

JPET #199802 PIP

## Title Page

### **Autophagy inhibition for chemosensitization and radiosensitization in cancer: do the preclinical data support this therapeutic strategy?**

Molly L. Bristol, Sean M. Emery, Paola Maycotte, Andrew Thorburn, Shweta Chakradeo and David A. Gewirtz

Departments of Pathology (MLB), Pharmacology and Toxicology (SME, SC, DAG) and Massey Cancer Center (DAG), Virginia Commonwealth University, Richmond, VA; Department of Pharmacology, University of Colorado School of Medicine, Aurora, Colorado (PM, AT).

JPET #199802 PIP

Running Title Page

**Running title:** Autophagy inhibition, chemosensitization and radiosensitization

Correspondence should be addressed to Dr. David A. Gewirtz

Massey Cancer Center

401 College St.

Virginia Commonwealth University

Richmond, VA 23298

Phone: 804-828-9523

Fax: 804-827-1134

e-mail: gewirtz@vcu.edu

Number of Text pages: 27

Number of Tables: 0

Number of Figures: 3

Number of References: 51

Number of words in Abstract: 245

Number of words in Introduction: 438

Number of words in Discussion: 799

**Abbreviations:** CQ – chloroquine; 3-MA- 3-methyl adenine; IR – ionizing radiation.

JPET #199802 PIP

## ABSTRACT

Recognition of the cytoprotective functions of autophagy that occur in tumor cells exposed to various forms of chemotherapy or radiation has generated intense interest in the possibility that pharmacological interference with autophagy could provide a clinical strategy for overcoming therapeutic resistance. Multiple clinical trials are currently in progress to evaluate the antimalarial agent chloroquine (generally in its clinical formulation as hydroxychloroquine) and its impact on various forms of cancer therapy. In this commentary/review, we focus on the relatively limited number of studies in the literature where chloroquine has been tested in combination with chemotherapy or radiation in experimental tumor-bearing animal models. We also present recent data from our own laboratories, in cell culture experiments as well as *in vivo* studies, which demonstrate that neither chloroquine nor silencing of an autophagy regulatory gene was effective in conferring radiation sensitivity in an experimental model of breast cancer. The capacity for sensitization by chloroquine appears to be quite wide-ranging, with dramatic effects for some drugs/tumor models and modest or minimal effects in others. One possible caveat is that with only a few exceptions, experiments have generally been performed in xenograft models, thereby eliminating the involvement of the immune system, which might ultimately prove to play a central role in determining the effectiveness of autophagy inhibition in chemosensitization or radiosensitization. Nevertheless, a careful review of the current literature suggests that caution is likely to be warranted in translating preclinical findings relating to autophagy inhibition as an adjunctive therapeutic strategy.

## Introduction

In recent years, it has been recognized that one potential mechanism of resistance to chemotherapy as well as radiotherapy in cancer could be the promotion of protective autophagy; this recognition has generated interest in the possibility that interference with autophagy could enhance sensitivity to treatment (Abedin et al., 2007; Amaravadi et al., 2007; Apel et al., 2008; Boya et al., 2005; Bristol et al., 2012; Djavaheri-Mergny et al., 2007; Kanzawa et al., 2004; Kondo et al., 2005; Paglin et al., 2001; Qadir et al., 2008; Sotelo et al., 2006; Wilson et al., 2011). An extensive number of studies in cell culture (for example, Amaravadi et al., 2007; Apel et al., 2008; Boya et al., 2005; Bristol et al., 2012; Kanzawa et al., 2004; Livesey et al., 2009; Ma et al., 2011; Paglin et al., 2001; Qadir et al., 2008; Solomon and Lee, 2009; Wilson et al., 2011; Zhao et al., 2005), as well as a more limited number of studies in animal models (Carew et al., 2010; Ding et al., 2011; Fu et al., 2009; Ghadami et al., 2012; Godbole et al., 2012; Guo et al., 2012; Hu et al., 2012; Jiang et al., 2010; Liang et al., 2012; Loehberg et al., 2012; Lopez et al., 2011; Mirzoeva et al., 2011; Pan et al., 2011; Rao et al., 2012; Shi et al., 2011; Sasaki et al., 2012; Wu et al., 2010; Xu et al., 2011) have been performed combining chloroquine or hydroxychloroquine with chemotherapeutic drugs or radiation. Furthermore, a number of clinical trials have been initiated to test this premise in patients (Sotelo et al., 2006; U.S. National Institutes of Health, 2012).

In view of the fact that therapeutic efficacy of both antitumor drugs and radiation may be highly dependent on the immune system (Michaud et al., 2011; Golden et al., 2012; Martins et al., 2012), and since, with few exceptions, studies in the current literature have been performed using tumor xenografts, we assessed the influence of treatment with chloroquine on sensitivity to

JPET #199802 PIP

radiation in the 4T1 syngeneic murine breast tumor model. Complementary experiments were performed in cell culture evaluating the impact of chloroquine as well as genetic silencing of the autophagy regulatory gene, *Atg12*, on sensitivity to radiation in murine 4T1 breast tumor cells. Given that our studies, both in cell culture as well as *in vivo*, appeared to indicate that either pharmacologic or genetic interference with autophagy fails to confer radiation sensitization, it appeared to be relevant to review the literature relating to animal model systems combining chloroquine with chemotherapy and radiotherapy in order to evaluate the preclinical evidence that might support the ongoing clinical trials.

## Materials and Methods

**Cell Culture and Treatment** 4T1 cells were obtained from ATCC. 4T1 derived cell lines were grown from frozen stocks in basal RPMI 1640 supplemented with 5% FBS, 5% BCS, 2 mmol/L l-glutamine, and penicillin/streptomycin (0.5 mL/100 mL medium). All cells were maintained at 37°C under a humidified, 5% CO<sub>2</sub> atmosphere. Cells were routinely subcultured by trypsinization (0.25% trypsin, 0.03% EDTA, Gibco) upon reaching confluence. All cell cultures were examined by microscope for bacterial and fungal contamination prior to experiments. Additionally, all cell lines were determined to be free of mycoplasma.

For in vitro radiation experiments, 4T1 cells were treated for 1 h with chloroquine before radiation in an X-ray irradiator (RS2000) at the indicated doses. After treatment, medium was changed and protein was collected 4 h after treatment for Western Blotting. For clonogenic assays, cells were plated in 12 well plates at a density of 300 cells per well, treated as described above and allowed to recover for 7 days after treatment. Cells were then fixed and stained with crystal violet (BD), the stain was solubilized with 30% acetic acid and absorbance was measured at 540 nm. 4T1 *Atg12* shRNA inducible cells were generated as described previously (Maycotte et al., 2012) . Briefly, 4T1 cells were transduced with lentiviruses containing a pTRIPZ non-silencing shRNA or an *Atg12* mouse shRNA cloned from a pGIPZ shRNAmir (V2LMM\_72549) plasmid (Open Biosystems). Cells were grown in DMEM with 10% tetracycline-free FBS (Hyclone), selected with puromycin and clones were isolated and validated for ATG12 knockdown. For shRNA induction, cells were treated with 1 µg/ml doxycycline (Clontech) for 72 h, replacing doxycycline every 24 h. For radiation treatment, cells were plated in 12 well plates at a density of 200 cells per well, treated ± doxycycline for 72 h, and irradiated at the

JPET #199802 PIP

indicated doses. After a 7 day recovery period, cells were fixed and clonogenic growth was evaluated as described above.

