Am J Cancer Res 2023;13(8):3741-3752
www.ajcr.us /ISSN:2156-6976/ajcr0149764

Original Article

Modulation of mitochondrial apoptosis by β2-adrenergic receptor blockage in colorectal cancer after radiotherapy: an *in-vivo* and *in-vitro* study

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Received February 20, 2023; Accepted July 17, 2023; Epub August 15, 2023; Published August 30, 2023

Abstract: Colorectal cancer (CRC) is one of the leading causes of malignancy-related deaths worldwide. Radiotherapy is often combined with surgery to treat patients with more advanced CRC. Despite impressive initial clinical responses, radiotherapy resistance is the main reason for most treatment failures in colorectal cancer. The G protein-coupled adrenergic receptor (AR) has shown to involve in the development and radiotherapy resistance of CRC. The β2-AR blockage (ICI-118,551) can use to inhibit the progression of CRC through downregulating EGFR-Akt-ERK1/2 signaling. Since catecholamines-activated the G protein-coupled AR activation has been shown to result in radioresistant, co-treatment with both β2-AR blockage and radiation may be improved the clinical outcome of CRC. We demonstrated that selective β2-AR blockage, but not selective β1-AR blockage, significantly enhanced radiation-induced apoptosis in CRC cells with wild-type *p53 in vitro*. The molecular mechanism of the apoptotic pathway was possibly triggered by a change in the mitochondrial membrane permeability and release of cytosolic cytochrome C through phospho-P53 mitochondrial translocation. We also found that a P53 knockout in the HCT116 cells was correlated with reversing β2-AR blockage-mediated apoptosis induction after radiation treatment. Furthermore, the β2-AR blockage significantly inhibited CRC cell-xenograft growth *in vivo*. Our study suggests that β2-AR blockage may be used as adjunct agent for improving the clinical outcomes of CRC following radiotherapy by inducing apoptosis in CRC cells.

Keywords: Colorectal cancer, β2-adrenergic receptor, radiotherapy, P53, mitochondrial

Introduction

Colorectal cancer (CRC) is one of the top ten most commonly diagnosed malignancy worldwide [1]. The incidence and mortality rates of CRC are 10% and 9.4%, respectively [2]. Approximately 25% to 30% of CRC patients are diagnosed with *in situ* liver metastases [3, 4]. In clinical practice, radiotherapy is often combined with surgery to raise the survival rates of CRC patients [5]. Despite radiotherapy being an effective treatment strategy, the underlying molecular mechanisms of radiotherapy resistance that often develops in CRC cases are unknown.

The DNA damage response and certain extracellular receptors are activated after cells are exposed to radiation. The *p53* gene plays a critical role in response to DNA damage, including DNA repair, cell cycle arrest, apoptosis, and cellular senescence [6, 7]. The expression and phosphorylation of P53 is upregulated by promoting its migration into the nucleus and preventing its degradation, after radiation exposure [8]. Mutation of the *p53* gene is observed in approximately 40%-50% of CRC cases, and mainly results in cancer progression and drug-resistant tumor cell generations [9]. Loss of one *p53* allele ($p53^{+/-}$) [10] or mutation of the gene [11] can lead to early development of tumors in mice. In recent years, it has been revealed that P53 mediates a novel transcription-independent signaling pathway in the cytoplasm [12]. These finding indicate that P53 directly partici-

pates in mitochondrial apoptosis, in which a fraction of stress-stabilized wild-type *p53* is rapidly translocated to the mitochondrial outer membrane, and initiates changes to the mitochondrial membrane potential, cytochrome C release, and caspase activation [12].

Chronic restraint stress is one of reasons that result in the failure of radiotherapy [13]. When the chronic restraint stress response is stimulated, epinephrine and norepinephrine are produced and released, binding directly to G protein-coupled adrenergic receptors (ARs) [14, 15]. Research by Hasegawa et al. has shown that chronic restraint stress can increase tumor development through β-AR activation [16]. Another study demonstrated that radiation also enhances phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein, and that knockdown of CREB by decoy oligonucleotides can significantly increase radiosensitivity in cancer cells [17]. The expression of CREB may be induced through the β-ARs-cAMP-PKA signaling pathway [18]. Works by Liao et al. have also shown that β-AR suppression by a β-AR blockage enhanced the radiosensitivity of gastric cancer [19]. Using a β-AR blockage in non-small cell lung cancer cells can further raise the sensitivity of tumor cells to radiotherapy and conventional chemotherapy *in vitro* [20].

The β2-AR receptor is a member of the β-AR family, which has been shown to participate in various processes involved in colorectal cancer and progression [21]. The treatment of HT29 colorectal carcinoma cells with epinephrine enhanced cell proliferation by β2-AR stimulation in a COX-2 dependent pathway, in one recent report [22]. Another study reported that norepinephrine-induced locomotion of SW480 colorectal carcinoma cells was mediated by the β2-AR signaling pathway [23]. Our previous study showed that the administration of a β2-AR blockage significantly suppressed the viability of CRC cells that expressed a wild-type Kirsten rat sarcoma virus (KRAS), by downregulating the EGFR-Akt/Erk1/2 signaling pathway and thus inducing apoptosis [24]. However, the synergistic effect of β2-AR blockage plus radiotherapy in CRC cells remains unclear. In this study, we investigated the therapeutic effect of a combination of a β2-AR blockage and radiation for CRC treatment, through the lens of the P53 signaling pathway.

Materials and methods

Cell lines

The CRC cell lines HCT116, LS174T, and HT29 were purchased from the Taiwan Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). The HCT116 cells were cultured in McCoy's 5A medium (Thermos Fisher Scientific, Massachusetts, USA). The LS174T cells were cultured in minimum essential medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The HT29 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (Thermos Fisher Scientific, Massachusetts, USA). Wild type HCT116 ($p53^{+/+}$) cell line and isogenic *p53*-null cell line (HCT116 ($p53^{-/-}$)) were purchased from Horizon Discovery Group (Cambridge, UK) [25] and cultured in RPMI 1640 medium (Thermos Fisher Scientific, Massachusetts, USA). All media were supplemented with 10% fetal bovine serum (Thermos Fisher Scientific, Massachusetts, USA) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), in accordance with the manufacturer's instructions.

Apoptosis analysis

To evaluate apoptosis, fluorescein isothiocyanate (FITC)-labeled annexin-V and PI (BD Biosciences Inc., California, USA) were used as double stains to assess early and late apoptosis in live and necrotic cells. Firstly, the CRC cells were seeded in 6-well plates with complete-medium-containing 10% FBS. After 18 hours, the cells were treated with radiation (2, 4 and 8 Gy), following which they were further treated with β1/2-, β1-, β2-AR blockages for 48 hrs. After treatment, the cells were washed and stained with annexin-V-FITC and PI, and analyzed by flow cytometry.

Immunocytochemistry

HCT116 cells were irradiated, fixed in 4% paraformaldehyde for 10 minutes at room temperature, and processed for immunocytochemistry. The cells were permeabilized with 0.25% Triton X-100 in phosphate buffered saline and incubated overnight at 4°C with one of the following monoclonal anti-mouse primary antibodies phosphorylation of P53 (Santa Cruz

Biotechnology, California, USA) and cytochrome C (BD Biosciences Inc., California, USA), followed by incubation with a secondary Alexa Fluor 488 conjugated antibody (Molecular Probes, Oregon, USA) for an hour at room temperature. The samples were then mounted in Vectashield Mounting Media supplemented with DAPI (Vector Laboratories Inc., California, USA) and inspected in a fluorescence confocal microscope (TCS SP5 II, Leica Microsystems, Germany). To study colocalization, mitochondria were labeled by incubation of cells with 200 nM Mitotracker Red (Molecular Probes, Oregon, USA) for 30 minutes at 37°C before fixation.

In vivo tumorigenicity assay

To evaluate the effect of radiation combined with β1/2- and β2-AR blockages on the growth of HCT116 xenograft tumors *in vivo*, 6-week-old nude mice (nu/nu) (BioLASCO, Taipei, Taiwan) were used with the approval of the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (Chiayi, Taiwan). For this assay, 1×10^6 HCT116 cells were suspended in 50 μL of phosphate buffered saline (PBS), mixed with an equal volume of Matrigel® (Corning Inc., Oneonta, NY, USA), and injected subcutaneously into the mice. After the resulting tumors had grown to approximately 100 mm^3, the mice were treated with radiation at 8 Gy and then injected with β1/2- and β2-AR blockages for three weeks. The tumor volumes were measured every three days, and they were not allowed to exceed volumes of 2000 mm^3. After 21 days, the tumors were excised, photographed, fixed, and frozen for further analysis.

Statistical analyses

The statistical analysis was performed by GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). Data was expressed as means (± standard deviation (SD)). The Bonferroni post-hoc test was used to compare data between more than two groups *in vivo*. Statistical significance was defined by $P < 0.001$.

Results

β2-AR, but not β1-AR, blockage induced apoptosis of CRC cells harboring wild-type p53 after radiotherapy

Our previous study showed that selective β2-AR blockage significantly inhibited the viability of CRC cell lines harboring wild-type *Kras* but not mutant *Kras* genes [24]. However, the synergistic effect of the combination of β1/2-AR blockages and radiation on CRC cell growth remains unknown. To further investigate whether the β1/2-AR signaling pathway was involved in the desensitization of CRC cells by radiotherapy, we used radiation combined with β1/β2-AR blockages. Propranolol (non-selective β1/2-AR blockage), atenolol (selective β1-AR blockage), and ICI-118,551 (selective β2-AR blockage) were incubated with HCT116 and LS174T cell lines harboring wild-type *p53*/mutant *Kras* and a HT29 cell line harboring mutant *p53*/wild-type *Kras* for 48 hrs. The results showed that propranolol (**Figure 1A** and Supplementary Figure 1A, 1D and 1G) and ICI-118,551 (**Figure 1C** and Supplementary Figure 1C, 1F and 1I), but not atenolol (**Figure 1B** and Supplementary Figure 1B, 1E and 1H), significantly increased the percentages of early and late apoptosis in HCT116 and LS174T cells. However, the apoptotic rate of HT29 cells was not raised by treatment with any of the various blockages in a dose-dependent manner. These results showed that the *p53* gene might be involved in the apoptosis pathway of CRC cells harboring wild-type *p53* when exposed to a combination of selective β2-AR blockage and radiotherapy.

