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Inhibition of autophagy stimulate molecular iodine-induced apoptosis in hormone independent breast tumors

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ABSTRACT

Estrogen receptor negative (ER^{-ve}) and p53 mutant breast tumors are highly aggressive and have fewer treatment options. Previously, we showed that molecular Iodine (I_2) induces apoptosis in hormone responsive MCF-7 breast cancer cells, and non-apoptotic cell death in ER^{-ve}–p53 mutant MDA-MB231 cells (Shrivastava, 2006). Here we show that I_2 (3 μM) treatment enhanced the features of autophagy in MDA-MB231 cells. Since autophagy is a cell survival response to most anti-cancer therapies, we used both *in vitro* and *in vivo* systems to determine whether ER^{-ve} mammary tumors could be sensitized to I_2-induced apoptosis by inhibiting autophagy. Autophagy inhibition with chloroquine (CQ) and inhibitors for PI3K (3MA, LY294002) and H+/ATPase (baflomycin) resulted in enhanced cell death in I_2 treated MDA-MB231 cells. Further, CQ (20 μM) in combination with I_2, showed apoptotic features such as increased sub-G1 fraction (~5-fold), expression of cleaved caspase-9 and -3 compared to I_2 treatment alone. Flowcytometry of I_2 and CQ co-treated cells revealed increase in mitochondrial membrane permeability ($p < 0.01$) and translocation of cathepsin D activity to cytosol relative to I_2 treatment. For *in vivo* studies ICRC mice were transplanted subcutaneously with MMTV-induced mammary tumors. A significant reduction in tumor volumes, as measured by MRI, was found in I_2 and CQ co-treated mice relative to I_2 or vehicle treated mice. These data indicate that inhibition of autophagy renders ER^{-ve} breast tumor cells more sensitive to I_2 induced apoptosis. Thus, I_2 together with autophagy inhibitor could have a potential tumorostatic role in ER^{-ve} aggressive breast tumors that may be evaluated in future studies.

1. Introduction

Breast cancer, a leading cause of cancer related deaths in women, is one of the most frequently diagnosed cancers. Breast tumors are considered estrogen dependent and consequently, anti-estrogens and aromatase inhibitors are used as hormonal treatment for hormone responsive tumors, i.e. those with estrogen receptor (ER) and progesterone receptor (PR) expression in the tumor tissue hormone treatment is not effective in patients with ER/PR negative tumors. Furthermore, in many cases, ER positive (ER^{+ve}) tumors initially respond to hormonal treatment but subsequently become resistant to endocrine therapy [1].

About 50% of breast cancers have p53 deficient/mutant tumor suppressor gene, a critical mediator of cell death. Aggressive nature of ER negative p53 mutant/deficient breast tumors require better therapeutic choices. It has been recently shown that deletion, depletion or inhibition of p53 induces autophagy [2], whether autophagy represents a mechanism for cell death or survival is unclear and could lead to therapeutic resistance [3]. In this regard, inhibition of autophagy, by chloroquine (CQ), has been found to enhance therapeutic efficacy in cMyc lymphoma [4]. However, the concurrent use of CQ has not been evaluated with breast cancer therapies.

Iodine has long been known to maintain the normal physiology of thyroid and breast tissue. Molecular iodine (I_2) has been found effective in diminishing mammary dysplasia and atypia resulting from iodine deficiency, symptoms of mammary fibrosis in women and occurrence of chemically induced mammary cancer in rats (50–70%) [5]. Earlier we demonstrated an estrogen receptor and p53 status independent cytotoxic effect of I_2 in breast cancer cells [6]. Furthermore, chronic I_2 supplementation in rats had no

Abbreviations: CQ, chloroquine; I_2, molecular iodine; MMTV, mouse mammary tumor virus; ER, estrogen receptor.

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demonstrable harmful secondary effects on either thyroid or general physiology [7].

In the present study we attempt to demonstrate that I_2 induces cell death as well as autophagic response. In addition, we evaluated if inhibition of autophagy by sub-therapeutic antimalarial dose of chloroquine (CQ) enhances I_2 mediated cell death by p53 independent and caspase mediated apoptosis, and whether CQ potentiates apoptotic and tumor regressive effects of I_2 treatment *in vivo*.