**Western Blotting** Cells were washed with PBS and lysed with RIPA buffer. Protein was quantitated using Bradford reagent (Bio-Rad). 20  $\mu$ g of protein were loaded in a 10% SDS-PAGE and PVDF membranes (Millipore) were probed with anti-ATG12 (Cell Signaling, D88H11) or actin antibodies (Sigma, A5441). For LC3 Western Blots, 7.5  $\mu$ g of protein were used and probed with anti-LC3 antibody (Novus Biologicals, NB100-2220).

**Animal Studies** Female BALB/c mice, 8 weeks old, were purchased from Jackson Laboratories and were maintained in groups of four per cage with access to food and water. The animals were acclimated at least two days before use and maintained throughout at standard conditions: 24 $\pm$ 2 $^{\circ}$ C temperature; 50 $\pm$ 10% relative humidity. All studies involving mice were approved by the Institute's Animal Care and Use Committee (IACUC).

Mice were challenged s.c. with 5 x 10<sup>4</sup> 4T1 mouse breast tumor cells, transfected with luciferase, in the right flank on day 0. On day 3, animals were injected with 150 mg/kg luciferin and the presence of primary tumors was determined by bioluminescence signal as imaged using an IVIS 50 (Xenogen, part of Caliper Life Sciences). Of note, the bioluminescent signal could be detected in the animal before primary tumors were palpable and could be measured with a caliper. Tumor-bearing mice were randomly assigned into groups and were injected intraperitoneally (ip) as indicated: (a) 25 mg/kg CQ, (b) 50 mg/kg CQ, (c) 100mg/kg CQ, or (d) PBS. The CQ doses chosen were based on the available literature, where studies have generally been performed using CQ at 50-60mg/kg (Carew et al., 2010; Ding et al., 2011; Fu et al., 2009;

JPET #199802 PIP

Ghadami et al., 2012; Godbole et al., 2012; Guo et al., 2012; Hu et al., 2012; Jiang et al., 2010; Liang et al., 2012; Loehberg et al., 2012; Lopez et al., 2011; Mirzoeva et al., 2011; Pan et al., 2011; Rao et al., 2012; Shi et al., 2011; Sasaki et al., 2012; Wu et al., 2010; Xu et al., 2011). Additional experiments were conducted where the mice were exposed to  $\gamma$ -irradiation using a  $^{137}\text{Cs}$  irradiator as indicated: (e) 5 Gy IR, (f) 10 Gy IR, (g) 15 Gy IR, (h) 10Gy IR + 14 days CQ ip. Each group consisted of 6-8 mice. Irradiation was performed on day 3 and chloroquine (50mg/kg) or saline treatment was initiated on day 3 and was repeated every day for 14 days. Tumor growth was monitored multiple times per week by bioluminescence. Upon sacrifice, tumors were resected and weighed.

**Statistical Analysis** All of the data are represented as means  $\pm$  SE. Statistical differences were determined using StatView statistical software. Comparisons were made using a one-way ANOVA followed by Tukey Kramer's post-hoc test. For density analysis, mean comparison was done with a Student's t-Test. *P values of*  $\leq 0.05$  were taken as indicating statistical significance.



## Results

**Studies in 4T1 cells in culture** Extensive evidence in the literature supports a role for autophagy as a cytoprotective tumor survival pathway that is induced by exposure to chemotherapy or radiation (Abedin et al., 2007; Amaravadi et al., 2007; Apel et al., 2008; Boya et al., 2005; Bristol et al., 2012; Djavaheri-Mergny et al., 2007; Kanzawa et al., 2004; Kondo et al., 2005; Paglin et al., 2001; Qadir et al., 2008; Sotelo et al., 2006; Wilson et al., 2011). In this work we treated 4T1 cells with radiation and different doses of chloroquine to block autophagy. Figure 1A shows induction of autophagy in 4T1 cells after radiation treatment (2, 5 and 10 Gy with and without exposure to chloroquine (10, 25 and 30 $\mu$ M) when evaluated by Western blotting for LC3. During autophagy, LC3, the mammalian homolog of Atg8, is cleaved and conjugated to phosphatidylethanolamine. This modified form, termed LC3II, is involved in the elongation of the autophagosome (Mizushima et al., 2010). However, LC3II is also degraded in the lysosome after autophagosome-lysosome fusion (Mizushima et al., 2010; Klionsky et al., 2012). Therefore, in order to measure autophagic flux, lysosomal inhibitors such as chloroquine are used to inhibit LC3II degradation, since in the absence of these inhibitors, an autophagy-inducing treatment can result in a modest increase or even a decrease in the amount of LC3II. The relevant parameter in this assay is the difference in the amount of LC3II in the presence and absence of lysosomal inhibitors (Klionsky et al., 2012). In Figure 1A, chloroquine by itself increased LC3II due to inhibition of basal autophagy, and LC3II was increased when compared to untreated controls at the three different radiation doses. A greater increase in LC3II was evident in irradiated cells in the presence of chloroquine, indicating the autophagic flux induced by irradiation, which can be blocked by chloroquine treatment. Despite autophagy induction via irradiation, blocking

JPET #199802 PIP

autophagy with chloroquine did not result in a reduction in cell viability compared to radiation treatment alone when measured by clonogenic survival (Figure 1B).

Genetic inhibition of autophagy via silencing of ATG12 was performed to confirm the results of the experiments utilizing pharmacological inhibition. In a previous report, we developed 4T1 cells with an inducible *Atg12* shRNA and complementary controls with non-silencing shRNA (Maycotte et al., 2012). Figure 2 presents the results of studies assessing radiation sensitivity in these 4T1 cells by a clonogenic survival assay. Doxycycline treatment decreased ATG12 protein levels (Figure 2A), with the consequent suppression of radiation-induced autophagy in 4T1 cells at all radiation doses used in this study (Figure 2B). However, these experiments clearly demonstrate lack of sensitization by *Atg12* silencing as the sensitivity to doses of 2, 5 and 10 Gy is essentially identical in the silenced and non-silenced cells with and without doxycycline (Figure 2C).

**Studies of 4T1 cells in an animal model** A quite extensive body of evidence has been generated in support of the presumption that chloroquine has the potential to sensitize tumor cells to radiation or chemotherapy, ostensibly via the inhibition of autophagy (Amaravadi et al., 2007; Apel et al., 2008; Boya et al., 2005; Bristol et al., 2012; Kanzawa et al., 2004; Livesey et al., 2009; Ma et al., 2011; Paglin et al., 2001; Qadir et al., 2008; Solomon and Lee, 2009; Wilson et al., 2011; Zhao et al., 2005). In conjunction with the genetic and pharmacological studies that were performed in cell culture to inhibit autophagy induced by radiation, experiments were designed to assess the impact of chloroquine on the response to radiation using the murine syngeneic 4T1 breast tumor cells in immunocompetent Balb/c mice. Varying doses of radiation alone (individual doses of 5, 10 and 15 Gy) as well as chloroquine alone (at concentrations of 25,

JPET #199802 PIP

50 and 100 mg/kg) were monitored for effects on tumor volume by bioluminescence and final tumor weight. A radiation-dose-dependent decrease in tumor size was detected by bioluminescence measurement (Figure 3A), which was supported by assessment of tumor weight at the termination of the study (Figure 3B). Chloroquine alone had no impact on tumor weight (3D); although the bioluminescence assay seemed to suggest acceleration of tumor growth, this was not significant for any dose of the drug (3C). When 50mg/kg chloroquine (the dose generally used *in vivo*) was combined with 10Gy radiation, both the bioluminescence (3E), and final tumor weight determination (3F) indicated that the impact of the combination treatment was no greater than for radiation alone. Of note, with all treatments, animal weights remained consistent throughout the study (not shown).