Selective β2-AR blockage enhanced the mitochondrial translocation of phosphorylated-P53 in HCT116 cells after radiotherapy

Since the apoptosis pathway was significantly triggered by co-treatment with both selective β2-AR blockage and radiotherapy in CRC cell lines harboring wild-type *p53*, the potential underlying molecular mechanisms were explored via the protein changes of these cells following the treatment. **Figure 2A** shows that both phosphorylated P53 (p-P53) and total P53 were present in higher levels in both the radiation alone and the radiation plus ICI-118,551 groups, compared to the ICI-118,551 alone group. These levels remained unchanged in the ICI-118,551 alone group in a dose-dependent manner with radiation treatment. In cases where co-treatment with ICI-118,551 and radiation was found to induce cellular apoptosis but p-P53 and total P53 levels remained unchanged, localization of p-P53 in HCT116 cells was directly confirmed by immunofluorescence. **Figure 2B** indicates that the p-P53 was activated and showed enhanced

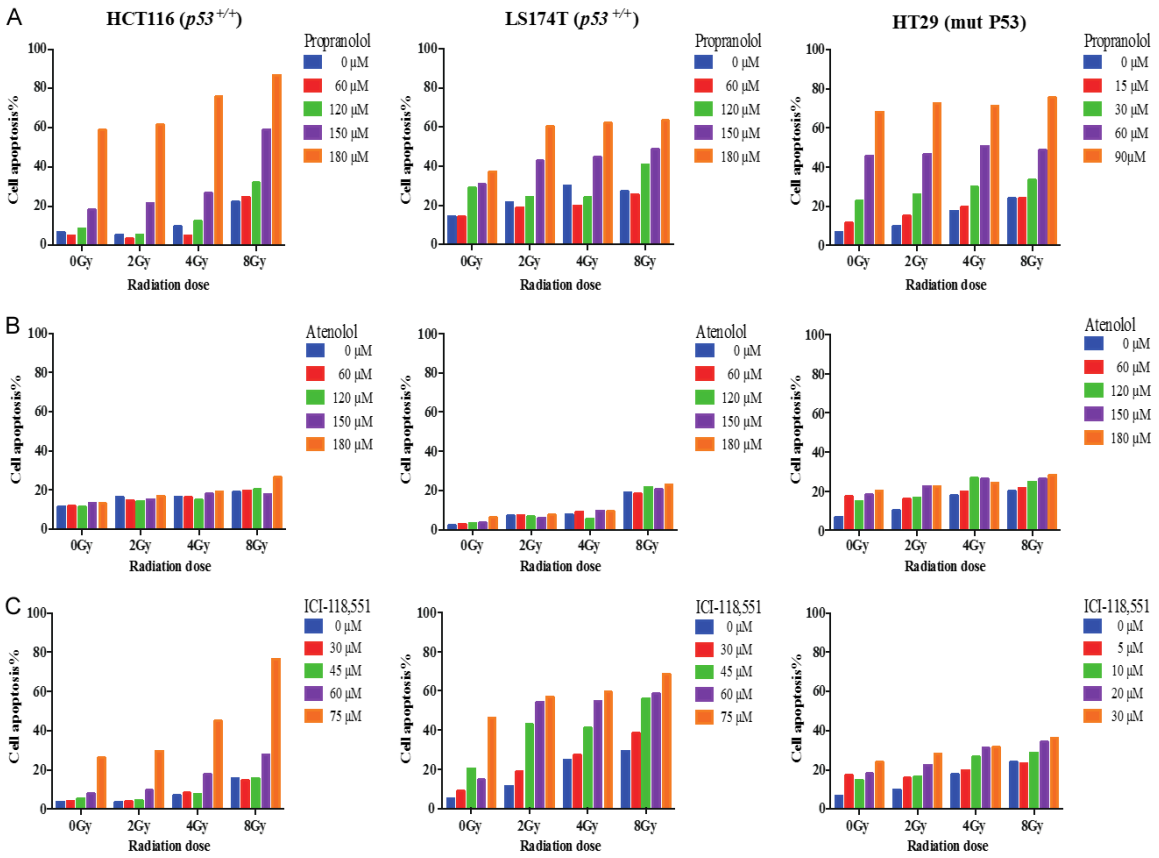

Figure 1. Selective β2-AR blockage enhanced apoptosis of colorectal cancer (CRC) cells with wild-type *p53* after radiation treatment. CRC cells were incubated with β1/2-, β1- and β2-AR blockages after radiation treatment for 48 hrs. After treatment, apoptosis of CRC cells was measured by flow cytometry with annexin V staining. A. Propranolol; B. Atenolol; C. ICI-118,551.

nuclear translocation when radiation was used alone, whereas radiation plus ICI-118,551 notably promoted mitochondrial translocation over nuclear. These findings showed that P53 mainly induced apoptosis of CRC cells by changing the mitochondrial membrane permeability. However, western blotting revealed that the expression of cellular myelocytomatosis (c-Myc) was significantly down-regulated in the radiation plus ICI-118,551 group, compared to the ICI-118,551 alone group (**Figure 2A**). c-Myc is a transcriptional factor that can regulate cell survival and proliferation [26, 27]. A previous study demonstrated that silencing of *c-myc* oncogene expression by siRNA inhibits cell proliferation in CRC cells *in vitro* and *in vivo* [28]. In contrast, overexpression of c-Myc in various cancer is related to radiotherapy resistance [29, 30]. For this reason, we used the transient transfection of the *c-myc* vector to enforce c-Myc expression in HCT116 cells. **Figure 3A** shows that the transient transfection of *c-myc* in HCT116 cells strongly upregulated c-Myc overexpression. On the other hand, the enforced c-Myc overexpression could not reverse the enchantment of HCT116 cell apoptosis in the radiation plus ICI-118,551 group (**Figure 3B** and Supplementary Figure 2). This finding indicated that c-Myc might not play a critical role in the apoptosis of CRC cells during treatment with a combination of both radiation and selective β2-AR blockage.

Selective β2-AR blockage promoted mitochondrial cytochrome C release in HCT116 cells after radiotherapy

A recent study reported that P53 was implicated in a mitochondrial function, where it enhanced cell apoptosis [31]. Our previous report further showed that catecholamine-β2-AR signaling-transactivated EGFR-Akt-ERK1/2 is a survival dependent mechanism in β2-AR-blockade-sensitive (wild-type *Kras* HT29 and Colo205) but not-insensitive (mutated *Kras*

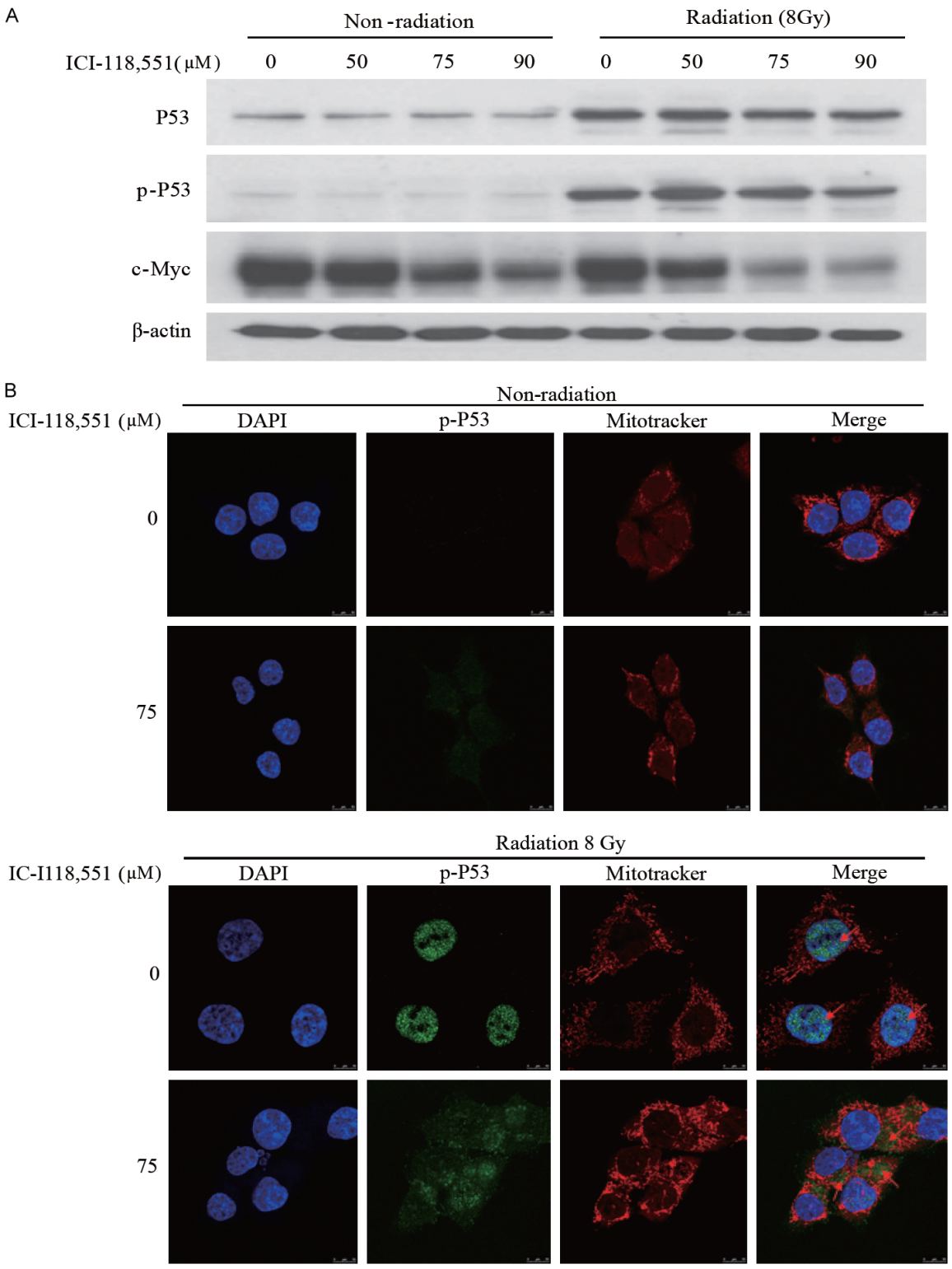

Figure 2. Co-treatment with selective β2-AR blockage and radiation promoted p-P53-migration to mitochondria in HCT116 cells. A. Selective β2-AR blockage did not affect the expression and phosphorylation of P53 protein, but did diminish c-Myc expression after radiation treatment. B. Selective β2-AR blockage enhanced p-P53 translocation to mitochondria after radiation treatment.

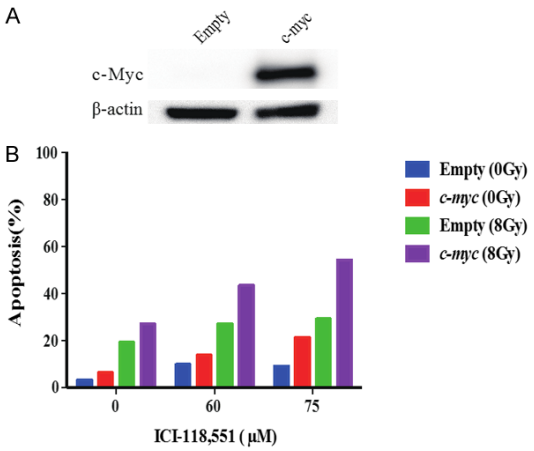

Figure 3. Co-treatment with selective β2-AR blockage and radiation non-dependently utilized c-Myc signaling to induce CRC cell apoptosis. A. After CRC cells were introducing *c-myc* gene for 48 hrs, the c-Myc proteins were strongly over-expressed. B. The enforced expressions of c-Myc did not reverse β2-AR blockage-mediated apoptosis enhancement of CRC cells after radiotherapy.

