII-X).

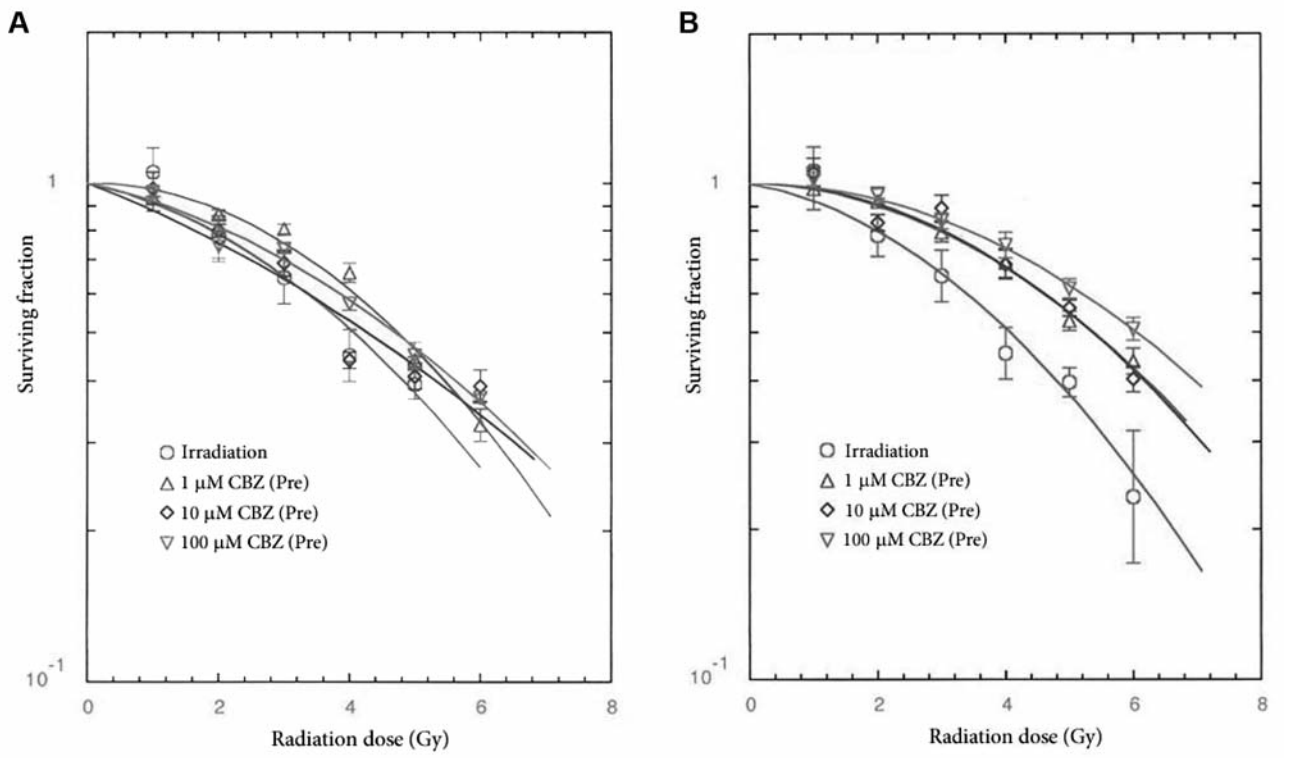

Figure 4. *Radiation protection/mitigation by carbamazepine (CBZ) is independent of p53. A: $p53^{+/+}$ and B: $p53^{-/-}$ murine bone marrow stromal cells were irradiated to doses of 0-8 Gy then plated in clonogenic assay and colonies greater than 50 cells were scored at 14 days. CBZ protected (pre) and mitigated (post) radiation damage in both the p53-containing and -deficient lines.*

Table VI. *Effect of carbamazepine (CBZ) on antioxidant stores in irradiated $Atg5^{+/+}$ compared to $Atg5^{-/-}$ (WT) cell lines.*