## Discussion

**Studies in the literature relating to sensitization by chloroquine** Multiple studies have been published indicating that genetic or pharmacological interference with autophagy can enhance the response to radiation (for example, Apel et al., 2008; Bristol et al., 2012; Chaachouay et al., 2011; Gaudin and Yielding, 1969; Jiang et al., 2010; Lim et al., 2012; Livesey et al., 2009; Pazmino and Yuhas, 1974; Solomon and Lee, 2009; Wilson et al., 2011; Zhao et al., 2005). In contrast, our studies indicate that 4T1 cells could *not* be sensitized to radiation *in vitro* by pharmacological inhibition of autophagy with chloroquine treatment, by silencing of the autophagy related gene ATG12 or by chloroquine *in vivo*. These findings stand in dramatic contrast to much of the previous literature cited above as well as findings from our own laboratories demonstrating that ionizing radiation promotes a cytoprotective form of autophagy in MCF-7 and ZR-75-1 breast tumor cells that can be inhibited by drugs such as chloroquine or

JPET #199802 PIP

by the silencing of autophagy-regulatory genes, with the consequent enhancement of radiation sensitivity (Bristol et al., 2012; Wilson et al., 2011).

These findings raise the compelling question as to whether the efforts to utilize autophagy inhibition as a clinical strategy for chemosensitization and radiosensitization might be somewhat premature in the absence of sufficient and rigorous supporting preclinical data. In reviewing the available literature, there are clearly studies where the potential impact of chloroquine was realized in terms of the impact on tumor growth; however, in a number of studies, only very modest effects are evident in animal models assessing chemosensitization by chloroquine. Furthermore, few studies have demonstrated prolongation of survival in tumor bearing animals. A review of the relevant literature is presented below.

In recent studies by Ghadimi et al (Ghadimi et al., 2012), chloroquine clearly enhanced sensitivity to a dual PI3K/mTOR inhibitor in a xenograft model of malignant peripheral nerve sheath tumors. In contrast, Mirzoeva et al demonstrated minimal sensitization to an agent with a similar mechanism of action in a xenograft model of pancreatic cancer (Mirzoeva et al., 2011). These differences could be related to the tumor models utilized, as Lopez et al (Lopez et al., 2011) reported quite pronounced sensitization to an HDAC inhibitor in studies that were also performed using malignant peripheral nerve sheath tumors; in the latter report, the combination with chloroquine also reduced lung tumor metastases.

With regard to the combination of chloroquine with some of the more conventional antitumor drugs, Pan et al show potentiation of the effectiveness of both doxorubicin and melphalan in a xenograft model of multiple myeloma (Pan et al., 2011). In contrast, quite modest and limited

JPET #199802 PIP

effects were evident in models of hepatocellular carcinoma utilizing oxaliplatin (Ding et al., 2011), Sorafenib (Shi et al., 2011) or cisplatin, although the combination with 5-FU did appear to be quite effective (Guo et al., 2012). Again, one might ascribe these diverse findings to the generally high degree of resistance to therapy in hepatocellular carcinoma that may not be susceptible to sensitization solely through the suppression of autophagy; that is, drug resistance could arise, in part, from drug degradation by hepatic enzymes.

With regards to a number of other agents of varying mechanisms of action, enhanced effectiveness of a Src family kinase inhibitor was observed when combined with chloroquine in a xenograft model of prostate cancer (Wu et al., 2010), to IL-2 in a model of murine colorectal carcinoma (Liang et al., 2012), and to Bevacizumab in glioblastoma (Hu et al., 2012); the latter two findings are of particular interest and relevance and will be addressed in more detail below. Conversely, only quite modest improvement of the response to chemotherapeutic agents was detected for chloroquine in combination with an mTOR inhibitor in MCF-7 xenografts (Loehberg et al., 2012), with voranistat in colon carcinoma (Carew et al., 2010), with perifosine in lung cancer xenografts (Fu et al., 2009), with a dual PI3K/Akt/mTOR inhibitor in NSCLC xenografts (Xu et al., 2011), with a retinoic acid metabolism blocker in breast tumor cells (Godbole et al., 2012), and with panobinostat in breast cancer (Rao et al., 2012); in the latter studies, the lack of impact could be ascribed to the use of an unusually low dose of chloroquine of 10 mg/kg, which was apparently necessitated by the high degree of toxicity that was observed with chloroquine alone in this work.

A particularly relevant issue is that it is frequently unclear as to whether chloroquine has achieved levels in the tumor cells that are likely to be therapeutically effective in terms of the

JPET #199802 PIP

inhibition of autophagy. Although many investigators assess the promotion of apoptosis in the tumor cells, suppression of autophagy has not been uniformly tested in all of the cited studies, which may relate in large part to the lack of uniformly accepted protocols for monitoring autophagy suppression *in vivo*.

The studies by Liang et al are noteworthy in being virtually the only ones that demonstrated a clear increase in long-term survival for the combination treatment with chloroquine (Liang et al., 2012). It is possible that this may be related to the experiments having been performed in a syngeneic, immune competent system. However, the use of immune-competent animals was necessitated by the fact that these studies involved a component of the immune system; therefore at this juncture it cannot be convincingly argued that immune-competent animals would necessarily prove to be more predictive of the impact of autophagy inhibition on chemotherapeutic sensitivity than xenograft models. In fact, the studies by Hu et al. (2012), where genetic inhibition of autophagy demonstrated marked effects on tumor growth delay as well as survival of the tumor bearing animals, were performed in xenograft models.

With respect to the studies of Hu (Hu et al., 2012) combining chloroquine with Bevacizumab in glioblastoma, while an increase in survival of the tumor-bearing animals is evident, it is critical to note that this occurred *only* under the conditions where the implanted tumor cells had been genetically engineered for silencing of ATG7; surprisingly, no survival studies were presented for the combination treatment with chloroquine, raising the possibility that chloroquine might have failed to achieve the desired outcome in this experimental system. As with virtually all of the data reported in the literature, the outcome of the combination treatment with chloroquine was essentially one of tumor growth delay without any substantive decline in tumor size over

JPET #199802 PIP

time. In contrast, when utilizing tumor xenografts with silencing of ATG7, Hu et al provided clear evidence of tumor cell killing ( a reduction in tumor size over time), which may account for the observed increase in survival of the tumor bearing animals (Hu et al., 2012).