HCT116 and DLD1) CRC cells. Moreover, ICI-118,551 could also promote cell apoptosis induction and enhance cytochrome C release from mitochondria to the cytosol in wild-type *Kras* HT29 and Colo205 cell lines [24]. For this reason, we assessed whether mitochondrial translocation of p-P53 contributed to mitochondrial dysfunction upon co-treatment of ICI-118,551 and radiation in CRC cell harboring wild-type *p53*. **Figure 4A** shows that when HCT116 cells were exposed to either radiation or ICI-118,551 alone, the MitoTracker (red) stain and the cytochrome C (green) stain were co-localized in cellular mitochondria but that radiation plus ICI-118,551 resulted in the loss of the mitochondrial membrane potential and diffusion of cytochrome C. **Figure 4B** and Supplementary Figure 3 indicate that the numbers of mitochondrial cytochrome C release in ICI-118,551 plus Radiation group were more than ICI-118,551 and radiation alone. These findings indicated that co-treatment with ICI-118,551 and radiation promoted the induction of apoptosis in CRC cells, through mitochondrial dysfunction and cytosolic cytochrome C release.

Co-treatment with ICI-118,551 and radiotherapy resulted in the induction of apoptosis in HCT116 cells through a P53 signaling pathway

Previous studies have shown that chronic restraint-induced stress can decrease P53 function and promote carcinogenesis in p53-heterozygous mice exposed to radiation [13, 32]. Co-treatment with selective β2-AR blockage and radiation did not enhance the expression and phosphorylation of P53 in a dose-dependent manner, but it could raise the CRC cells apoptosis ratio. Here, we verified the physiological significance of β2-AR and the P53 signaling pathway on apoptosis in CRC cells following radiotherapy, HCT116 cells with wild type *p53* (*p53*$^{+/+}$) and isogenic P53 knockout (*p53*$^{-/-}$) were used. **Figure 5A** shows that p-P53 and total P53 were not detected in HCT116 cells with *p53*$^{-/-}$ after radiation plus ICI-118,551 treatment. Furthermore, ICI-118,551 treatment did not affect the apoptosis of *p53*$^{-/-}$ HCT116 cells more than HCT116 cells with wild-type *p53* (**Figure 5B** and Supplementary Figure 4). This data showed that presence of P53 was important for apoptosis induction in CRC cells sensitized to radiation through selective β2-AR blockage.

β2-AR blockage significantly inhibited HCT116-xenograft tumor growth after radiotherapy in vivo

In vitro, co-treatment with β1/β2- and β2-AR blockages and radiotherapy induced cell apoptosis in CRC through mitochondrial dysfunction and cytochrome C release into the cytoplasm. In light of this promising result, the therapeutic efficacy of propranolol/ICI-118,551 and radiation co-treatment in HCT116-xenograft tumor growth was also investigated *in vivo*. **Figure 6A** and **6B** show the effects on HCT116-xenograft tumors of treatment with propranolol/ICI-118,551 in combination with radiation, respectively. Both propranolol and ICI-118,551 substantially inhibited increase in the volume and weight of HCT116-xenograft tumors during the three weeks following radiation treatment (**Figure 6C-F**). This indicated that selective β2-AR blockage treatment was able to efficiently sensitize HCT116-tumor growth to radiotherapy *in vivo*.

Discussion

CRC is an aggressive cancer that causes high global mortality [33]. Radiotherapy is one of the standard treatments in local relapse or oligo-metastases of CRC, but patients nonetheless often present with recurrences and metastasis [34]. Moreover, chronic restraint stress also affects radiotherapy efficiency and results in

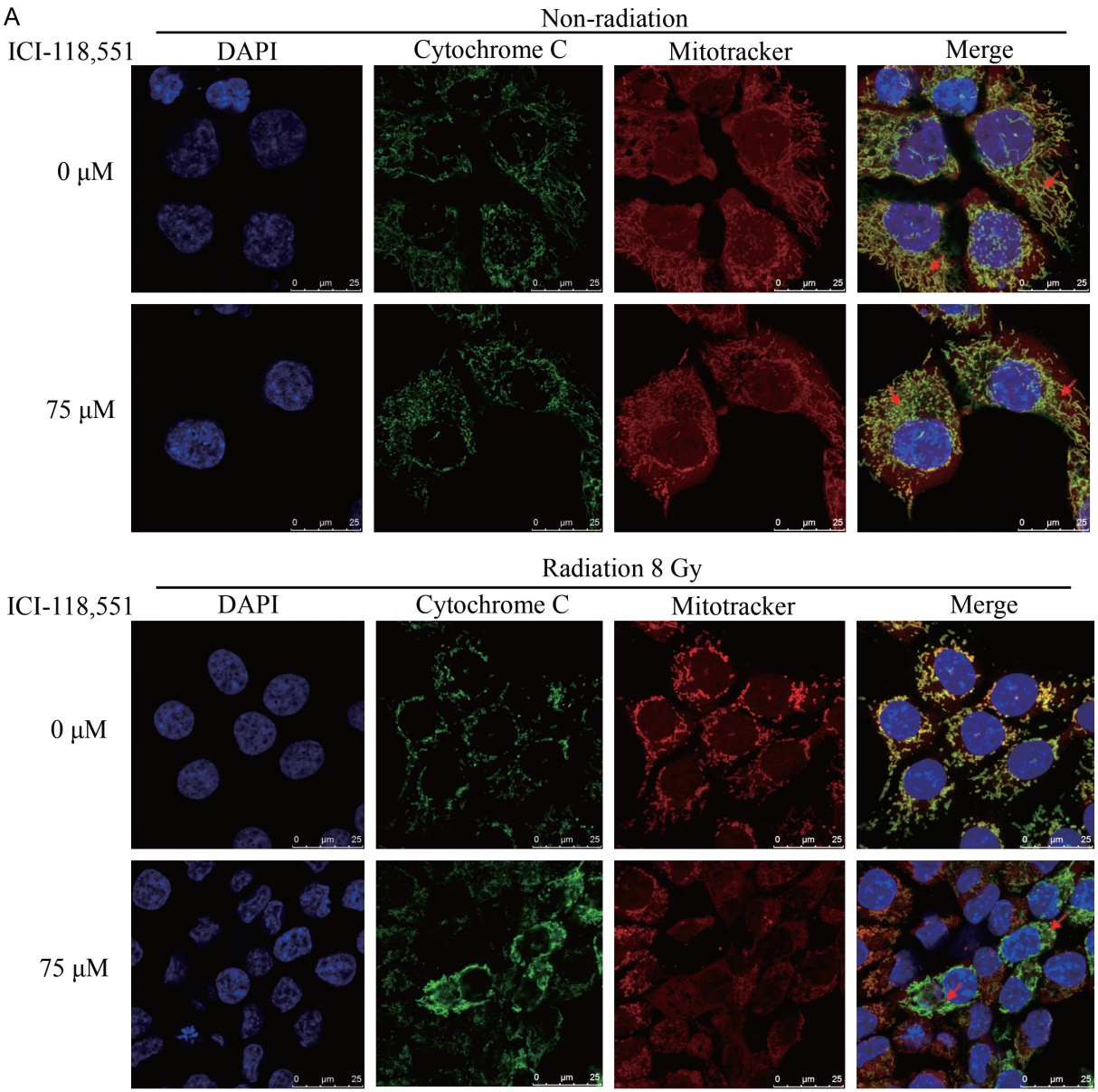

Figure 4. Co-treatment with selective β2-AR blockage and radiation induced cytochrome C release from mitochondria into the cytoplasm in HCT116 cells *in vitro*. A. HCT116 cells were treated with or without ICI-118,551 (75 μM)/radiation for 48 hrs. After treatment, the cells were fixed and stained with anti-cytochrome C antibody (green) and Mitotracker (red). Arrowheads indicate cells wherein cytochrome C was released. B. The cytochrome C-released cells were counted. Each value represents the mean ± SD of quintuplicate fields. $P < 0.001$ versus ICI-118,551 (75 μM) and radiation alone. Similar results were obtained in two-independent experiments.

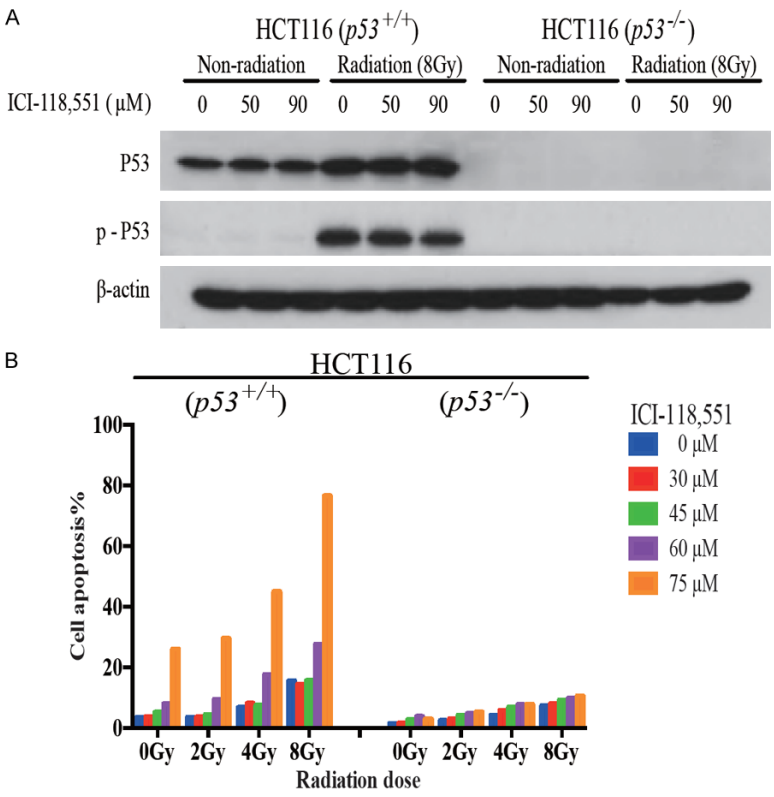

Figure 5. P53 participated mitochondrial apoptosis in HCT116 cells with wild-type P53 after treatment with combination of selective β2-AR blockage and radiation *in vitro*. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with ICI-118,551 (75 μM) in combination with radiation for 48 hrs. A. After treatment, cell lysates were analyzed by Western Blotting. In HCT116 $p53^{-/-}$ cells, the expression and phosphorylation of P53 were undetected. B. After treatment, knockout of the *p53* gene in HCT116 cells could reverse ICI-118,551-mediated apoptosis induction following radiation treatment.

treatment failure [35]. However, the underlying mechanisms and protective effects of chronic restraint stress and radiation in CRC cells remain unclear. In this study, our results demonstrated that selective β2-AR blockage, but not β1-AR blockage, in combination with radiotherapy, markedly induced CRC cell apoptosis via P53-dependent signaling. We proved that this mechanism was mainly induced by the translocation of P53 into mitochondria, not into nucleoli, which in turn resulted in mitochondrial membrane diffusion and cytochrome C release in selective β2-AR blockage-sensitive CRC cells (HCT116 and LS174T). Furthermore, our data also showed that the administration of selective β2-AR blockage in combination with radiation significantly suppressed the viability of CRC cells with wild-type *p53/Kras* mutation more than those with *p53* mutation/wild-type *Kras*. These findings were different from those of our previous study, where selective β2-AR blockage alone suppressed the proliferation of CRC cells with *p53* mutation/wild-type *Kras* but not that of CRC cells with wild-type *p53/Kras* mutation [24]. Thus, it seems that blocking of the β-adrenoceptor and the efficiency of radiotherapy exhibit some potential correlation in CRC cells with wild-type *p53/Kras* mutation, and that both P53 and *Kras* may be key factors for sensitization of CRC cells following radiation treatment.