Cell Line	Total antioxidants (Trolox equivalents)				GSH (μM)			
	0 Gy		24 h after 4 Gy		0 Gy		6 h after 6 Gy	
	0 μM CBZ	10 μM CBZ	0 μM CBZ	10 μM CBZ	0 μM CBZ	10 μM CBZ	0 μM CBZ	10 μM CBZ
WT 1			0.026	0.039		0.217	2.111	<0.010
WT 3			0.064	0.050			0.067	<0.010
KO 2	0.49	0.67	0.039	0.043	<0.010	<0.010	<0.010	<0.010
KO 4			0.042	0.133			<0.010	<0.010

Clonal cell lines of $Atg5^{+/+}$ (WT1 and 3) and $Atg5^{-/-}$ (KO2 and 4) were irradiated to doses from 0, 4 or 6 Gy as described in the methods. Total antioxidants (Trolox units) and levels of glutathione (GSH) were measured using a commercial kit at 24 h following 4 Gy or 6 h after irradiation of 6 Gy, respectively.

Discussion

An aggressive search for small-molecule radiation protectors and mitigators has been necessitated by both the need for such agents in clinical radiotherapy (35) and in radiation counter-terrorism (36). In clinical radiotherapy, the availability of novel modalities of intensity modulated radiotherapy (37), stereotactic radiosurgery (38), and high-dose rate brachytherapy (39) still does not prevent the normal tissue toxicity of ionizing irradiation and often prevents radiation dose-escalation protocols. We were encouraged by the discovery that carbamazepine was a radioprotector and mitigator (2). Carbamazepine is a Food and Drug Administration approved drug for clinical use for epilepsy,

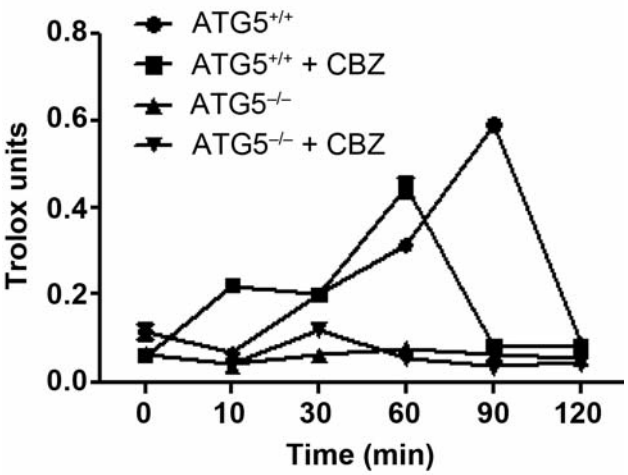

Figure 5. *Carbamazepine (CBZ) increases antioxidant levels in irradiated Atg5$^{+/+}$ but not Atg5$^{-/-}$ cells. Cells from Atg5$^{+/+}$ and Atg5$^{-/-}$ cell lines were treated with 10 μM CBZ for 1 hour prior to 6 Gy irradiation, harvested at different time points and antioxidant levels measured. Baseline antioxidants remained higher in autophagy-proficient Atg5$^{+/+}$ cells. CBZ-treated Atg5$^{+/+}$ cells had an increase in antioxidants at 10 minutes, peaking at 60 minutes after irradiation.*