In comparing the pharmacological experiments with the genetic silencing studies in the animal models, it can be argued that the studies by Hu (Hu et al., 2012) do in fact appear to provide proof of principle for autophagy inhibition as a potentially effective therapeutic strategy. However, it can also be concluded that chloroquine is unlikely to be uniformly effective in suppressing autophagy in tumors *in vivo*. Interestingly, although there is fairly extensive literature on the pharmacokinetics of chloroquine in humans, there is relatively limited information from animal studies. Pharmacokinetic studies of chloroquine in both animal models and in human subjects indicate that chloroquine has quite a long half-life (Mzayek et al., 2007; Lim et al., 2009; Karunajeewa et al., 2010; Moore et al., 2011), indicating that drug clearance is unlikely to be a limiting factor in the potential capacity of chloroquine to suppress of autophagy. However, the maximal concentrations of chloroquine achieved in the plasma fall only within the range of 1.5 and 3 $\mu$ M, even with daily administration of the drug, concentrations which are unlikely to be sufficient to effectively interfere with autophagy (based on the concentrations required for autophagy inhibition in cell culture). It should however be noted that chloroquine accumulates in the lysosomes within cells and plasma levels may therefore not accurately reflect the levels in tumor tissues, especially when chloroquine treatment occurs over a long period of time. Overall, the variable effectiveness of chloroquine in enhancing sensitivity to chemotherapy and radiation in animal studies may relate to differences in the capacity of tumor models to accumulate chloroquine as well as differential sensitivity of tumors to autophagy inhibition by

JPET #199802 PIP

chloroquine. Consequently, a more potent autophagy inhibitor that might be developed for clinical use could actually prove to be quite effective as an adjunctive therapy.

Finally, although chloroquine is perhaps the most widely used drug to inhibit autophagy *in vitro*, and its effects on cell death have been directly attributed to its inhibition of autophagy, the mechanism by which chloroquine induces cell death could involve other functions independent of its ability to block autophagy that are less well characterized. Among these, chloroquine can decrease sequestration of anti-cancer drugs such as doxorubicin and mitoxantrone in endosomes by raising endosomal pH, thereby increasing their availability and cytotoxicity (Solomon and Lee, 2009). It can also promote cell cycle arrest (Jiang et al., 2010), intercalate into DNA (Solomon and Lee, 2009), activate ATM and p53 and induce apoptosis (Bakkenist and Kastan, 2003). These actions are likely to confuse interpretation of studies in the literature where different outcomes are observed for the combination treatment of chloroquine with chemotherapeutic drugs or radiation.

**Relevance of the current experimental findings** We observed that pharmacological inhibition of autophagy with chloroquine and genetic silencing of the autophagy gene, ATG12, failed to influence radiation sensitivity in 4T1 breast tumor cells *in vitro*. In complementary experiments, the autophagy inhibitor, chloroquine, failed to influence sensitivity to radiation in 4T1 cells grown in a syngeneic animal model of breast cancer. The lack of radiation sensitization *in vitro* complements earlier studies where chloroquine failed to sensitize tumor cells to cisplatin (Maycotte et al, 2012), a chemotherapeutic agent that, like radiation, has DNA as its primary target. The lack of radiosensitization *in vivo* may be due to additional factors such as the fact that 4T1 cells proliferate rapidly and palpable tumors quickly become necrotic and ulcerated,



JPET #199802 PIP

preventing long term observational studies. In one similar study where the effect of chloroquine on radiation sensitivity was investigated in 4T1 cells (Jiang et al, 2010), and where the animals were injected with twice the number of cells as in the current work, no impact of chloroquine treatment on radiation-inhibited tumor growth was detected until 37 days post-challenge. This may relate to the development of hypoxia, as proposed Rouschop et al. (2012) and that is further discussed below.

Studies of radiation sensitization by chloroquine that were performed in HCT-116 colon carcinoma and U373 glioblastoma cells (Rouschop et al, 2010) provide unequivocal evidence of tumor growth delay by the combination treatment, which is ascribed to a reduction of hypoxia-induced autophagy by chloroquine. Of note, the growth curves for radiation alone and for chloroquine + radiation did not begin to diverge until after 14 days, which presumably reflects the time required for the tumors to become hypoxic. This may explain, in part, the lack of sensitization to radiation in our own animal studies, where the experiments were necessarily terminated after 15-18 days due to animal health and safety concerns.

It is further of interest that in the study by Rouschop et al. (2010) sensitization to radiation *did not occur* in cell culture under either normoxic or hypoxic conditions, which is consistent with our findings in 4T1 cells. We also observed that neither chloroquine nor 3-MA conferred radiation sensitization in Hs578t breast tumor cells (unpublished observations). Taken together, these studies argue that radiation does not uniformly promote a cytoprotective form of autophagy that can be exploited for the purpose of tumor cell sensitization. This inconsistency in the effectiveness of chloroquine clearly also appears to be the case for chemotherapeutic drugs, where the effectiveness or lack thereof of this approach is likely to be drug and tumor specific.

JPET #199802 PIP

**Conclusions** It is worth considering that studies in xenograft models alone may be insufficient for accurately determining the capacity of autophagy inhibition to sensitize tumors to chemotherapy or radiation as a recent elegant study has shown that therapeutic success may be highly dependent on the immune system. In this work, anthracyclines and oxaliplatin did not alter the proliferation of tumors growing in athymic mice but only in immune-competent animals; furthermore, autophagy was determined to be essential for the release of immunogenic ATP from dying cells (Michaud et al., 2011; Martins et al., 2012); these findings indicate that syngeneic models of cancer and xenografts are likely to demonstrate uniquely different responses to therapeutic approaches involving autophagy induction or inhibition.

In additional preliminary studies in both non-small cell lung cancer cell lines and head and neck cancer cell lines, we have observed that autophagy inhibition by chloroquine does not consistently enhance radiation sensitivity (unpublished observations). Taken together with the studies in animal models discussed in this review, it can be predicted that autophagy inhibition is unlikely to be uniformly effective in increasing radiation or chemotherapeutic sensitivity across the tumor spectrum. However, this is not unusual or unexpected given that the sensitivities of malignancies of different origins to chemotherapeutic approaches can differ by orders of magnitude.

It is possible, but as yet untested, that the strength of autophagy induction in each system might determine its susceptibility to autophagy inhibition and sensitization. However, as autophagy is a dynamic process and as autophagy related proteins are expressed and regulated differently among tissues (Klionsky et al., 2012), it is currently quite difficult to quantify and compare the degree of induction caused by different drugs, especially *in vivo*.

JPET #199802 PIP

Accepting the premise that autophagy inhibition can potentially serve to promote chemosensitization and radiosensitization, it follows that it will be critical to identify biomarkers that will distinguish between malignancies that are likely to benefit from autophagy inhibition as a strategy for chemosensitization or radiation sensitization and those tumors which are unlikely to benefit from this therapeutic approach. Furthermore, it will be necessary to establish that the drug(s) used to modulate autophagy are capable of achieving and maintaining concentrations in the tumor cells that will suppress autophagy, possibly throughout the course of treatment. However, in considering the variability and uncertainty in the current literature relating to autophagy inhibition as a therapeutic strategy, caution appears to be warranted in extrapolating to the clinic from the limited preclinical data in animal models of cancer that have combined chloroquine with chemotherapy or radiotherapy.

### **Acknowledgements**

The parental 4T1 cells were generously provided by Dr. Fred Miller at the Karmanos Cancer Institute at Wayne State University School of Medicine. The 4T1-luc cells were generously provided by Dr. Kazuaki Takabe at Virginia Commonwealth University.