Our previous report showed the correlation of β2-AR and *Kras/p53* status in CRC cells by selective β2-AR blockage treatment [24]. The β-AR blockage, as a radiosensitizer, can also enhance gastric cancer cell apoptosis and decrease cell survival via the NF-κB-VEGF signaling pathway following radiation treatment [19, 36]. However, in this study selective β2-AR blockage, but not β1-AR blockage, significantly promoted apoptosis in CRC cells though P53-dependent mechanism. These results were consistent with the findings of a previous report that chronic restraint stress diminished the function of Trp53 and increased radiocarcinogenesis in Trp53-heterozygous ($Trp53^{+/-}$) mice [37]. The mechanism of apoptotic induction in CRC cells was mainly promoted by P53 translocation to mitochondria, inducing mitochondrial membrane changes and cytochrome C release. The P53 status of tumor cells have been found to be predictive biomarkers for neoadjuvant radiation response in CRC patients [38]. Here, the impacts of β2-AR and P53 on radiation-induced tumour sensitization were investigated in CRC cells. In $p53^{-/-}$ HCT116 cells, the selective β2-AR blockage lost its ability to promote radiation-induced tumour sensitization. These results provide direct evidence that β2-AR is involved in the regulation of radiation-induced tumour

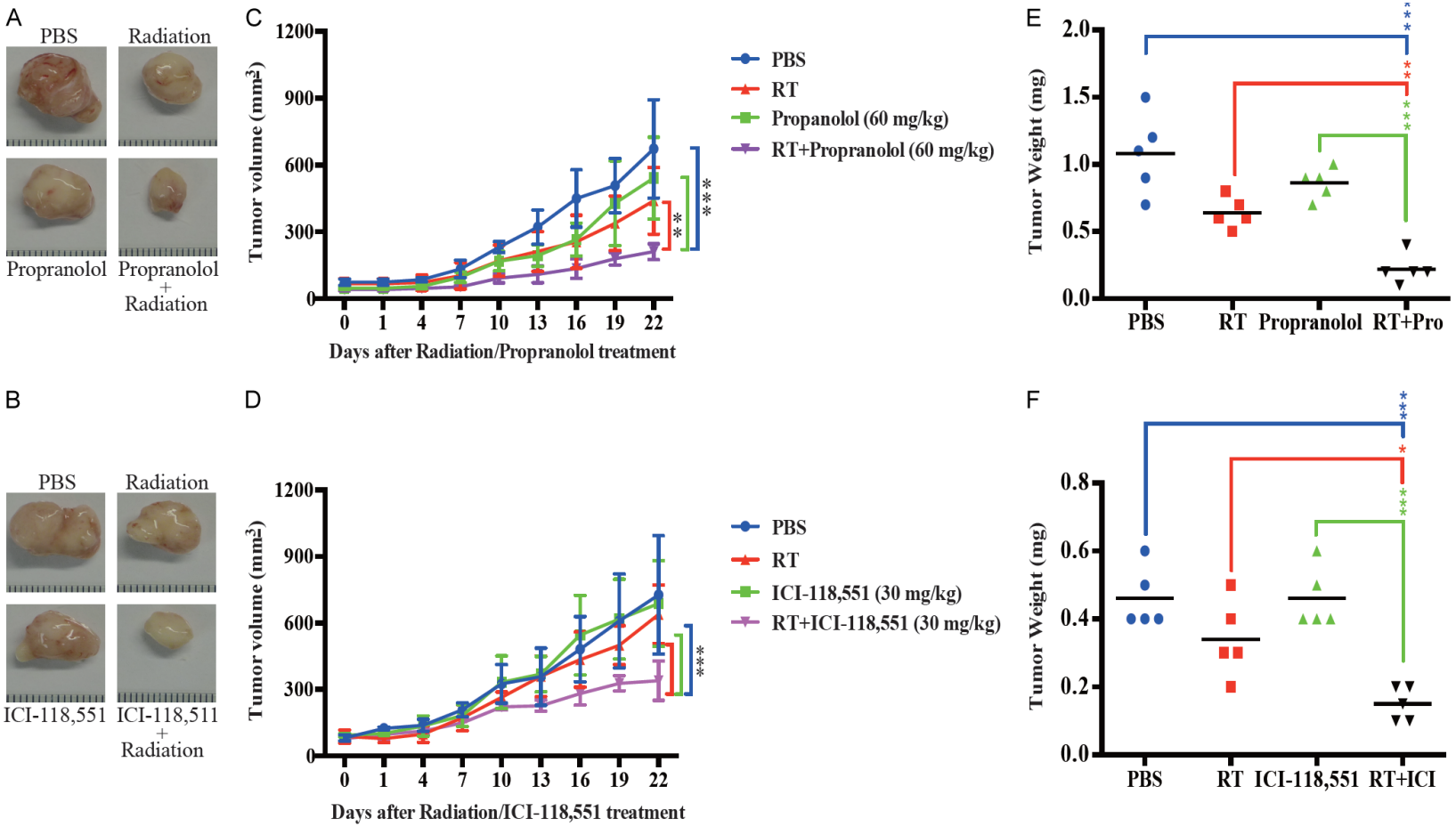

Figure 6. β1/2- and β2-AR blockages in conjunction with radiation significantly inhibited HCT116-xenograft growth *in vivo*. HCT116-bearing mice were treated with 8 Gy of radiation, and the irradiated tumors were further treated with propranolol (60 mg/kg/day) and ICI-118,551 (30 mg/kg/day) by intraperitoneal injection for three weeks. A, B. Representative images of excised tumors from each group. C, D. Tumor volumes were measured to reflect tumor growth. E, F. Tumor weights were compared on the last day of treatment. $^{***}P < 0.001$ compared to PBS.

sensitization of CRC cells along with the wild-type P53 protein, and that the mechanisms may involve directly targeting the P53 signaling pathway.

Our results showed that co-treatment with selective β2-AR blockage and radiation could significantly enhance apoptosis in CRC cells with wild-type *p53*, but not in CRC cells with mutated *p53*. Moreover, selective β2-AR blockage treatment did not promote P53 overexpression in CRC cells after radiation treatment. P53 is a regulator of cellular responses that has two mechanisms of P53-dependent apoptosis induction when cancer cells are treated with radiation. One mechanism involves transcription-dependent apoptotic P53 activity in the nucleolus, and the other is transcription-independent triggering of the intrinsic apoptotic pathway in mitochondria [39]. This molecular mechanism of mitochondrial apoptosis mainly causes mitochondrial outer membrane permeabilization, followed by mitochondrial intermembrane space proteins and cytochrome C release into the cytosol to activate caspases [40]. Cancer cells absent in P53, or with loss-of-function *p53* mutations may have decreased release of cytochrome C into the cytoplasm, through suppression of PUMA and NOXA expression [41]. Our results showed that HT29 cells with *p53* loss-of-function mutations and HCT116 with *p53* knockout mutations were significantly diminished in β2-AR blockage-mediated viability reduction after radiation treatment, but this did not occur in HCT116 and LS174T cells with wild-type *p53*. This finding was similar to that of a previous report [37]. However, the mitochondrial apoptotic mechanism by which the selective β2-AR blockage and radiation combination enhances P53 translocation remains to be elucidated.

Kras is an oncogene that is found in approximately 35% to 40% of mutant *Kras*-driven CRC cancer [42]. In CRC cases, *Kras* mutations have been recognized as an important marker for therapeutic response to chemotherapy [43] and radiotherapy [44]. Furthermore, co-treatment with *Kras*-MEK inhibitors and radiation can reduce NSCLC-xenograft tumor growth by raising antitumor immunity. Thus, the *Kras* status in CRC is crucial in both chemo- and radiotherapy. Moreover, tyrosine kinase inhibitors, including Crizotinib [45] and Iressa [46], enhance the radiation response of CRC cells with mutated *Kras*, by inactivating it. Our results indicated that selective β2-AR blockage, in combination with radiation, significantly inhibited viability and induced apoptosis of *p53* wild-type*/Kras* mutant (HCT116 and LS174T) cells, but did not affect *p53* mutant*/Kras* wild-type (HT29) cells. This finding suggested that suppression of chronic restrain stress using selective β2-AR blockage possibly interfered with *Kras* activation to regulate survival signaling in CRC, but the detailed mechanism behind this β2-AR and *Kras* signaling pathway remains to be determined.

In summary, the results of our study demonstrated that selective β2-AR blockage mediated mitochrondia-migration of p-P53 to significantly trigger apoptosis in CRC cells harboring wild-type *p53*/Kras mutant. Moreover, β2-AR blockage could be a potential therapeutic strategy in HCT116-xenograft tumors *in vivo*. These results indicate that β2-AR blockage may be used as adjunct agents for improving the clinical outcomes of CRC following radiotherapy by inducing apoptosis in CRC cells.

Acknowledgements

This study were funded by the Chang Gung Memorial hospital at Chiayi, Taiwan (CMRPG6K0021, CMRPG6L0011, and CMRPD6M0021) and the Ministry of Science and Technology, R.O.C. [MOST108-2320-B-182-010, MOST109-2320-B-182-012 and National Science and Technology Council 112-2320-B-182-016]. The authors thank the Laboratory Animal Center of Chang Gung Memorial Hospital, Chiayi, Taiwan for providing the facilities and animal care required for this study. We would like to acknowledge the BD FACSCanto II flow cytometer and Leica SP5II confocal microscope services provided by the Precious Instrumentation Core Laboratory, Chang Gung Memorial Hospital, Chiayi.

Disclosure of conflict of interest

None.