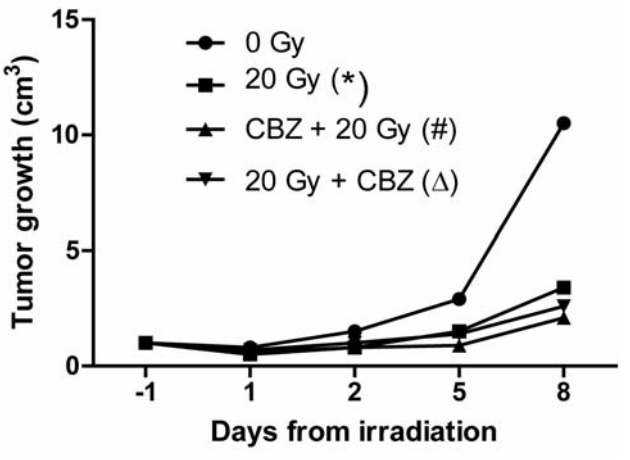

Figure 6. *Effect of carbamazepine (CBZ) on irradiation-mediated size reduction of 3LL carcinoma cell line-derived orthotopic tumors in vivo. We injected 10^6 3LL cells into the hind leg of C57BL/6 HNsd mice and tumors grew to a solid mass for 7 days. Mice then received intraperitoneal injection of CBZ at 20 mg/kg in 0.1/ml and tumors were then irradiated to 20 Gy and monitored for tumor growth. Tumors in mice receiving 20 Gy only to the tumor (*) grew significantly slower than tumors in 0 Gy mice (p=0.0019). Tumors in mice treated with CBZ before 20 Gy (#) grew slower than the tumors in 0 Gy mice but not tumors receiving 20 Gy only (p=0.0001 or 0.2431, respectively). Tumors in mice administered 20 Gy and followed by CBZ (Δ) had a decreased growth rate compared to tumors in 0 Gy mice but not in 20 Gy irradiated mice (p=0.0005 or 0.5439, respectively). CBZ treatment did not protect tumors from size reduction due to irradiation.*

Table VII. *Effect of carbamazepine (CBZ) on radiosensitivity of Lewis lung carcinoma in 3LL cells assayed by clonogenic radiation survival curve in vitro.*

CBZ Treatment	D_0 (Gy)	ñ
0 μM	2.0±0.4	5.7±2.4
10 μM Pre-Irradiation	2.0±0.1	3.3±1.0
	(*p*=0.9441)	(*p*=0.3520)
10 μM Post-Irradiation	2.0±0.2	3.2±1.3
	(*p*=0.09522)	(*p*=0.3795)

We evaluated 4-well plates with 500 or 1000 cells per plate irradiated to doses of 0-8 Gy. The plates were screened 14 days after irradiation for colonies of >50 cells as described in the Materials and Methods. The *p*-values compare cell survival of 3LL cell lines treated with CBZ before or after irradiation to those treated with irradiation alone, using linear quadratic or single-hit, multi-target models.

trigeminal neuralgia, and bipolar disorder, and is a commonly prescribed drug with a well-known safety and side-effect profile. Serious but rare hematologic complications after chronic use have been identified (7-8). Therefore, while attractive for potential clinical use (2), its safety in irradiated humans must be carefully evaluated.

The present study indicates that carbamazepine is a radiation protector and mitigator for normal murine tissues, but not of tumor cells, both *in vitro* and *in vivo*. The data establish that radiobiologic effects of carbamazepine are not associated with changes in mitochondrial membrane permeability or radiation-induced apoptosis. Since carbamazepine increases radio-resistance of the mouse hematopoietic progenitor cell line 32Dcl3 in clonogenic survival curve assays (2), the mechanism may be subtle and not detectable at the level of initial apoptosis of single cells. Consistent with this data was the observation that carbamazepine protected both p53$^{-/-}$ and p53$^{+/+}$ cell lines from ionizing irradiation. The combined evidence indicates that carbamazepine acts by an apoptosis-independent mechanism.

Since carbamazepine is a pro-autophagy agent (1), we evaluated two other autophagy-promoting drugs, lithium chloride and VPA, as radiation-dose modifiers. These drugs have similar clinical indications and effects on the inositol cycle, and are also known to up-regulate autophagy (40). Neither lithium chloride nor VPA were effective radiation protectors or mitigators *in vitro*. Both Atg5$^{-/-}$ cells, which demonstrated absence of autophagy in western blot assay for LC3, and Atg5$^{+/+}$ MEF cells were significantly radioprotected and mitigated by carbamazepine in clonogenic survival curve assays. The data indicate that the mechanism of radiation protection and mitigation by carbamazepine is independent of autophagy.

We demonstrated that Atg5$^{-/-}$ cells had a lower baseline level of antioxidants compared to the autophagy proficient Atg5$^{+/+}$ wild-type cells. This result was consistent with other

Table VIII. *Lack of radioprotection or mitigation of radiation-induced effects in human cell lines by carbamazepine (CBZ).*