### **Authorship Contributions**

Designed and performed experiments: Bristol, Emery, Maycotte, Chakradeo

Contributed to the writing of the manuscript: Bristol, Emery, Maycotte, Gewirtz, Thorburn

JPET #199802 PIP

## References

- Abedin MJ, Wang D, McDonnell MA, Lehmann U, Kelekar A. (2007) Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell Death Differ* **14**:500-510.
- Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB. (2007) Autophagy inhibition enhances therapy-induced apoptosis in a myc-induced model of lymphoma. *J Clin Invest* **117**:326-336.
- Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. (2008) Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. *Cancer Res* **68**:1485-1494.
- Bakkenist CJ and Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**:499-506.
- Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Metivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. (2005) Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol* **25**:1025-1040.
- Bristol ML, Di X, Beckman MJ, Wilson EN, Henderson SC, Maiti A, Fan Z, Gewirtz DA. (2012) Dual functions of autophagy in the response of breast tumor cells to radiation: Cytoprotective autophagy with radiation alone and cytotoxic autophagy in radiosensitization by vitamin D 3. *Autophagy* **8**:1-15.
- Carew JS, Medina EC, Esquivel JA, 2nd, Mahalingam D, Swords R, Kelly K, Zhang H, Huang P, Mita AC, Mita MM, Giles FJ, Nawrocki ST. (2010) Autophagy inhibition enhances vorinostat-induced apoptosis via ubiquitinated protein accumulation. *J Cell Mol Med* **14**:2448-2459.
- Chaachouay H, Ohneseit P, Toulany M, Kehlbach R, Multhoff G, Rodemann HP. (2011) Autophagy contributes to resistance of tumor cells to ionizing radiation. *Radiother Oncol* **99**:287-292.
- Ding ZB, Hui B, Shi YH, Zhou J, Peng YF, Gu CY, Yang H, Shi GM, Ke AW, Wang XY, Song K, Dai Z, Shen YH, Fan J. (2011) Autophagy activation in hepatocellular carcinoma contributes to the tolerance of oxaliplatin via reactive oxygen species modulation. *Clin Cancer Res* **17**:6229-6238.
- Djavaheri-Mergny M, Botti J, Codogno P. (2007) Autophagy and autophagic cell death. *Apoptosis, Senescence, and Cancer* **93**:107.
- Fu L, Kim YA, Wang X, Wu X, Yue P, Lonial S, Khuri FR, Sun SY. (2009) Perifosine inhibits mammalian target of rapamycin signaling through facilitating degradation of major components in the mTOR axis and induces autophagy. *Cancer Res* **69**:8967-8976.

JPET #199802 PIP

Gaudin D and Yielding KL. (1969) Response of a "resistant" plasmacytoma to alkylating agents and x-ray in combination with the "excision" repair inhibitors caffeine and chloroquine. *Proc Soc Exp Biol Med* **131**:1413-1416.

Ghadimi MP, Lopez G, Torres KE, Belousov R, Young ED, Liu J, Brewer KJ, Hoffman A, Lusby K, Lazar AJ, Pollock RE, Lev D. (2012) Targeting the PI3K/mTOR axis, alone and in combination with autophagy blockade, for the treatment of malignant peripheral nerve sheath tumors. *Mol Cancer Ther* **11**:1758-1769.

Godbole AM, Purushottamachar P, Martin MS, Daskalakis C, Njar VC. (2012) Autophagy inhibition synergistically enhances anticancer efficacy of RAMBA, VN/12-1 in SKBR-3 cells, and tumor xenografts. *Mol Cancer Ther* **11**:898-908.

Golden EB, Pellicciotta I, Demaria S, Barcellos-Hoff MH and Formenti SC (2012) The convergence of radiation and immunogenic cell death signaling pathways. *Front Oncol* **2**:88.

Guo XL, Li D, Hu F, Song JR, Zhang SS, Deng WJ, Sun K, Zhao QD, Xie XQ, Song YJ, Wu MC, Wei LX. (2012) Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells. *Cancer Lett* **320**:171-179.

Hu YL, DeLay M, Jahangiri A, Molinaro AM, Rose SD, Carbonell WS, Aghi MK. (2012) Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. *Cancer Res* **72**:1773-1783.

Jiang PD, Zhao YL, Deng XQ, Mao YQ, Shi W, Tang QQ, Li ZG, Zheng YZ, Yang SY, Wei YQ. (2010) Antitumor and antimetastatic activities of chloroquine diphosphate in a murine model of breast cancer. *Biomed Pharmacother* **64**:609-614.

Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* **11**:448-457.

Karunajeewa HA, Salman S, Mueller I, Baiwog F, Gomorrai S, Law I, Page-Sharp M, Rogerson S, Siba P, Ilett KF and Davis TM (2010) Pharmacokinetics of chloroquine and monodesethylchloroquine in pregnancy. *Antimicrob Agents Chemother* **54**:1186-1192.

Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, Ahn HJ, Ait-Mohamed O, Ait-Si-Ali S, Akematsu T, Akira S, Al-Younes HM, Al-Zeer MA, Albert ML, Albin RL, Alegre-Abarrategui J, Aleo MF, Alirezaei M, Almasan A, Almonte-Becerril M, Amano A, Amaravadi R, Amarnath S, Amer AO, Andrieu-Abadie N, Anantharam V, Ann DK, Anoopkumar-Dukie S, Aoki H, Apostolova N, Arancia G, Aris JP, Asanuma K, Asare NY, Ashida H, Askanas V, Askew DS, Auberger P, Baba M, Backues SK, Baehrecke EH, Bahr BA, Bai XY, Bailly Y, Baiocchi R, Baldini G, Balduini W, Ballabio A, Bamber BA, Bampton ET, Banhegyi G, Bartholomew CR, Bassham DC, Bast RC, Jr., Batoko H, Bay BH, Beau I, Bechet DM, Begley TJ, Behl C, Behrends C, Bekri S, Bellaire B,

JPET #199802 PIP

Bendall LJ, Benetti L, Berliocchi L, Bernardi H, Bernassola F, Besteiro S, Bhatia-Kissova I, Bi X, Biard-Piechaczyk M, Blum JS, Boise LH, Bonaldo P, Boone DL, Bornhauser BC, Bortoluci KR, Bossis I, Bost F, Bourquin JP, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady NR, Brancolini C, Brech A, Brenman JE, Brennan A, Bresnick EH, Brest P, Bridges D, Bristol ML, Brookes PS, Brown EJ, Brumell JH, et al. (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8**:445-544.

Kondo Y, Kanzawa T, Sawaya R, Kondo S. (2005) The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* **5**:726-734.

Liang X, De Vera ME, Buchser WJ, Romo de Vivar Chavez A, Loughran P, Beer Stolz D, Basse P, Wang T, Van Houten B, Zeh HJ, 3rd, Lotze MT. (2012) Inhibiting systemic autophagy during interleukin 2 immunotherapy promotes long-term tumor regression. *Cancer Res* **72**:2791-2801.

Lim HS, Im JS, Cho JY, Bae KS, Klein TA, Yeom JS, Kim TS, Choi JS, Jang IJ and Park JW (2009) Pharmacokinetics of hydroxychloroquine and its clinical implications in chemoprophylaxis against malaria caused by *Plasmodium vivax*. *Antimicrob Agents Chemother* **53**:1468-1475.

Lim Y, Hedayati M, Merchant AA, Zhang Y, Yu HH, Kastan MB, Matsui W, Deweese TL. (2012) Chloroquine improves survival and hematopoietic recovery after lethal low-dose-rate radiation. *Int J Radiat Oncol Biol Phys* . **84**:800-806

Livesey KM, Tang D, Zeh HJ, Lotze MT. (2009) Autophagy inhibition in combination cancer treatment. *Curr Opin Investig Drugs* **10**:1269-1279.