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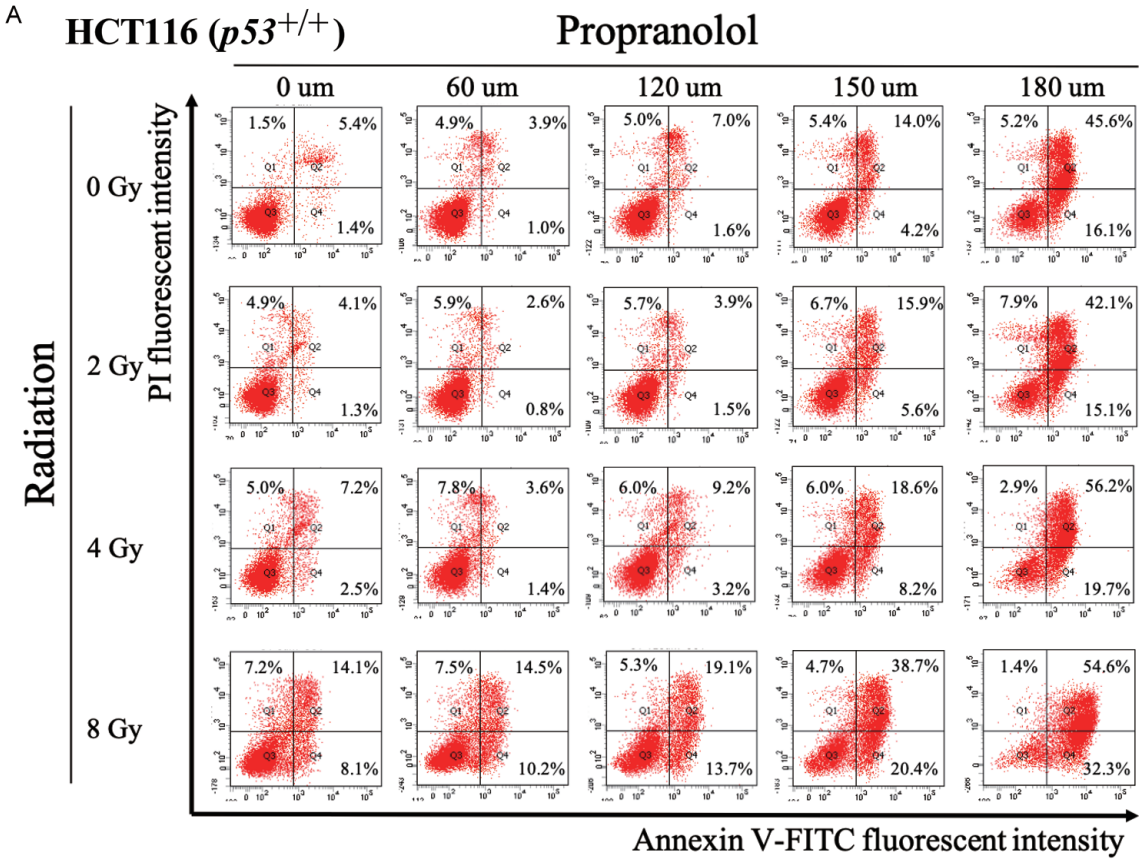
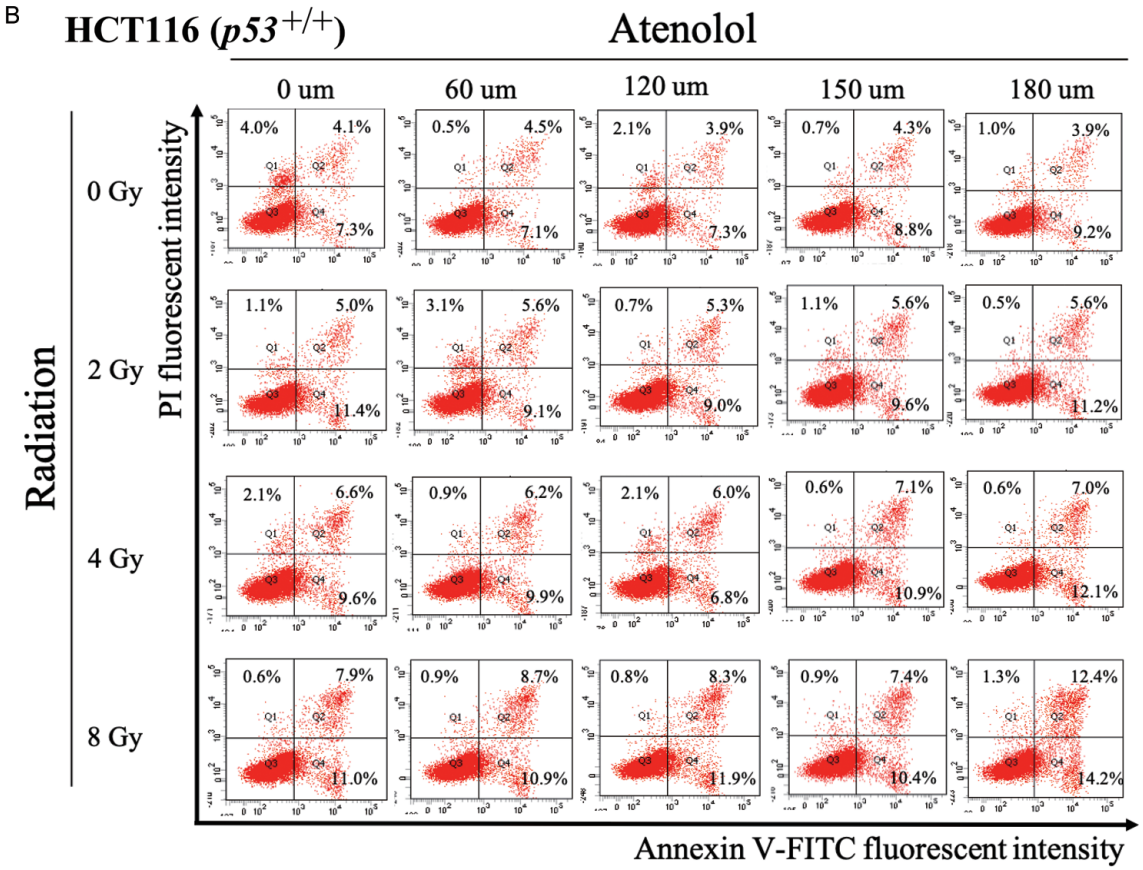
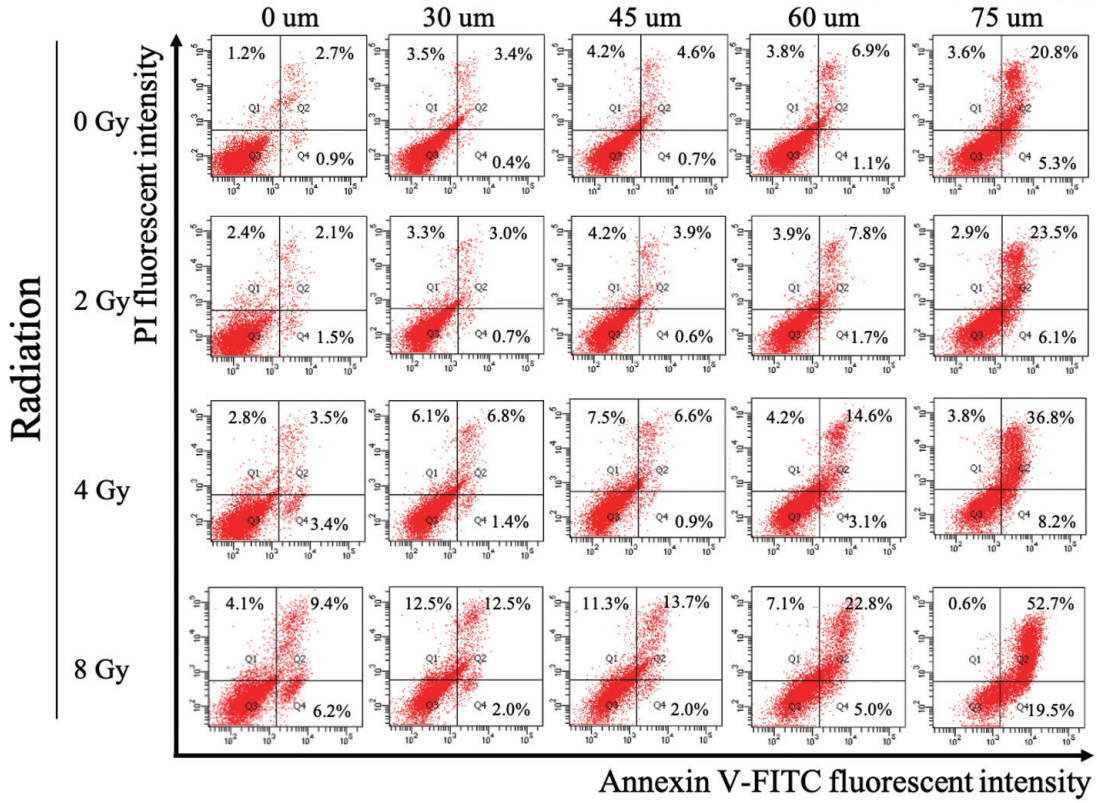
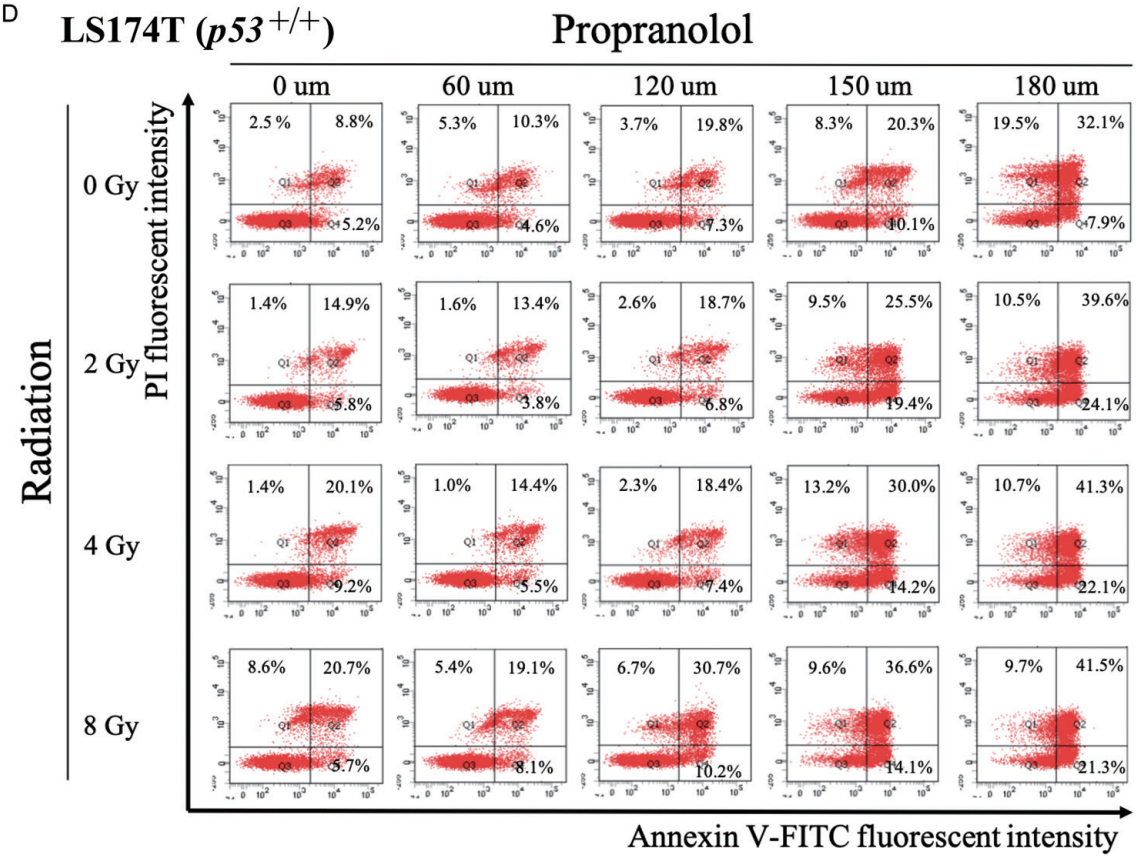