2. Materials and methods

2.1. Cytotoxicity assay

Human breast cancer cell line MDA-MB231 was obtained and maintained as described previously [6]. Cells were treated with either vehicle, 3 μM I_2 alone or combination of 3 μM I_2 and 20 μM chloroquine (Sigma). The stock solution of 75 μM iodine was prepared as previously described [6]. In parallel experiments following inhibitors were used; baflomycin (Sigma, 20 nM); 3 methyladenine (3MA) (Sigma, 1 mM) and LY294002 (Sigma, 10 μM). Cell viability was assessed by trypan blue (0.01%) dye-exclusion assay.

2.2. Live cell staining

We performed Acridine orange (Sigma) and Hoechst (Molecular Probes) staining as described previously [8]. In separate set of experiments, cells were processed for immunocytochemistry using mouse anti-microtubule-Associated Protein-1 light chain-3 antibody, LC-3 (1:250, a kind gift of Dr. Tamotsu Yoshimori) as described previously [8].

2.3. Transmission electron microscopy

Standard electron microscopy procedures were followed as described previously using FET Tecnai-12 Twin electron microscope [8].

2.4. Flow cytometric analysis

Sub diploid peak analysis and Mitochondrial Transmembrane Potential (MPT) measurement, using fluorescent probe dihexyloxacarbocyanine iodide (DiOC6, Molecular Probes), were performed through FACScan (Becton Dickinson) as described previously [6].

2.5. Cathepsin D enzymatic activity

Enzymatic activity in whole cell lysates and lysosomal free cytosolic fractions was assayed using hemoglobin substrate as described by Schultz et al. [9]. A unit of cathepsin D activity is defined as the amount of enzyme necessary to cause an absorbance change of 1.0 at 280 nm following substrate incubation. Protein content was measured using Bradford method.

2.6. Western blotting

Immunoblotting was performed as described previously [6]. Primary antibodies against, rabbit Beclin-1 and mouse LC-3 (a kind gift of Dr. Tamotsu Yoshimori), rabbit cleaved caspase-3 and -9 (Abcam), mouse Bcl-2 (Santacruz) and mouse α tubulin (Santacruz) were used according to recommended dilutions.

2.7. Mammary tumor mouse model

ICRC female mice were maintained and all animal procedures were performed according to Institutional Guidelines for Animal Care and Research. Murine mammary tumor virus (MMTV)-induced ICRC mice were used for tumor transplantation. Tumor pieces of 3–4 mm^3 from a spontaneous mammary tumor harboring ICRC mice were subcutaneously transplanted in 20 female ICRC mice. After 2 weeks of tumor transplantation, mice were treated with either vehicle ($n = 5$), I_2 (0.5 mg/kg body weight orally; $n = 7$), or I_2 plus chloroquine (40 mg/kg body weight, intramuscular $n = 8$). Body weights were measured weekly throughout the study.