CBZ concentration	Pre or post irradiation	HeLA		KM101		IB3	
		D_0 (Gy)	ñ	D_0 (Gy)	ñ	D_0 (Gy)	ñ
0 μM	Pre	1.7±0.1	7.6±2.5	NA	NA	NA	NA
	Post	1.79±0.2	5.1±1.3	1.3±0.1	3.0±0.3	1.6±0.1	7.1±4.6
1 μM	Pre	1.9±0.3	7.6±4.0	NA	NA	NA	NA
	Post	1.6±0.2	7.3±2.7	1.3±0.1	3.2±0.3	1.6±0.1	5.5±2.7
10 μM	Pre	1.8±0.2	9.0±4.1	NA	NA	NA	NA
	Post	1.8±0.2	4.7±1.0	1.2±0.1	3.5±0.5	1.8±0.1	4.6±2.2
100 μM	Pre	1.8±0.1	6.6±1.6	NA	NA	NA	NA
	Post	1.4±0.2	6.1±2.2	1.2±0.1	2.2±0.1	1.7±0.1	6.6±4.6

Cell lines were irradiated to doses of 0-8 Gy in the presence of CBZ one hour pre-irradiation (protection) or plated in CBZ (mitigation). Colonies were scored on day 7 as described in the Materials and Methods. Data are summarized as mean±SEM. NA, not assayed pre-irradiation.

Table IX. *Effect of carbamazepine (CBZ) pre or post irradiation on colony formation by human cord blood mononuclear cells.*

Experimental conditions	Pre or post irradiation	CFU-GM		BFU-E		CFU-GEMM	
		D_0	ñ	D_0	ñ	D_0	ñ
Cells only		1.60±0.28	1.71±0.41	1.40±0.19	3.31±0.73	2.08±0.05	1.22±0.12
DMSO	Pre	1.57±0.20	2.73±1.73	1.61±0.18	1.81±0.30	2.13±0.10	1.22±0.13
		p1=0.9340	p1=0.5971	p1=0.4598	p1=0.1307	p1=0.6221	p1=0.9858
	Post	1.68±0.20	1.43±0.25	1.30±0.17	11.98±9.55	2.11±0.14	1.24±0.04
		p1=0.8202	p1=0.5923	p1=0.7331	p1=0.4600	p1=0.8004	p1=0.9445
CBZ 1 μM	Pre	1.69±0.07	1.38±0.35	1.46±0.01	3.31±1.30	2.02±0.23	1.35±0.16
		p1=0.7558	p1=0.5738	p1=0.7872	p1=0.9983	p1=0.8293	p1=0.5526
		p2=0.5831	p2=0.4868	p2=0.4760	p2=0.3213	p2=0.6781	p2=0.5572
	Post	1.84±0.06	1.16±0.16	1.66±0.07	1.63±0.23	2.11±NA	1.00±NA
		p1=0.4346	p1=0.2797	p1=0.2762	p1=0.0941	p1=NA	p1=NA
		p3=0.4833	p3=0.4015	p3=0.1191	p3=0.3917	p3=NA	p3=NA
CBZ 10 μM	Pre	1.50±0.21	2.46±1.46	1.46±0.16	2.69±1.14	2.06±NA	1.34±NA
		p1=0.7864	p1=0.6463	p1=0.8141	p1=0.6693	p1=NA	p1=NA
		p2=0.8179	p2=0.9120	p2=0.5594	p2=0.4960	p2=NA	p2=NA
	Post	1.91±0.07	1.00±0.00	1.67±0.03	1.61±0.22	2.37±NA	1.00±NA
		p1=0.3354	p1=0.2287	p1=0.2436	p1=0.0908	p1=NA	p1=NA
		p3=0.3436	p3=0.2224	p3=0.0973	p3=0.3910	p3=NA	p3=NA
CBZ 50 μM	Pre	1.63±0.06	1.83±0.19	1.47±0.04	3.01±0.23	-	-
		p1=0.9123	p1=0.8009	p1=0.7313	p1=0.7161		
		p2=0.7747	p2=0.6567	p2=0.4812	**p2=0.0322**		
	Post	1.72±0.27	1.27±0.14	1.70±0.32	1.71±0.40	-	-
		p1=0.7715	p1=0.3724	p1=0.4553	p1=0.1296		
		p3=0.9184	p3=0.6041	p3=0.3249	p3=0.3949		
CBZ 100 μM	Pre	1.74±0.04	1.26±0.18	1.50±0.24	3.09±2.01	2.05±NA	1.00±NA
		p1=0.7258	p1=0.4742	p1=0.7693	p1=0.9091	p1=NA	p1=NA
		p2=0.5576	p2=0.5581	p2=0.7116	p2=0.4676	p2=NA	p2=NA
	Post	1.55±0.19	1.00±0.00	1.32±0.18	2.38±0.66	-	-
		p1=0.9014	p1=0.2287	p1=0.8053	p1=0.4480		
		p3=0.6786	p3=0.2224	p3=0.9514	p3=0.4933		