A
HCT116 ($p53^{+/+}$)
Propranolol
0 um
60 um
120 um
150 um
180 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
1.5% 5.4% 1.4%
4.9% 3.9% 1.0%
5.0% 7.0% 1.6%
5.4% 14.0% 4.2%
5.2% 45.6% 16.1%
4.9% 4.1% 1.3%
5.9% 2.6% 0.8%
5.7% 3.9% 1.5%
6.7% 15.9% 5.6%
7.9% 42.1% 15.1%
5.0% 7.2% 2.5%
7.8% 3.6% 1.4%
6.0% 9.2% 3.2%
6.0% 18.6% 8.2%
2.9% 56.2% 19.7%
7.2% 14.1% 8.1%
7.5% 14.5% 10.2%
5.3% 19.1% 13.7%
4.7% 38.7% 20.4%
1.4% 54.6% 32.3%


B
HCT116 ($p53^{+/+}$)
Atenolol
0 um
60 um
120 um
150 um
180 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
4.0% 4.1% 7.3%
0.5% 4.5% 7.1%
2.1% 3.9% 7.3%
0.7% 4.3% 8.8%
1.0% 3.9% 9.2%
1.1% 5.0% 11.4%
3.1% 5.6% 9.1%
0.7% 5.3% 9.0%
1.1% 5.6% 9.6%
0.5% 5.6% 11.2%
2.1% 6.6% 9.6%
0.9% 6.2% 9.9%
2.1% 6.0% 6.8%
0.6% 7.1% 10.9%
0.6% 7.0% 12.1%
0.6% 7.9% 11.0%
0.9% 8.7% 10.9%
0.8% 8.3% 11.9%
0.9% 7.4% 10.4%
1.3% 12.4% 14.2%

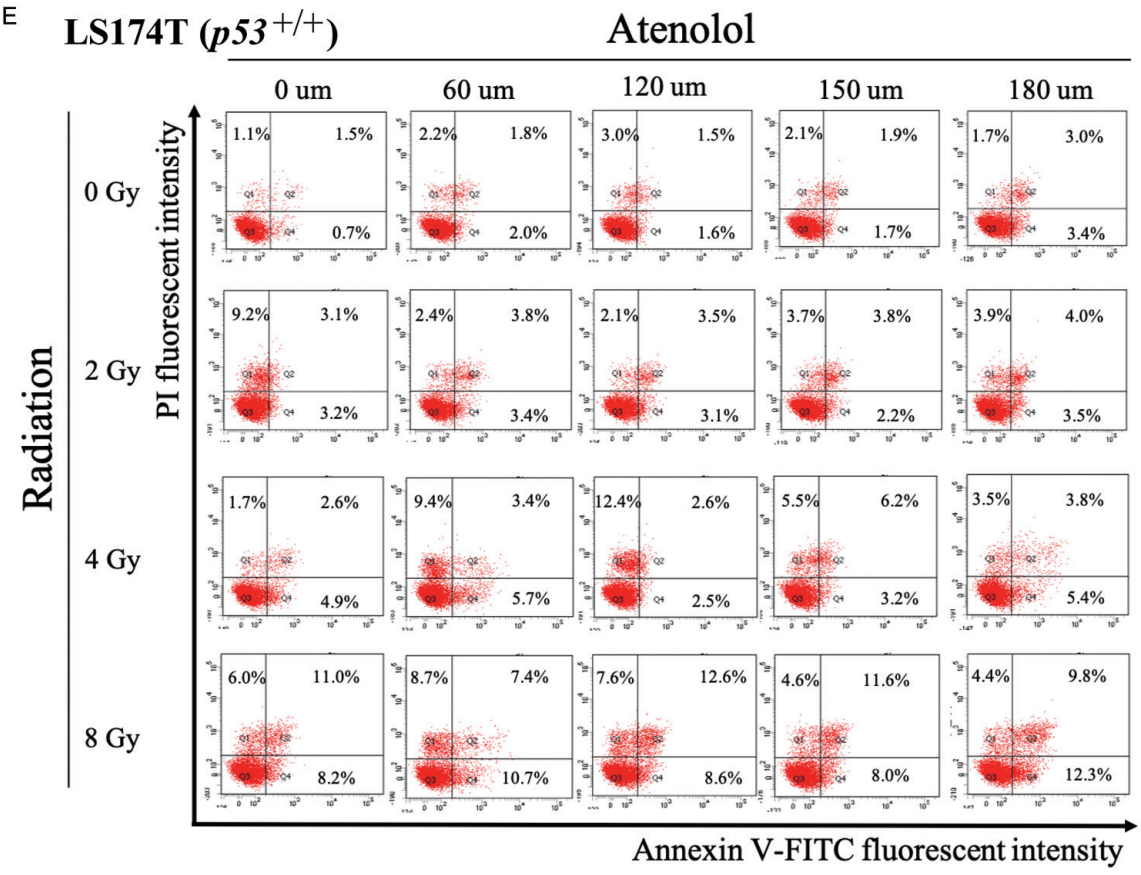
C


HCT116 (p53+/+)
ICI-118,551
0 um
30 um
45 um
60 um
75 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
1.2% 2.7% 0.9%
3.5% 3.4% 0.4%
4.2% 4.6% 0.7%
3.8% 6.9% 1.1%
3.6% 20.8% 5.3%
2.4% 2.1% 1.5%
3.3% 3.0% 0.7%
4.2% 3.9% 0.6%
3.9% 7.8% 1.7%
2.9% 23.5% 6.1%
2.8% 3.5% 3.4%
6.1% 6.8% 1.4%
7.5% 6.6% 0.9%
4.2% 14.6% 3.1%
3.8% 36.8% 8.2%
4.1% 9.4% 6.2%
12.5% 12.5% 2.0%
11.3% 13.7% 2.0%
7.1% 22.8% 5.0%
0.6% 52.7% 19.5%

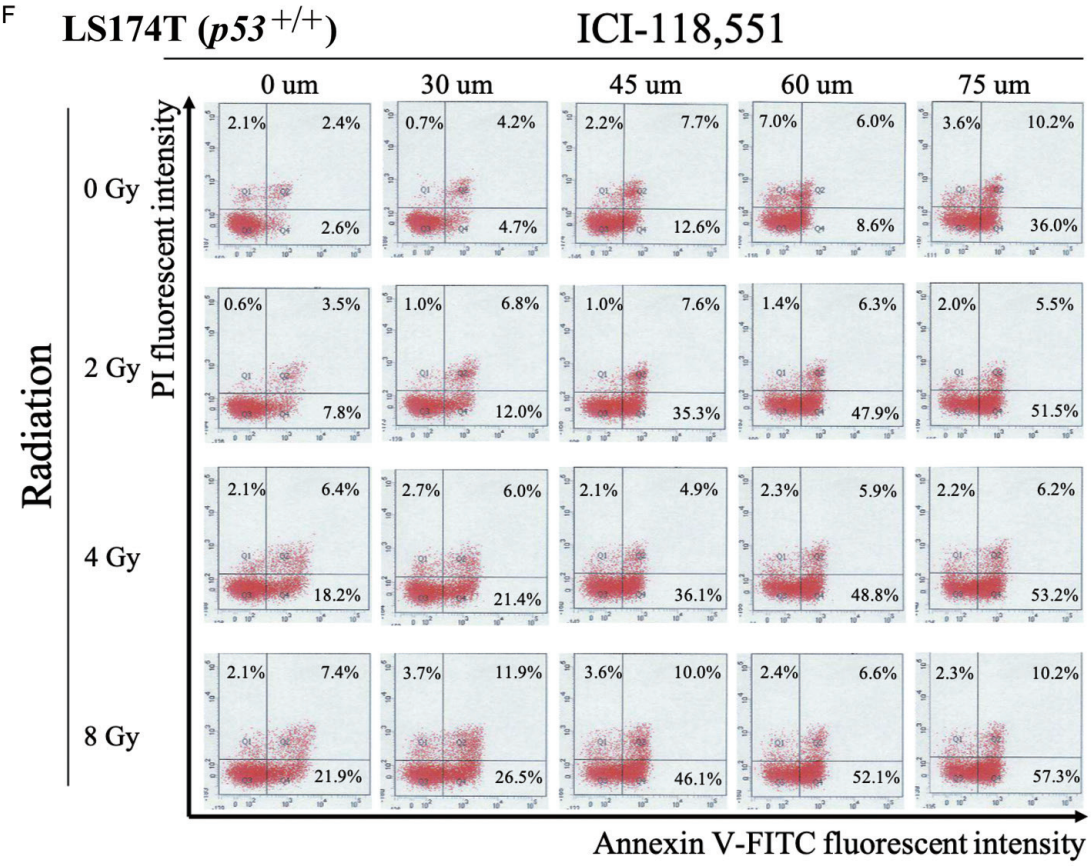
D


LS174T (p53+/+)
Propranolol
0 um
60 um
120 um
150 um
180 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
2.5% 8.8% 5.2%
5.3% 10.3% 4.6%
3.7% 19.8% 7.3%
8.3% 20.3% 10.1%
19.5% 32.1% 7.9%
1.4% 14.9% 5.8%
1.6% 13.4% 3.8%
2.6% 18.7% 6.8%
9.5% 25.5% 19.4%
10.5% 39.6% 24.1%
1.4% 20.1% 9.2%
1.0% 14.4% 5.5%
2.3% 18.4% 7.4%
13.2% 30.0% 14.2%
10.7% 41.3% 22.1%
8.6% 20.7% 5.7%
5.4% 19.1% 8.1%
6.7% 30.7% 10.2%
9.6% 36.6% 14.1%
9.7% 41.5% 21.3%