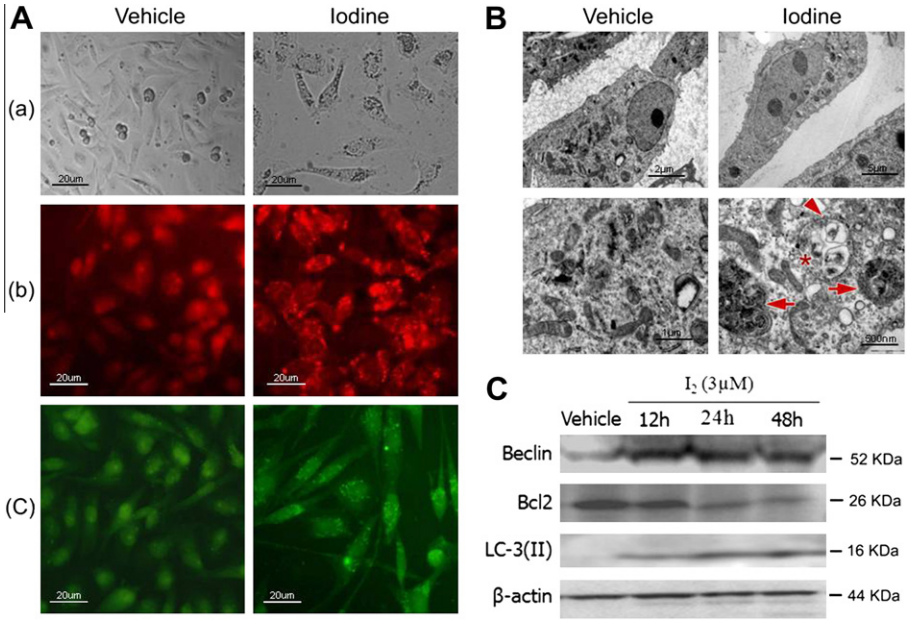

Fig. 1. Molecular iodine (I_2) induces cell death and activates autophagy in MDA-MB231 cells. (A) Representative microscopic images evaluating autophagic features in MDA-MB231 cells treated with 3 μM I_2 relative to vehicle; (a) morphological alterations (phase contrast, 60×); (b) acridine orange staining for acidic vesicles accumulation and (c) LC-3 immunostaining (fluorescence microscopy, 60×). (B) Representative electron-micrograph shows ultra-structural alterations in response to I_2, autophagosome (astric); autolysosome formation (arrow); vesicular fusion of autophagosomes (arrow heads), (a) 1 μM (b) 5 nM. (C) Representative immunoblot of whole cell lysate for Beclin, BCl_2 and cleaved LC3 expression.