Loehberg CR, Strissel PL, Dittrich R, Strick R, Dittmer J, Dittmer A, Fabry B, Kalender WA, Koch T, Wachter DL, Groh N, Polier A, Brandt I, Lotz L, Hoffmann I, Koppitz F, Oeser S, Mueller A, Fasching PA, Lux MP, Beckmann MW, Schrauder MG. (2012) Akt and p53 are potential mediators of reduced mammary tumor growth by cloroquine and the mTOR inhibitor RAD001. *Biochem Pharmacol* **83**:480-488.

Lopez G, Torres K, Lev D. (2011) Autophagy blockade enhances HDAC inhibitors' pro-apoptotic effects: Potential implications for the treatment of a therapeutic-resistant malignancy. *Autophagy* **7**:440-441.

Ma XH, Piao S, Wang D, McAfee QW, Nathanson KL, Lum JJ, Li LZ, Amaravadi RK. (2011) Measurements of tumor cell autophagy predict invasiveness, resistance to chemotherapy, and survival in melanoma. *Clin Cancer Res* **17**:3478-3489.

Martins I, Michaud M, Sukkurwala AQ, Adjemian S, Ma Y, Shen S, Kepp O, Menger L, Vacchelli E, Galluzzi L, Zitvogel L and Kroemer G (2012) Premortem autophagy determines the immunogenicity of chemotherapy-induced cancer cell death. *Autophagy* **8**:413-415.

JPET #199802 PIP

Maycotte P, Aryal S, Cummings CT, Thorburn J, Morgan MJ, Thorburn A. (2012) Chloroquine sensitizes breast cancer cells to chemotherapy independent of autophagy. *Autophagy* **8**:200-212.

Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, Rello-Varona S, Tailler M, Menger L, Vacchelli E, Galluzzi L, Ghiringhelli F, di Virgilio F, Zitvogel L and Kroemer G (2011) Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* **334**:1573-1577.

Mirzoeva OK, Hann B, Hom YK, Debnath J, Aftab D, Shokat K, Korn WM. (2011) Autophagy suppression promotes apoptotic cell death in response to inhibition of the PI3K-mTOR pathway in pancreatic adenocarcinoma. *J Mol Med (Berl)* **89**:877-889.

Mizushima N, Yoshimori T and Levine B (2010) Methods in mammalian autophagy research. *Cell* **140**:313-326.

Moore BR, Page-Sharp M, Stoney JR, Ilett KF, Jago JD and Batty KT (2011) Pharmacokinetics, pharmacodynamics, and allometric scaling of chloroquine in a murine malaria model. *Antimicrob Agents Chemother* **55**:3899-3907.

Mzayek F, Deng H, Mather FJ, Wasilevich EC, Liu H, Hadi CM, Chansolme DH, Murphy HA, Melek BH, Tenaglia AN, Mushatt DM, Dreisbach AW, Lertora JJ and Krogstad DJ (2007) Randomized dose-ranging controlled trial of AQ-13, a candidate antimalarial, and chloroquine in healthy volunteers. *PLoS Clin Trials* **2**:e6.

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* **61**:439-444.

Pan Y, Gao Y, Chen L, Gao G, Dong H, Yang Y, Dong B, Chen X. (2011) Targeting autophagy augments in vitro and in vivo antimyeloma activity of DNA-damaging chemotherapy. *Clin Cancer Res* **17**:3248-3258.

Pazmino NH and Yuhas JM. (1974) Chloroquine: Nonselective inhibition of recovery from radiation injury in tumors and normal tissues. *Radiat Res* **60**:54-61.

Qadir MA, Kwok B, Dragowska WH, To KH, Le D, Bally MB, Gorski SM. (2008) Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. *Breast Cancer Res Treat* **112**:389-403.

Rao R, Balusu R, Fiskus W, Mudunuru U, Venkannagari S, Chauhan L, Smith JE, Hembruff SL, Ha K, Atadja P, Bhalla KN. (2012) Combination of pan-histone deacetylase inhibitor and autophagy inhibitor exerts superior efficacy against triple-negative human breast cancer cells. *Mol Cancer Ther* **11**:973-983.

JPET #199802 PIP

Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkoul K, Keulers T, Mujcic H, Landuyt W, Voncken JW, Lambin P, van der Kogel AJ, Koritzinsky M, Wouters BG. (2010) The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *J Clin Invest* **120**:127-141.

Sasaki K, Tsuno NH, Sunami E, Kawai K, Hongo K, Hiyoshi M, Kaneko M, Muroto K, Tada N, Nirei T, Takahashi K, Kitayama J. (2012) Resistance of colon cancer to 5-fluorouracil may be overcome by combination with chloroquine, an in vivo study. *Anticancer Drugs* . **23**:675-682

Shi YH, Ding ZB, Zhou J, Hui B, Shi GM, Ke AW, Wang XY, Dai Z, Peng YF, Gu CY, Qiu SJ, Fan J. (2011) Targeting autophagy enhances sorafenib lethality for hepatocellular carcinoma via ER stress-related apoptosis. *Autophagy* **7**:1159-1172.

Solomon VR and Lee H. (2009) Chloroquine and its analogs: A new promise of an old drug for effective and safe cancer therapies. *Eur J Pharmacol* **625**:220-233.

Sotelo J, Briceno E, Lopez-Gonzalez MA. (2006) Adding chloroquine to conventional treatment for glioblastoma multiforme: A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* **144**:337-343.

U.S. National Institutes of Health. (2012).

Wilson EN, Bristol ML, Di X, Maltese WA, Koterba K, Beckman MJ, Gewirtz DA. (2011) A switch between cytoprotective and cytotoxic autophagy in the radiosensitization of breast tumor cells by chloroquine and vitamin D. *Horm Cancer* **2**:272-285.

Wu Z, Chang PC, Yang JC, Chu CY, Wang LY, Chen NT, Ma AH, Desai SJ, Lo SH, Evans CP, Lam KS, Kung HJ. (2010) Autophagy blockade sensitizes prostate cancer cells towards src family kinase inhibitors. *Genes Cancer* **1**:40-49.

Xu CX, Zhao L, Yue P, Fang G, Tao H, Owonikoko TK, Ramalingam SS, Khuri FR, Sun SY. (2011) Augmentation of NVP-BEZ235's anticancer activity against human lung cancer cells by blockage of autophagy. *Cancer Biol Ther* **12**:549-555.

Zhao H, Cai Y, Santi S, Lafrenie R, Lee H. (2005) Chloroquine-mediated radiosensitization is due to the destabilization of the lysosomal membrane and subsequent induction of cell death by necrosis. *Radiat Res* **164**:250-257.



JPET #199802 PIP

## Footnotes

Work in Dr. Gewirtz's laboratory was supported, in part, by a grant from the National Institutes of Health [5R01CA135043]; Molly Bristol was supported, in part, by a predoctoral training grant from the Department of Defense [W81XWH-09-1-0020]; Sean Emery was supported, in part, by a predoctoral training grant from the Department of Defense [W81XWH-11-1-016]. Work in Dr. Thorburn's laboratory was supported by grants from the National Institutes of Health [R01CA150925 and RO1CA111421].

JPET #199802 PIP

## Legends for Figures

**Figure 1.** Chloroquine treatment does not affect clonogenic survival in 4T1 cells treated with radiation. 4T1 cells were irradiated at 2, 5 or 10 Gy +/- CQ (10, 25 or 30  $\mu$ M) and evaluated for autophagy induction by Western Blotting for LC3 (A) or allowed to recover for 7 days and clonogenic growth was assessed (B). Graphs in A show density analysis of the mean  $\pm$  SE of 3 independent experiments. \* Different from untreated control, # different from control + CQ ( $p < 0.05$ ). Graph in B shows quantification of clonogenic assays, where control was normalized to 100%. Graph shows mean  $\pm$  SE of 3 independent experiments performed in triplicate. No statistical differences were found between untreated and CQ treated samples ( $p < 0.05$ ).