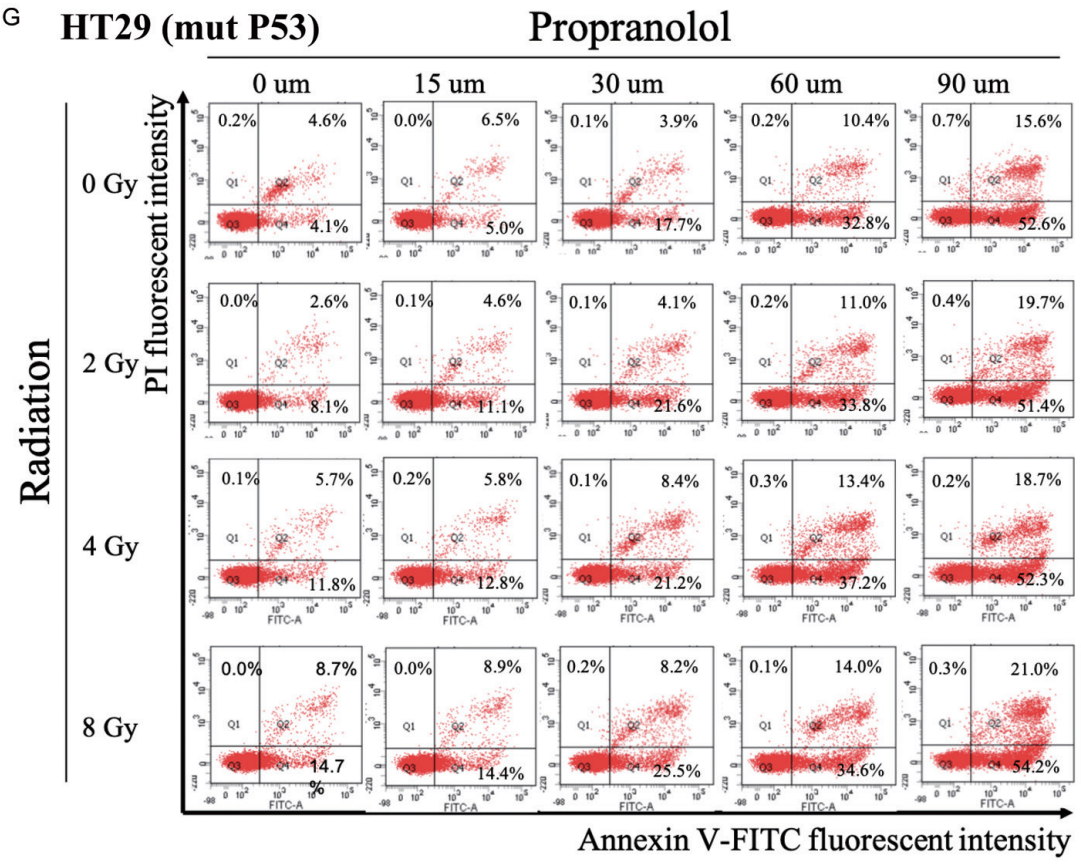
E


LS174T ($p53^{+/+}$)
Atenolol
0 um
60 um
120 um
150 um
180 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
1.1% 1.5% 0.7%
2.2% 1.8% 2.0%
3.0% 1.5% 1.6%
2.1% 1.9% 1.7%
1.7% 3.0% 3.4%
9.2% 3.1% 3.2%
2.4% 3.8% 3.4%
2.1% 3.5% 3.1%
3.7% 3.8% 2.2%
3.9% 4.0% 3.5%
1.7% 2.6% 4.9%
9.4% 3.4% 5.7%
12.4% 2.6% 2.5%
5.5% 6.2% 3.2%
3.5% 3.8% 5.4%
6.0% 11.0% 8.2%
8.7% 7.4% 10.7%
7.6% 12.6% 8.6%
4.6% 11.6% 8.0%
4.4% 9.8% 12.3%

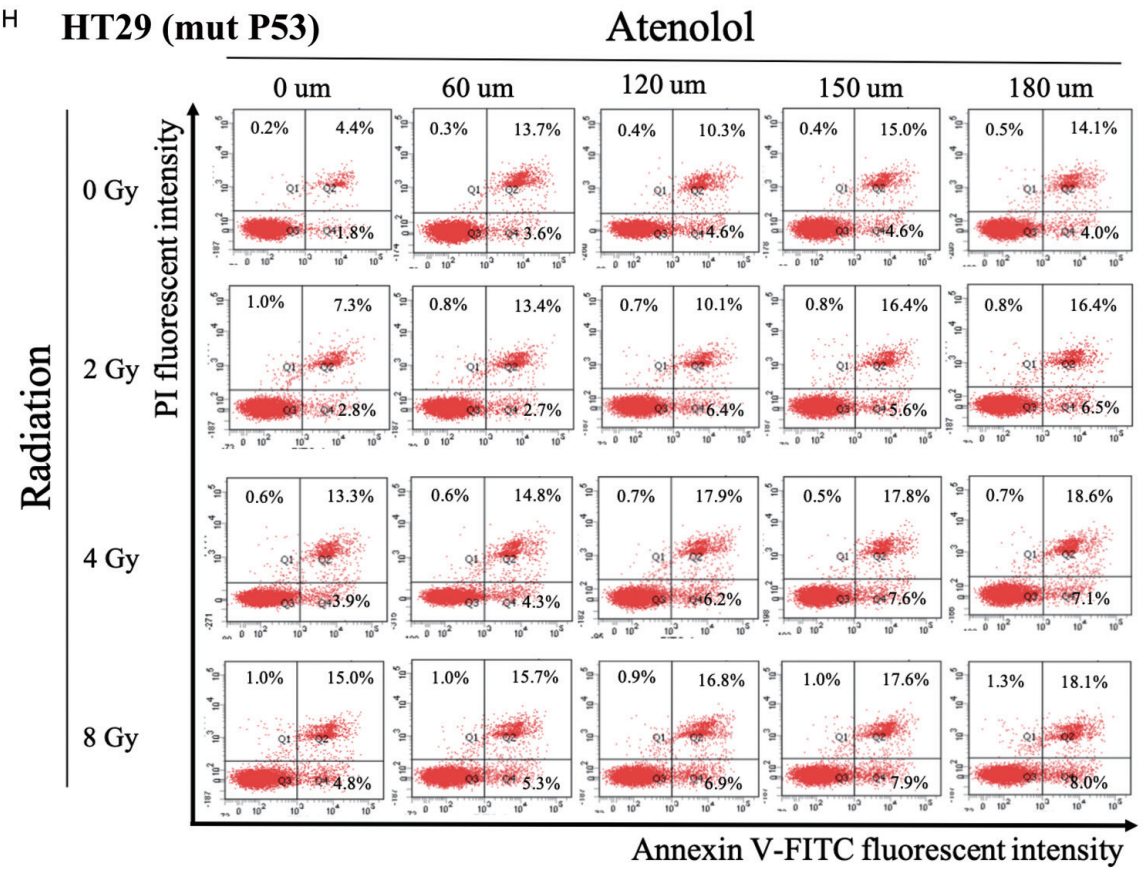
F

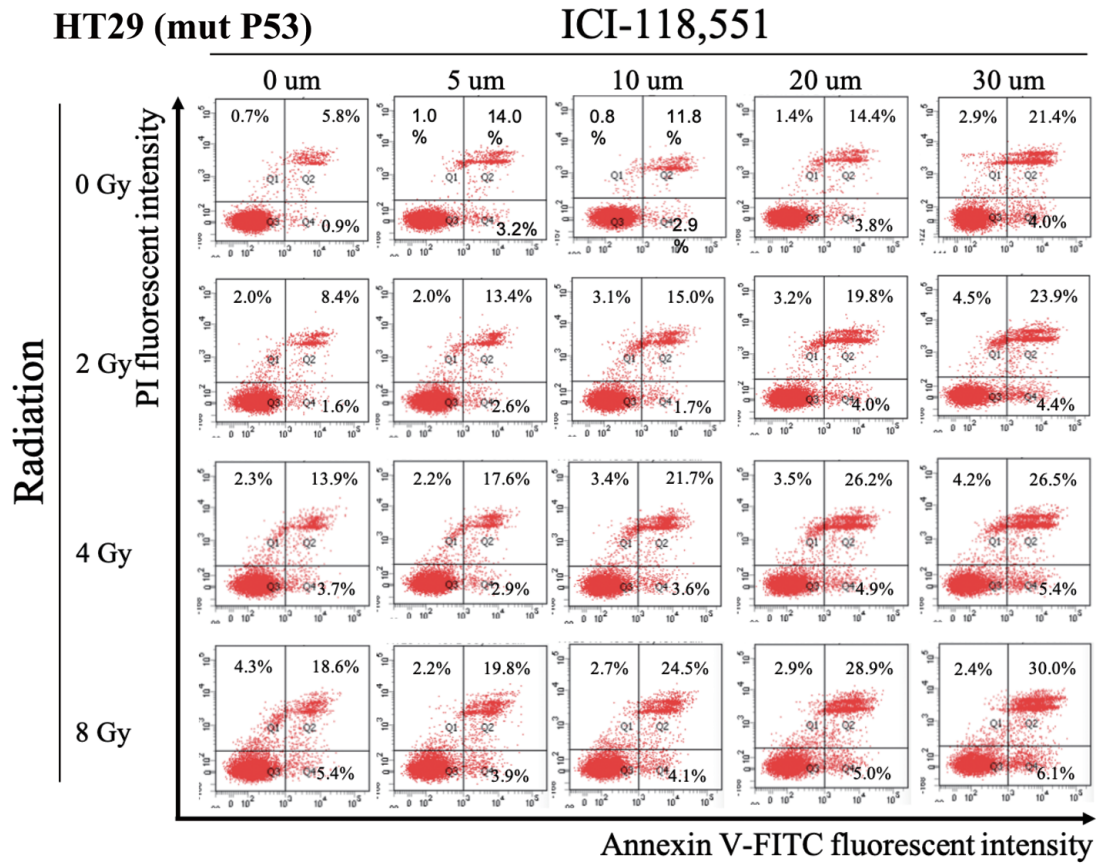

LS174T ($p53^{+/+}$)
ICI-118,551
0 um
30 um
45 um
60 um
75 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
2.1% 2.4% 2.6%
0.7% 4.2% 4.7%
2.2% 7.7% 12.6%
7.0% 6.0% 8.6%
3.6% 10.2% 36.0%
0.6% 3.5% 7.8%
1.0% 6.8% 12.0%
1.0% 7.6% 35.3%
1.4% 6.3% 47.9%
2.0% 5.5% 51.5%
2.1% 6.4% 18.2%
2.7% 6.0% 21.4%
2.1% 4.9% 36.1%
2.3% 5.9% 48.8%
2.2% 6.2% 53.2%
2.1% 7.4% 21.9%
3.7% 11.9% 26.5%
3.6% 10.0% 46.1%
2.4% 6.6% 52.1%
2.3% 10.2% 57.3%