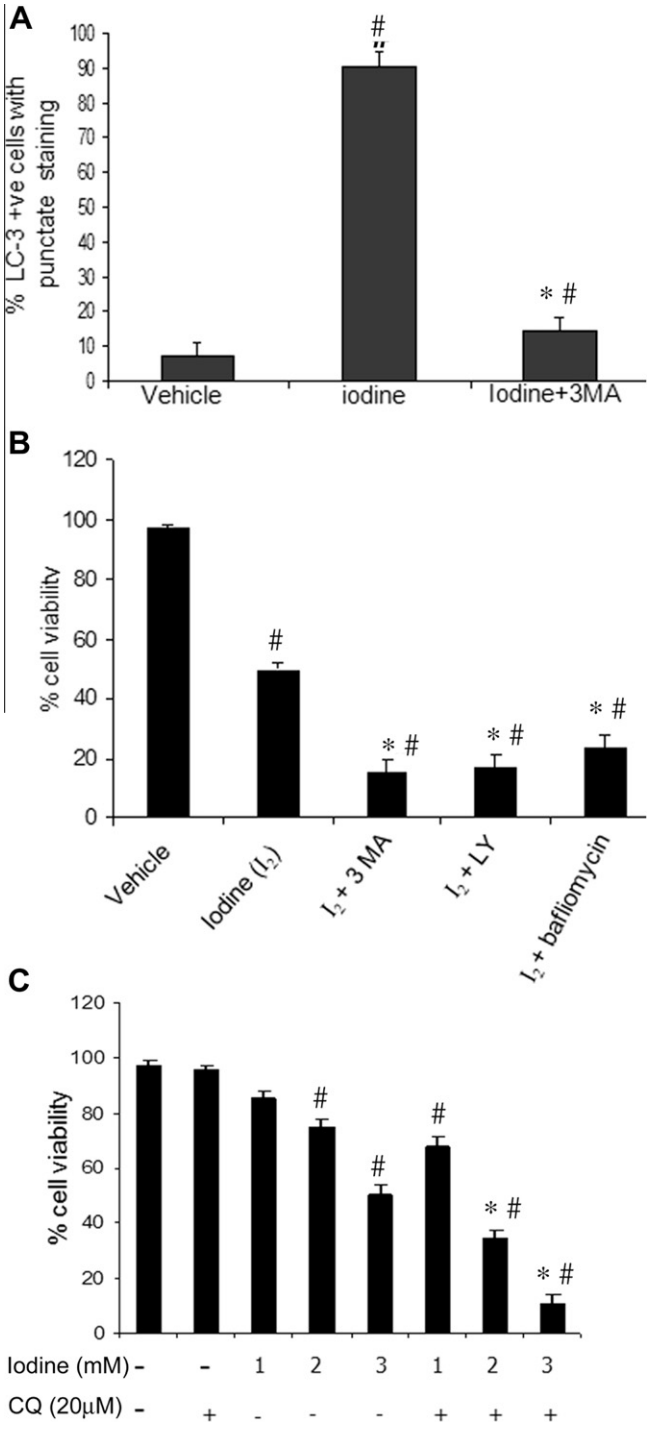

Fig. 2. Autophagy in response to molecular iodine (I_2) treatment provides survival advantage. (A) Mean of LC3 positive MDA-MB231 cells counted under fluorescence microscope in five different fields after immunostaining. MDA-MB231 cell viability (percentage) using trypan blue assay after 48 h of I_2 treatment with or without; 1 mM 3MA, 10 μM LY 294002 or 20 nM baflomysin (B), and 20 μM chloroquine (C). *Indicates $p < 0.05$ versus I_2 and # versus vehicle.

2.8. Magnetic resonance imaging

Tumor volumes were measured weekly using magnetic resonance imaging with micro-imaging accessories at 9.4 T (Bruker Biospin NMR with wide bore spectrometer). Tumor size was assessed in each serial MR image by Region of Interest (ROI) based measurements as described by Mayr et al. [10].

2.9. Immunohistochemistry

Formalin-fixed paraffin-embedded tumoral tissues were processed by standard method using monoclonal rabbit anti-cleaved caspase-3 antibody (1:500, Abcam).

2.10. Statistical analysis

SPSS software was used for Statistical analysis. Results were reported as mean ± S.E. of three independent experiments. Groups were compared by Student's *t* test where $p < 0.05$ was considered significant. The tumor volume comparison was evaluated by Mann–Whitney *U*-test and one way ANOVA.

3. Results and discussion

3.1. Molecular iodine induces cell death and autophagy in MDA-MB231 cells

Previously, we found that I_2 induces apoptosis in hormone responsive (ER positive) and p53 positive MCF-7 cells, however, in MDA-MB231 breast cancer cells I_2 induces non-apoptotic cell death [6]. In this study we confirmed that MDA-MB231 breast cancer cells are resistant to apoptotic effects of iodine. High levels of mutant p53 gene in these cells, stabilized by elevated phospholipase D (PLD) activity, may contribute to the suppression of apoptosis [11]. However, for the first time we now provide evidence of autophagy activation in MDA-MB231 cells in response to I_2 treatment, as indicated by increased vacuolation, accumulation of acidic vacuoles, autophagosome formation evident by punctate immunostaining of LC-3 as well as increased cleaved LC-3 levels and enhanced lysosomal activity (Figs. 1A, C and 4D). Electron microscopy observations conclusively point towards autophagic features (Fig. 1B). Direct interaction of antiapoptotic Bcl-2 protein with Beclin-1 has been shown as a mechanism to inhibit autophagy in yeast and mammalian cells [12]. Our observations of significant increase in Beclin-1 and down-regulation of Bcl-2 proteins in response to I_2 (Fig. 1C) are suggestive of a similar mechanism with possible complex formation of Beclin-1 with PI3-kinases [13].

3.2. Autophagy as a defense mechanism against iodine induced cytotoxicity

To answer the question whether autophagy contributes to the effectiveness of tumor therapy or is a defense mechanism in dying cells, we performed fluorescent imaging in the presence of PI3 kinase inhibitor-3MA. LC3 immunostained cells showed fewer numbers of punctuated stained cells on 3MA plus I_2 treatment as compared to I_2 treatment alone (Fig. 2A), suggesting disruption of autophagosome formation. In addition, inhibition of autophagy with PI3K (3MA, LY294002) or H+/ATPase (baflomycin) inhibitors enhance the cytotoxic response of I_2 (Fig. 2B). These data suggest that autophagy is acting as survival mechanism while extensive damage may be responsible for cytotoxic response in I_2 treated cancer cells. Induction of autophagy has been demonstrated in the surviving fraction of MCF7 cells following irradiation and tamoxifen therapy [3], and in response to herceptin in Her2 positive breast tumors [14]. Our data along with these observations [3,14] suggest that inclusion of autophagy inhibitor may improve therapeutic efficacy of anti-cancer drugs in breast cancer.