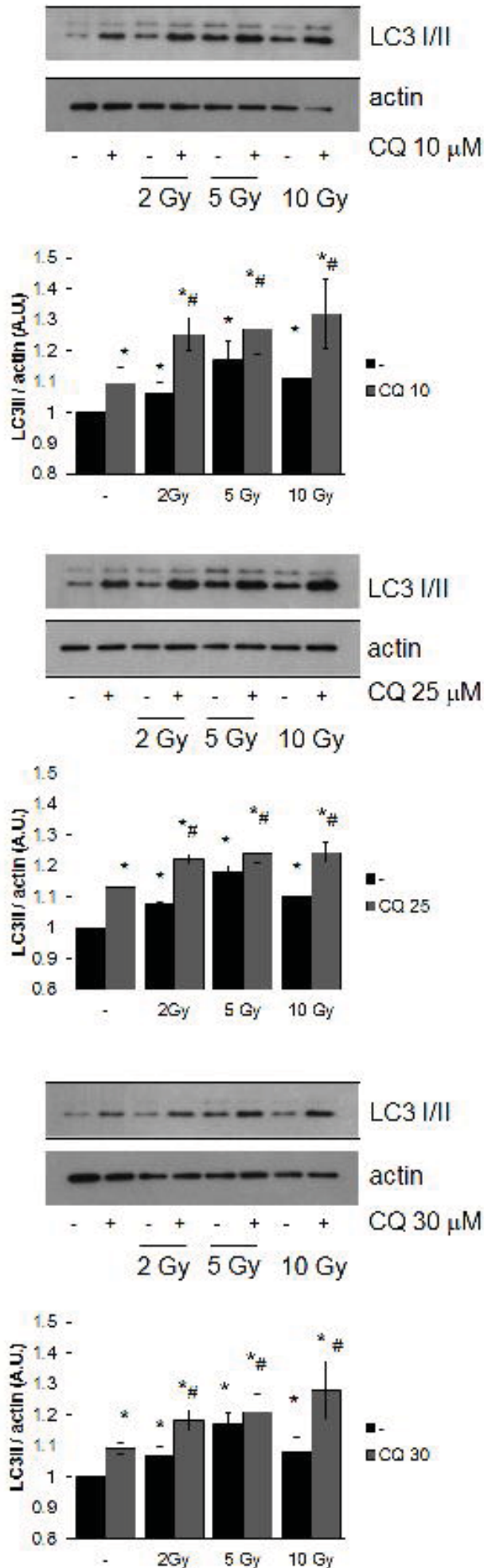
**Figure 2.** Atg12 knockdown does not affect clonogenic survival in 4T1 cells treated with radiation. 4T1 cells expressing an *Atg12* or non-silencing shRNA were treated with doxycycline (doxy) at the indicated concentrations for 72 h and tested for Atg12 knockdown (2A). Autophagic flux measured by LC3II Western Blot was evaluated in *Atg12* shRNA expressing cells treated  $\pm$  irradiation, 1  $\mu$ M doxycycline or 10  $\mu$ M chloroquine (2B). Survival was assessed as clonogenic growth of cells treated  $\pm$  doxycycline 1  $\mu$ g/mL, exposed to radiation (Rad) and allowed to recover for 7 days (2C). Graphs show mean  $\pm$  SE of 3 independent experiments performed in triplicate. Graphs in B show density analysis of the mean  $\pm$  SE of 3 independent experiments. \* Different from untreated control, # different from same treatment without doxycycline ( $p < 0.05$ ).

**Figure 3.** Influence of IR, CQ, or the combination on bioluminescence and final tumor weight. Animals were injected with luciferin and bioluminescence was monitored at the indicated times

JPET #199802 PIP

(A-IR, C-CQ; E-CQ + IR). Animals were sacrificed and tumors were resected and weighed (B-IR; D-CQ; F-CQ + IR). \*  $p < 0.05$ ; \*\*  $p < 0.001$ .