G

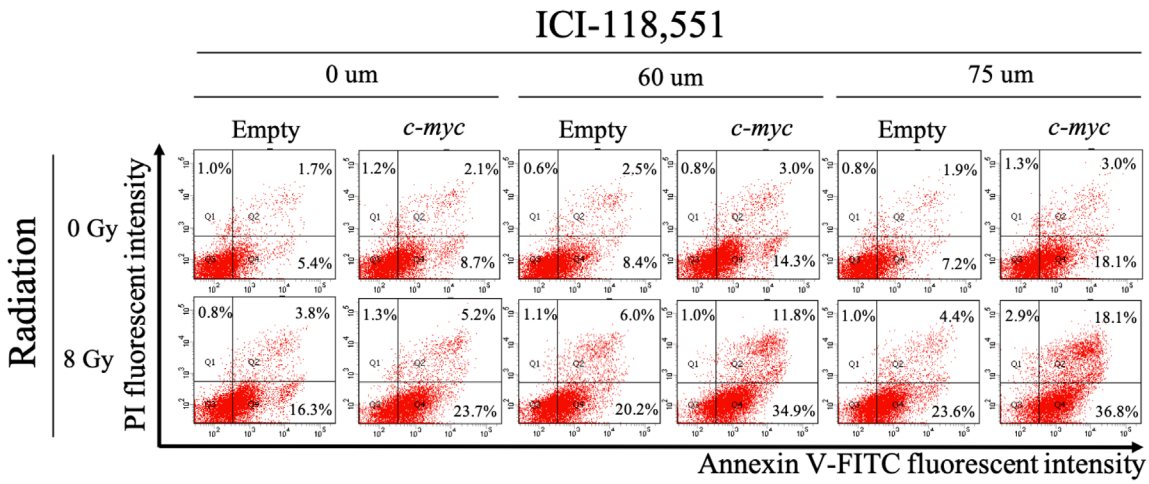

HT29 (mut P53)
Propranolol
0 um
15 um
30 um
60 um
90 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
0.2% 4.6% 4.1%
0.0% 6.5% 5.0%
0.1% 3.9% 17.7%
0.2% 10.4% 32.8%
0.7% 15.6% 52.6%
0.0% 2.6% 8.1%
0.1% 4.6% 11.1%
0.1% 4.1% 21.6%
0.2% 11.0% 33.8%
0.4% 19.7% 51.4%
0.1% 5.7% 11.8%
0.2% 5.8% 12.8%
0.1% 8.4% 21.2%
0.3% 13.4% 37.2%
0.2% 18.7% 52.3%
0.0% 8.7% 14.7 %
0.0% 8.9% 14.4%
0.2% 8.2% 25.5%
0.1% 14.0% 34.6%
0.3% 21.0% 54.2%

H

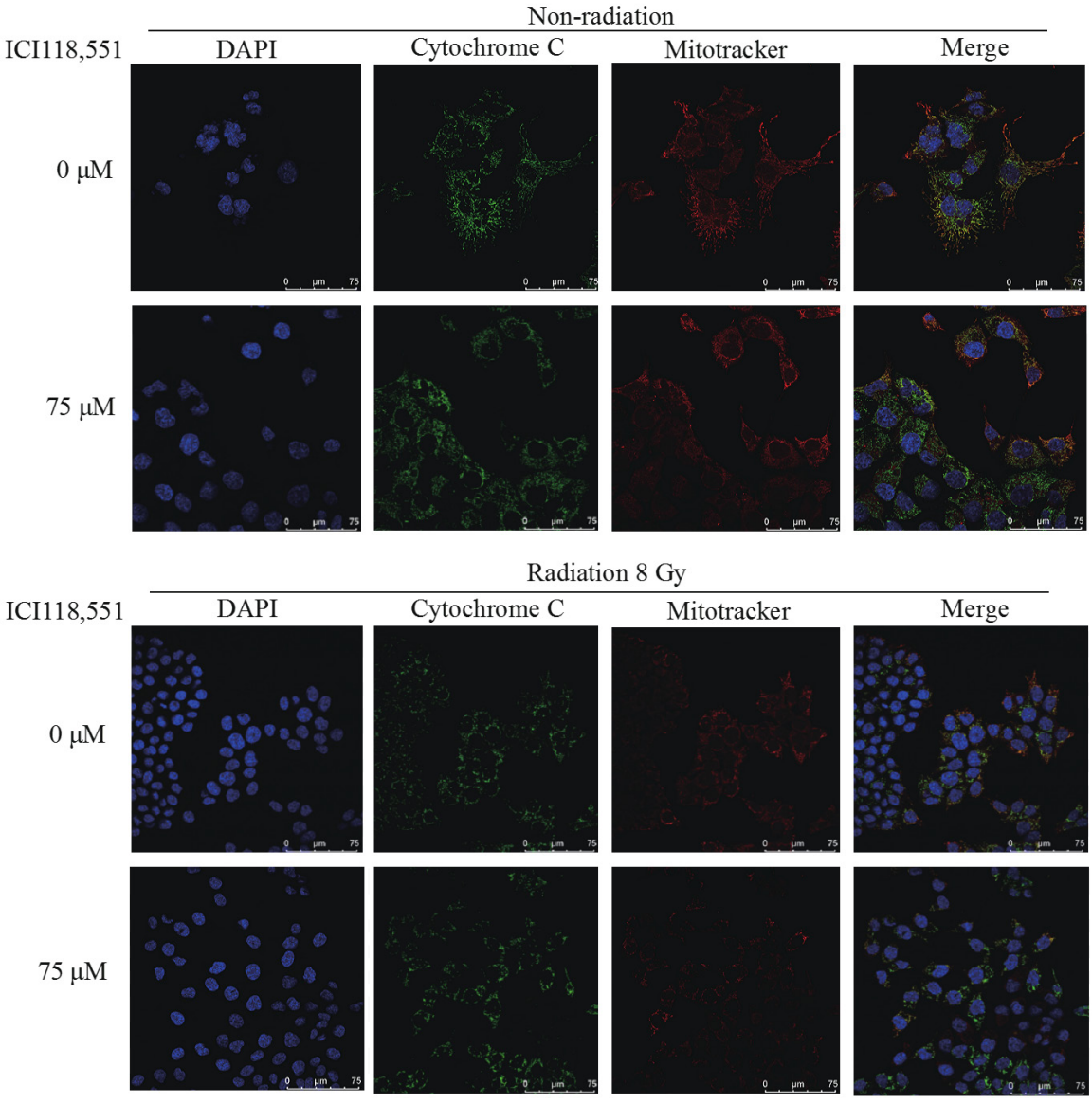

HT29 (mut P53)
Atenolol
0 um
60 um
120 um
150 um
180 um
Radiation
0 Gy
2 Gy
4 Gy
8 Gy
PI fluorescent intensity
Annexin V-FITC fluorescent intensity
0.2% 4.4% 1.8%
0.3% 13.7% 3.6%
0.4% 10.3% 4.6%
0.4% 15.0% 4.6%
0.5% 14.1% 4.0%
1.0% 7.3% 2.8%
0.8% 13.4% 2.7%
0.7% 10.1% 6.4%
0.8% 16.4% 5.6%
0.8% 16.4% 6.5%
0.6% 13.3% 3.9%
0.6% 14.8% 4.3%
0.7% 17.9% 6.2%
0.5% 17.8% 7.6%
0.7% 18.6% 7.1%
1.0% 15.0% 4.8%
1.0% 15.7% 5.3%
0.9% 16.8% 6.9%
1.0% 17.6% 7.9%
1.3% 18.1% 8.0%



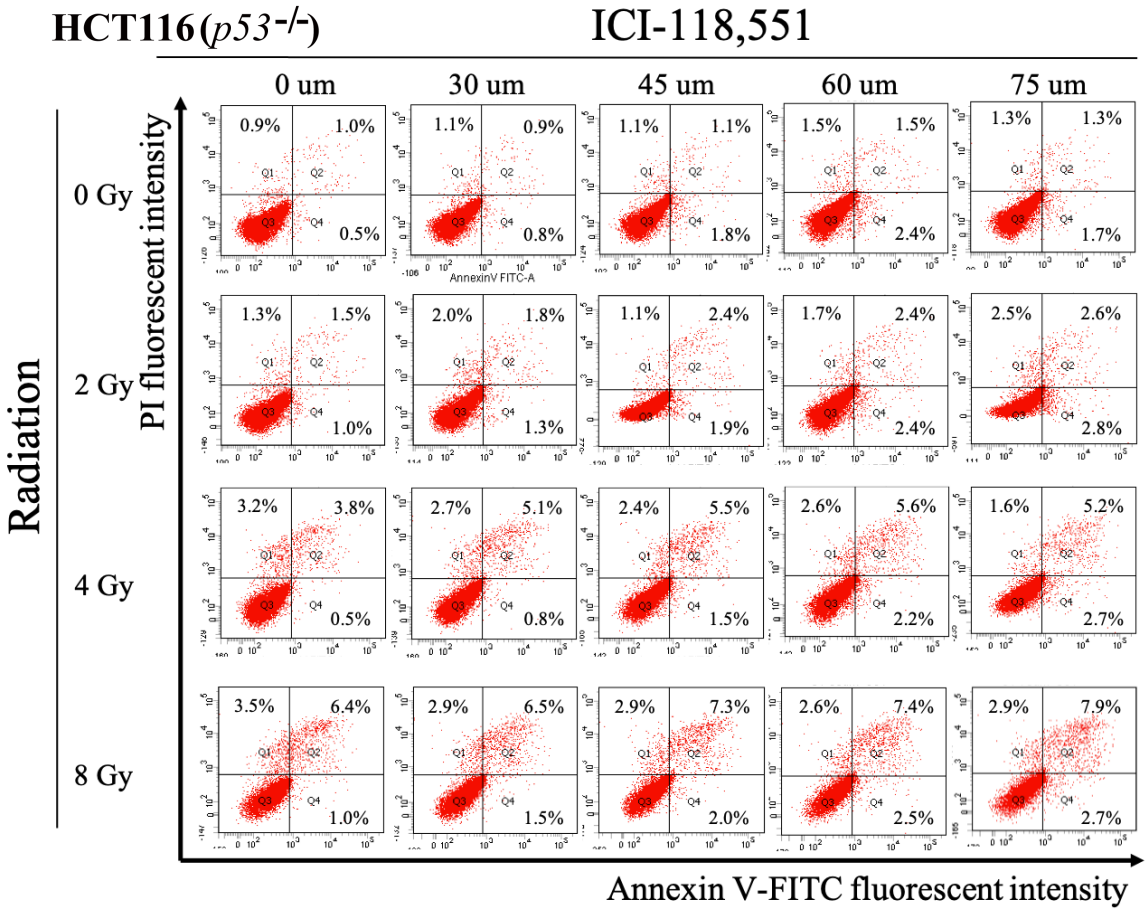
Supplementary Figure 1. A. Co-treatment with propranolol and radiation enhanced apoptosis in HCT116 cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. B. Co-treatment with atenolol and radiation did not enhanced apoptosis in HCT116 cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. C. Co-treatment with ICI-118,551 and radiation enhanced apoptosis in HCT116 cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. D. Co-treatment with propranolol and radiation enhanced apoptosis in LS174T cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. E. Co-treatment with atenolol and radiation did not enhanced apoptosis in LS174T cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. F. Co-treatment with ICI-118,551 and radiation enhanced apoptosis in LS174T cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. G. Co-treatment with propranolol and radiation did not enhanced apoptosis in HT29 cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. H. Co-treatment with atenolol and radiation did not enhanced apoptosis in HT29 cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry. I. Co-treatment with ICI-118,551 and radiation did not enhanced apoptosis in HT29 cells for 48 hrs. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry.



Supplementary Figure 2. The enforced expressions of c-Myc did not reverse ICI-118,551-mediated apoptosis enhancement of CRC cells after radiotherapy. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry.



Supplementary Figure 3. Selective β2-AR blockage increased cytochrome C release from mitochondria into the cytoplasm in HCT116 cells in vitro after radiotherapy.



Supplementary Figure 4. After treatment, knockout of the p53 gene in HCT116 cells could reverse ICI-118,551-mediated apoptosis induction following radiation treatment. Apoptotic cells were determined by dual staining of PI and Annexin-V and analyzed by flow cytometry.