Chloroquine (CQ) has been shown to block autophagy by its lysosomotropic property that raises intralysosomal pH and subsequent accumulation of ineffective autolysosomes [4]. Therefore, we tested whether CQ, a widely used antimalarial drug, can be used to enhance the cytotoxic response of I_2. Using a dose of 20 μM CQ, we

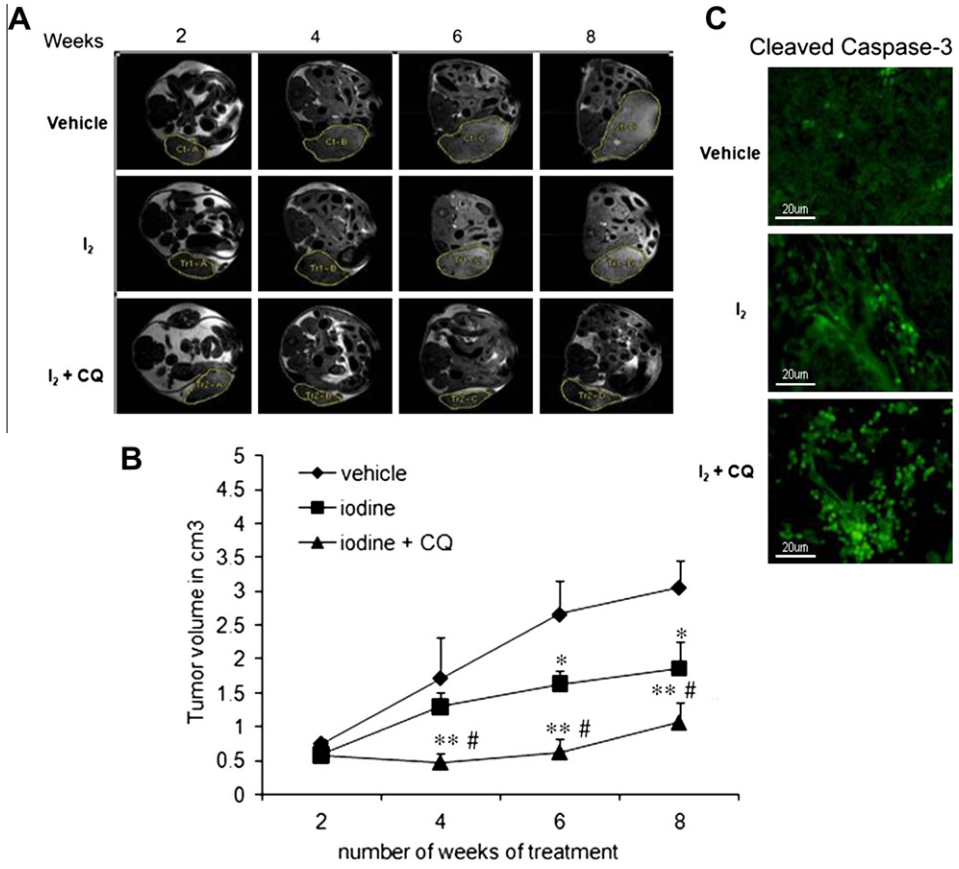

Fig. 3. Inhibition of autophagy by chloroquine (CQ) potentiates tumor regressive and apoptotic action of iodine. (A) Representative MRI image showing tumors (encircled areas) from MMTV-induced mammary tumor harboring ICRC mice treated with vehicle, I_2 or I_2 plus CQ. (B) Line graph showing tumor volume as measured by MRI. (C) Representative cleaved caspase-3 staining in tumor tissue excised from mice at the end of the study (60×). *Indicates $p < 0.05$, **indicate $p < 0.01$ versus vehicle and # versus I_2.

found that, while treatment with CQ alone had no effect on growth of MDA-MB231 cells, but in combination with I_2 it markedly increased the cytotoxic response of I_2 (Fig. 2C). At higher dose, i.e. 50–60 μM, CQ alone has been shown to inhibit proliferation and cause apoptosis in various cancer cell lines, including MDA-MB231 cells [15–17]. However, given the known side effects of CQ at such a high concentration, there is little if any, clinical significance of these *in vitro* studies [18].