We evaluated nucleated cells from 3 separate human umbilical cord blood samples as described in the Materials and Methods. Colonies were analyzed at day 14 as described in the methods. Data are summarized as mean±SEM. *P*-values were calculated with the two-sided two-sample *t*-test, where p1 is the *p*-value for comparison with the cells only control; p2 is for the comparison with the pre-irradiation dimethyl sulfoxide (DMSO) group; and p3 is for the comparison with the post-irradiation DMSO group. Significant *p*-values are shown in bold.

Table X. *Effect of carbamazepine (CBZ) pre or post irradiation on colony formation by human cord blood CD34+ cells.*

Experimental conditions	Pre or post irradiation	CFU-GM		BFU-E		CFU-GEMM	
		D_0	ñ	D_0	ñ	D_0	ñ
Cells only		1.73±0.10	1.07±0.07	1.68±0.06	1.70±0.28	2.07±0.21	1.00±0.0
DMSO	Pre	2.59±0.29	1.04±0.04	2.33±0.07	1.44±0.44	2.03±0.32	1.00±0.0
		p1=0.0248	p1=0.7214	**p1=0.0008**	p1=0.6225	p1=0.9151	p1=1.0000
	Post	2.15±0.16	1.08±0.80	2.25±0.28	1.00±0.0	2.30±0.56	1.00±0.0
		p1=0.0776	p1=0.9354	**p1=0.0398**	p1=0.0845	p1=0.6494	p1=1.0000
CBZ 1 μM	Pre	1.50±0.05	1.36±0.36	2.75±0.09	1.07±0.07	2.14±0.30	1.00±0.0
		p1=0.1971	p1=0.3007	**p1=0.0005**	p1=0.2004	p1=0.8522	p1=1.0000
		p2=0.0640	p2=0.5333	**p2=0.0327**	p2=0.5571	p2=0.8241	p2=1.0000
	Post	1.66±0.08	2.07±1.07	2.22±0.39	1.04±0.04	no colonies	no colonies
		p1=0.6647	p1=0.5212	p1=0.3902	p1=0.1857	p1=NA*	p1=NA
		p3=0.1114	p3=0.4537	p3=0.9552	p3=0.5000	p3=NA	p3=NA
CBZ 10 μM	Pre	2.13±0.38	1.00±0.0	2.26±0.31	1.21±0.15	2.03±0.35	1.00±0.0
		p1=0.2236	p1=0.3910	**p1=0.0489**	p1=0.3056	p1=0.9162	p1=1.0000
		p2=0.3932	p2=0.4226	p2=0.8058	p2=0.7099	p2=0.9975	p2=1.0000
	Post	1.64±0.26	3.71±2.71	1.95±0.29	1.29±0.29	1.69±NA	1.00±NA
		p1=0.6732	p1=0.5083	p1=0.2389	p1=0.4120	p1=NA	p1=NA
		p3=0.2293	p3=0.5095	p3=0.5278	p3=0.5000	p3=NA	p3=NA
CBZ 50 μM	Pre	1.80±0.02	1.63±0.06	2.16±0.22	1.30±0.30	2.43±0.08	1.00±0.0
		p1=0.6715	**p1=0.0073**	**p1=0.0392**	p1=0.4183	p1=0.3248	p1=1.0000
		p2=0.1275	**p2=0.0028**	p2=0.4397	p2=0.8244	p2=0.4074	p2=1.0000
	Post	1.22±0.18	35.75±33.30	2.57±NA	1.00±NA	no colonies	no colonies
		p1=0.0468	p1=0.4871	p1=NA	p1=NA	p1=NA	p1=NA
		p3=0.0587	p3=0.4872	p2=NA	p2=NA	p2=NA	p2=NA
CBZ 100 μM	Pre	1.94±0.44	1.41±0.41	2.20±0.45	1.00±0.0	2.22±0.12	1.00±0.0
		p1=0.5423	p1=0.2797	p1=0.4488	p1=0.0845	p1=0.6622	p1=1.0000
		p3=0.2785	p3=0.5291	p3=0.7397	p3=0.4226	p3=0.6768	p2=1.0000
	Post	1.13±NA	15.13±NA	no colonies	no colonies	no colonies	no colonies
		p1=NA	p1=NA	p1=NA	p1=NA	p1=NA	p1=NA
		p3=NA	p3=NA	p3=NA	p3=NA	p3=NA	p3=NA