A



B

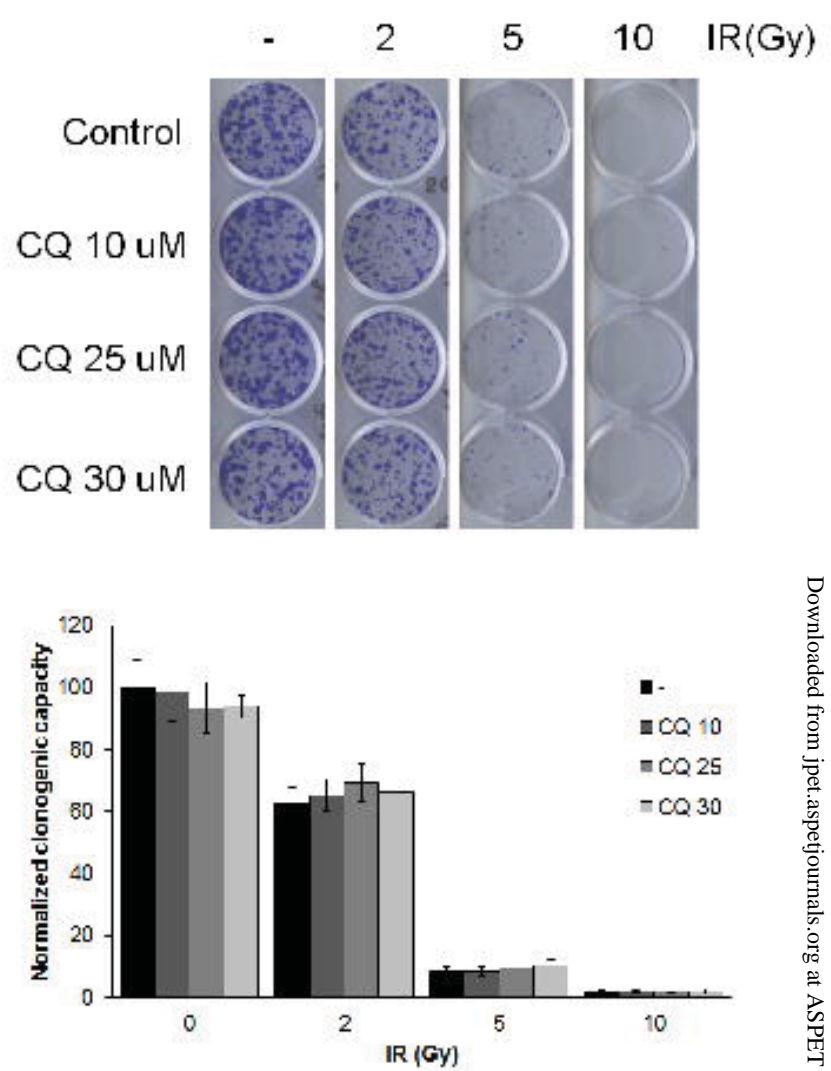


Figure 1

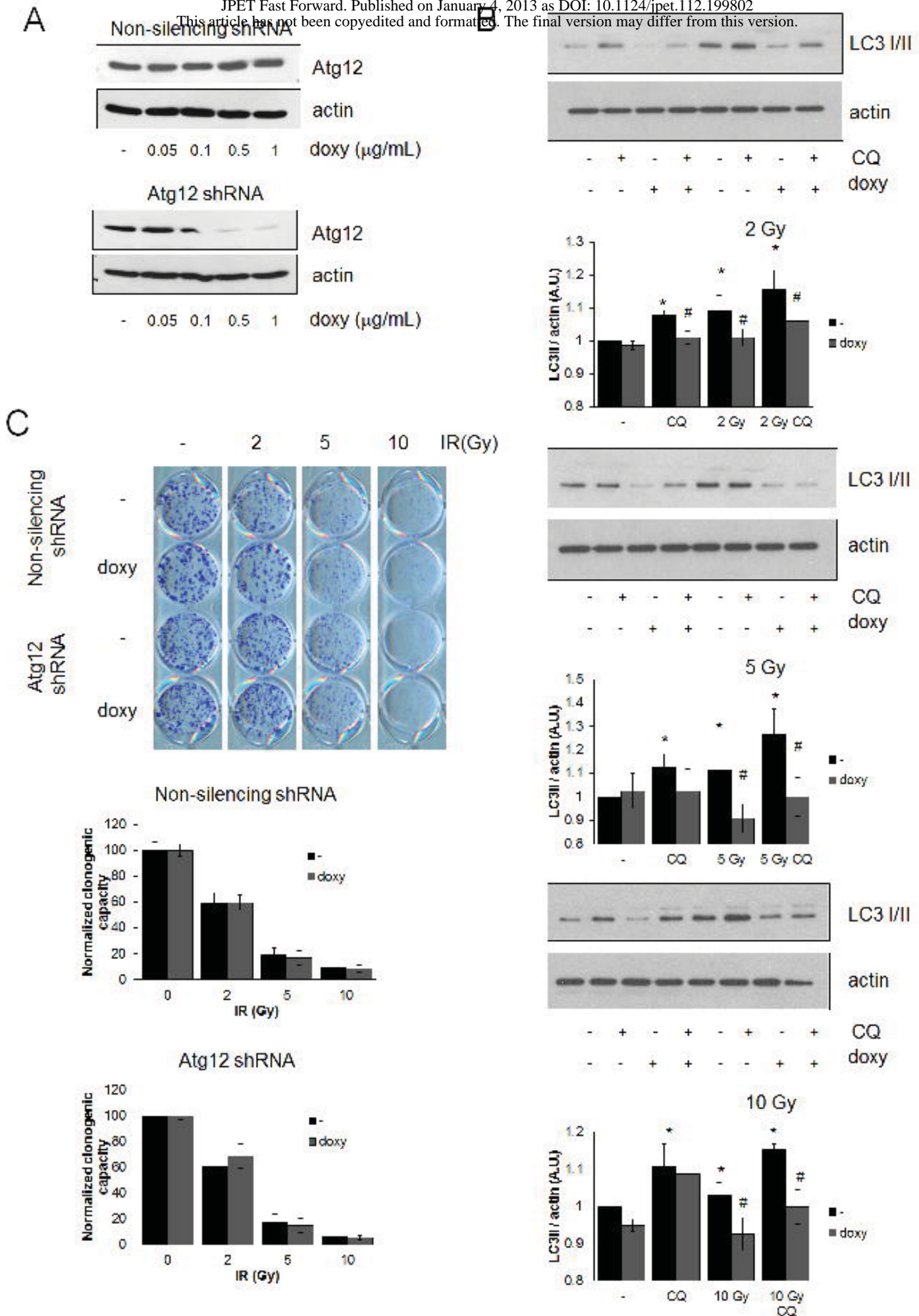


Figure 2

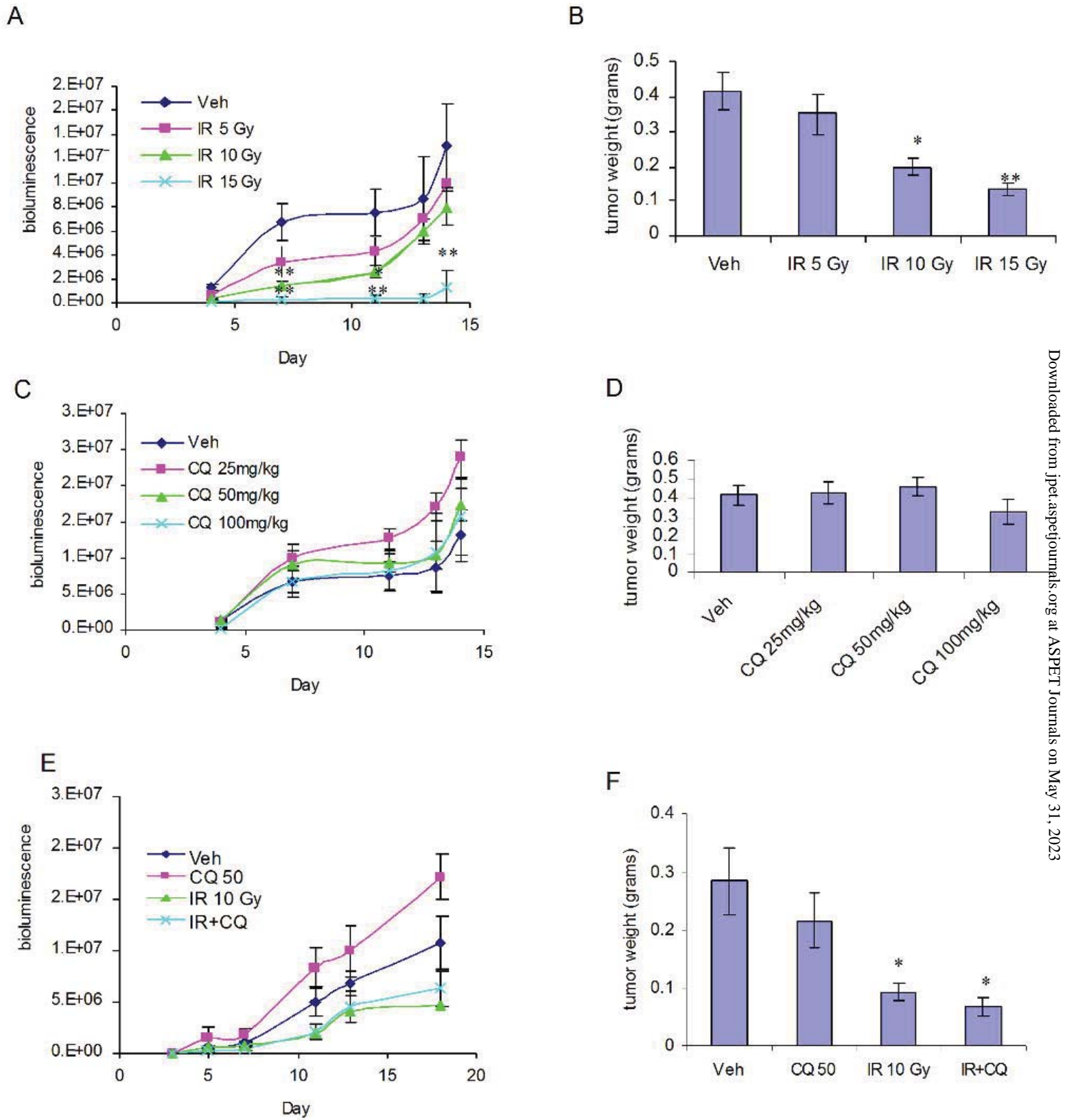


Figure 3