3.3. Autophagy inhibition by CQ potentiates the tumor regressive and apoptotic potential of iodine in ER negative tumors

We further tested whether combination of CQ could benefit the action of I_2 on hormone independent tumors or tumors that are resistant to conventional treatments. We employed MMTV-induced mammary tumor in an animal model system for breast cancer mimicking the human disease. Chiplunkar and Karande [19] have shown the expression of MMTV antigens in the mammary epithelium of ICRC mice strains during mammary tumorigenesis as they have higher rate of mammary tumor occurrence. The loss of estrogen and progesterone receptors after successive passage was confirmed by immunohistochemistry in implanted MMTV-induced tumors (data not shown). Tumor volume was significantly smaller in both, I_2 and CQ plus I_2 groups ($p < 0.05$ and $p < 0.01$, respectively by one-way ANOVA) compared to vehicle group where steady tumor growth was observed throughout the course of study (Fig. 3A and B). Moreover, relative to I_2 group, tumor volume was significantly smaller in I_2 plus CQ group ($p < 0.05$ by Mann–Whitney). CQ was able to enhance I_2 induced apoptosis as indicated by increased cleaved caspase-3 positivity (Fig. 3C). These *in vivo* observations are in good agreement with our *in vitro* studies in MDA-MB231 breast cancer cells. In agreement with the previous studies [5,7], no apparent side effects were observed after chronic I_2 supplementation (data not shown) suggesting its potential tumorostatic role.

3.4. Co-treatment of iodine and chloroquine (CQ) induces p53 independent and mitochondria mediated apoptosis

We explored the mechanism by which CQ potentiates cytotoxic effect of I_2 in MDA-MB231 cells. We observed that chloroquine in combination with I_2 treatment not only inhibited autophagy but also induced apoptosis in p53 mutant MDA-MB231 cells. This was evidenced by increased accumulation of subG1 fraction in flow cytometry, and nuclear fragmentation (Fig. 4A) and activation of caspase-9 and -3 (Fig. 4B). Thus, induction of p53 independent apoptosis by CQ appears to potentiate cytotoxic effect of I_2.

Our findings indicate two plausible mechanisms for the induction of p53 independent apoptosis by combined I_2 and CQ treatment. Possibility that CQ may disrupt lysosomal membrane permeability and thereby releasing I_2-induced cathepsin D to the cytosol was supported by enhanced cathepsin D levels in lysosome free cytosolic fraction ($p < 0.05$, Fig. 4D). This may contribute to p53 independent caspase mediated apoptotic cell death [20]. Secondly, mild disruption of mitochondrial membrane permeability (MPT) on I_2 treatment may not be enough to cause apoptosis, however, substantially enhanced MPT on CQ co-treatment could augment apoptosis