*NA=Not available. Data are summarized as mean±SEM. *p*-values were calculated with the two-sided two-sample *t*-test, where p1 is the *p*-value for comparison with the cells only control; p2 is for the comparison with the pre-irradiation DMSO group; and p3 is for the comparison with the post-irradiation dimethyl sulfoxide (DMSO) group. Significant *p*-values are shown in bold.

data showing that ROS oxidize Atg4, a process that induces and is essential for autophagy (41). $Atg5^{-/-}$ MEF cells accumulate ROS since ROS oxidation of Atg4 (ROS consumption) is upstream of Atg5 (41). Since carbamazepine causes oxidative stress after acute administration (42), this oxidative stress might result in higher levels of ROS and thus more Atg4 oxidation, and increased autophagy (41). It is possible that carbamazepine might up-regulate autophagy by this mechanism, but this process was independent of radioprotection or mitigation.

Intracellular glutamate transport is known to be altered by gamma irradiation in astrocytes and neurons (43). Since PI3K is involved in the regulation of glutamate transport, carbamazepine may enhance the affinity of transporters for their substrates. PI3K inhibitors LY-294,002 and wortmannin inhibit carbamazepine enhancement of glutamate transport activity (42). Since class III PI3K is upstream of Atg4 oxidation and the completion of autophagy (41), it is possible that effects of irradiation were propagated through a class III PI3K pathway and that carbamazepine ameliorates radiation damage in a class III PI3K-dependent manner (44). Our data suggest that it is unlikely that carbamazepine is inhibiting class III PI3K. Beclin 1 is a physiologic activator of class III PI3K (45). When the inhibitory complex of Beclin 1 with Bcl2 and BclX is disrupted, Beclin 1 can up-regulate class III PI3K activity (46). One potential mechanism by which carbamazepine may activate class III PI3K might be by preventing Bc12 or BclX interaction with Beclin 1. Further studies will be required to evaluate this possible mechanism of radiation mitigation and protection by carbamazepine.

The potential clinical value of carbamazepine as a radiation protector and mitigator was further supported in the present studies by our observation that 3LL tumor cells were not protected or mitigated *in vitro* by carbamazepine, and that *in vivo* orthotopic mouse tumors derived from 3LL cells were not protected by carbamazepine from single fraction irradiation. The data suggested that normal tissue protection and mitigation by carbamazepine should not extend to tumor cells. If carbamazepine acts by a novel mechanism of radioprotection, it might be additive or synergistic with other known small-molecule radiation protectors and mitigators that function through known anti-apoptotic pathways (29).

The present data indicated that there was no detectably significant carbamazepine radiation protection or mitigation of human cells *in vitro*. Furthermore, in other retrospective analysis studies of the use of carbamazepine during intracranial radiotherapy of patients with trigeminal neuralgia (47) or in other patients receiving radiotherapy for head and neck or lung cancer (48), there was no detectable decrease in radiation side-effects. Further studies will be required to determine how carbamazepine functions as a radiation protector and mitigator in mouse cells and in mice. Investigating the role of class III PI3K in carbamazepine-mediated protection and mitigation may reveal targets for future drug design, modification, and development which may increase its spectrum of activity to include human cells. Such data might lead to potential use of a modified agent in clinical radiotherapy and in radiation counter-measures.

Acknowledgements

This study was supported by NIH/NIAID grant U19A1068021 and NIH T32AG21885. The authors gratefully acknowledge Dr. Noboru Mizushima for providing the $Atg5^{-/-}$ and $Atg5^{+/+}$ cell lines, and thank Dr. David Perlmutter for helpful discussions.

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Received December 12, 2011
Revised January 27, 2012
Accepted January 31, 2012