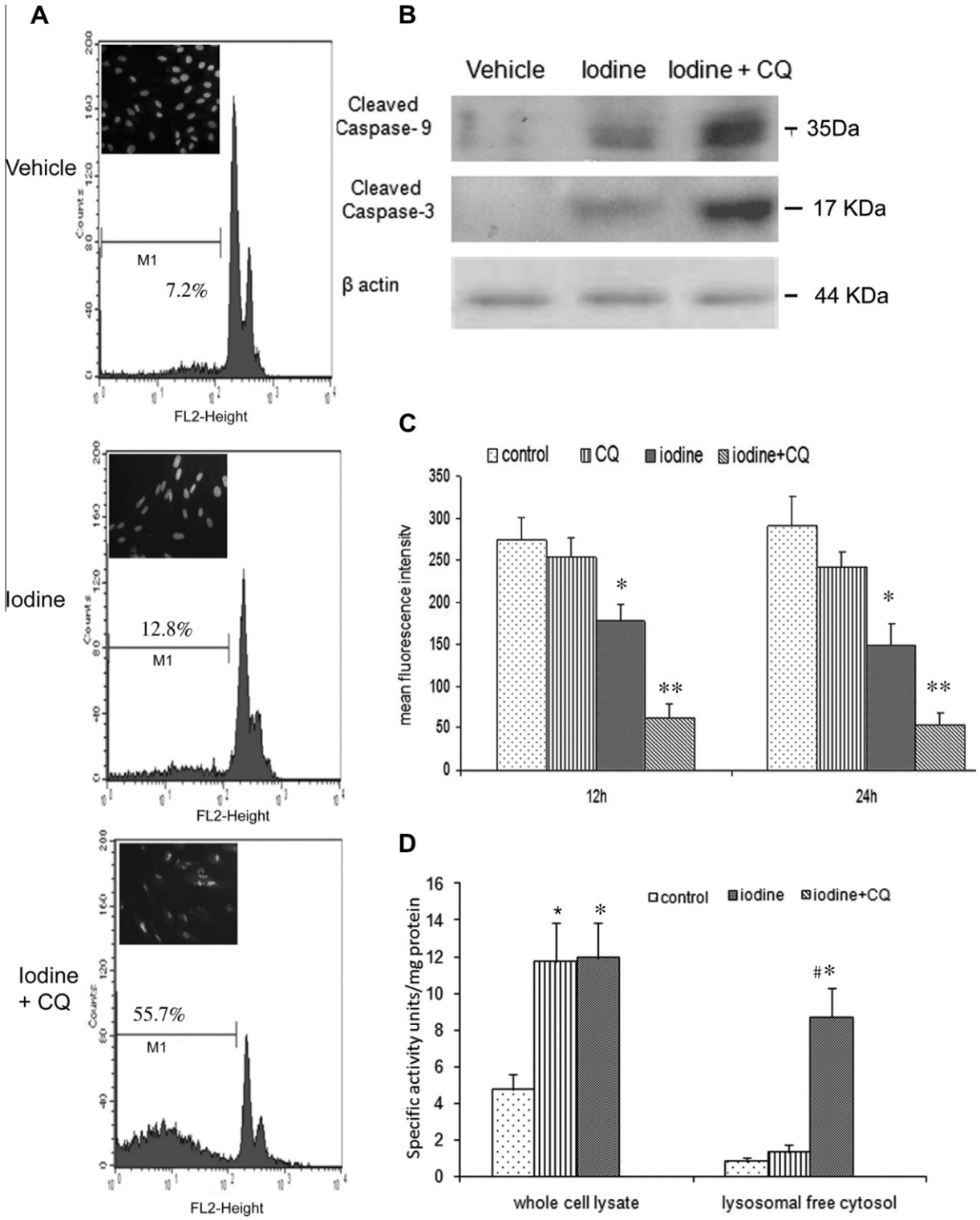

Fig. 4. Co-treatment of iodine and chloroquine (CQ) induces p53 independent and mitochondria mediated apoptosis. (A) SubG1 fraction analysis of MDA-MB231 cells treated with I_2 plus CQ or I_2 alone using flow cytometry following PI staining, upper left corner of the graph shows Hoechst stained cells from respective treatment. (B) Representative immunoblot of whole cell lysate for cleaved caspase-3 and -9 expression. (C) Flowcytometric analysis of mitochondrial permeability transition using DiOC6 dye. (D) Cathepsin D enzyme activity assay. *Indicates $p < 0.05$ and **indicate $p < 0.01$ versus vehicle and # versus I_2.

(Fig. 4C). Even lower doses of I_2 (1–2 μM) were found to enhance MPT when combined with CQ (data not shown).

Overall our results indicate that CQ potentiates I_2-induced cytotoxic effect on hormone independent breast cancers. Further studies on the potential synergistic benefits of I_2 and CQ combination along with existing therapeutic modalities need to be explored in hormone independent and p53 deficient breast tumors in carefully designed clinical research trials.

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