

# Synergistic Effect of Phloretin Combined With Radiotherapy on Lung Cancer

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## Research

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# Abstract

**OBJECTIVE:** The purpose of this study was to investigate the synergistic anti-tumor effects of phloretin (Ph) and radiotherapy (RT) on Lewis lung cancer (LLC), and the mechanisms involved.

**METHODS**—The proliferative rate of Lewis cells treated with phloretin was detected by MTT assay; Clone formation experiments were used to demonstrate the synergistic effect of phloretin and radiotherapy; Mice engrafted with the LLC cells were given intraperitoneal injections of saline, phloretin with or without RT. Tumor models were established by laterally transplanting LLC cells in the right thigh of C57BL/J mice. They were randomly divided into four groups, namely saline group, phloretin group, radiotherapy group, phloretin combined with radiotherapy group. 10Gy radiotherapy was performed during the administration. PET-CT was used to detect  $^{18}\text{F}$ -FDG uptake in tumor tissue; immunohistochemistry was used to detect Ki67 expression in tumor tissue; cell apoptosis was detected by flow cytometry.

**RESULTS:** Different concentrations of phloretin inhibited the proliferation of Lewis cells in a time and dose-dependent manner ( $P < 0.05$ ). The radiotherapy sensitization ratio (SER) of 50 $\mu\text{g}$  Phloretin was 1.645 in the LLC cells. The combination of Phloretin and RT increased survival compared to free Phloretin and RT ( $p < 0.05$ ), and prolonged tumor growth delay (TGD). Furthermore, the RT + Phloretin combination therapy significantly reduced  $^{18}\text{F}$ -FDG uptake, increased apoptosis and decreased the proliferation index (Ki67) in tumor cells compared to either monotherapy.

**CONCLUSION:** phloretin combined with radiotherapy has a synergistic anti-tumor effect, possibly by promoting apoptosis, as well as inhibiting proliferation rate and glucose transport.

## 1. Introduction

Globally, the number of lung cancer cases and deaths is rising. Smoking is the main risk factor for lung cancer[1]. In 2018, GLOBOCAN estimated that there were 2.09 million new lung cancer cases (11.6% of the total number of cancers) and 1.76 million death cases (18.4% of the total number of cancers), making it the most frequent cancer and cause of cancer death in human[2].

Although a variety of anti-cancer therapies have been developed, cancer mortality are still increasing worldwide. Phloretin (Ph) is a plant-derived metabolite with enormous pharmacological potential[3], which is a hydrophobic polyphenolic compound found in apples and other plants. Phloretin is predominantly present in the peel of the apple (80–420 mg/kg) and pulp (16–20 mg/kg)[4]. Phloretin exhibit the effects of anti-oxidant, anti-bacteria, anti-inflammatory, anti-proliferation, apoptosis induction, inhibition of tumor metastasis, anti-tumor and chemotherapy sensitization[5, 6]. Phloretin has anti-tumor effects on various tumors. Previous study has demonstrated that phloretin inhibits the proliferation rate of human oral cancer cells by inducing cell cycle arrest and cell death and also inhibits the migration of human oral cancer cells[7]. Phloretin inhibit the proliferation of EC-109 cells and indicated that phloretin exhibits anticancer behavior through the activation of the mitochondrial-apoptosis pathway[6]. Phloretin and Atorvastatin produce a powerful synergistic interaction in suppressing colon cancer cell growth via

the synergistic induction of apoptosis and the arrest of the cell cycle at the G2/M checkpoint[8]. Phloretin has significant anti-breast cancer effect is due to the inhibition of transmembrane glucose transport[9]. Phloretin inhibits the growth of human gastric cancer cells by inducing autophagy and cell cycle arrest, and at the same time inhibits the migration and invasion of human gastric cancer cells[3].

In this study, we explored and evaluated the potential synergistic inhibitory effects of Ph and RT on Lewis lung cancer cells. The synergistic mechanisms involving cell proliferation inhibition, sugar uptake inhibition and promotion of cell apoptosis have also been studied. The results of this study may provide a potential new treatment idea for the lung cancer population, especially through the combination of flavonoid functional ingredients and radiotherapy.

## **2. Materials And Methods**

### **2.1. Reagents and cell lines**

The Lewis cell line was provided by Cancer Center and State Key Laboratory of Biotherapy, West China Hospital of Sichuan, phloretin (purity, 97%, BR) was purchased from Dalian Meilun Company. Dimethyl sulfoxide (DMSO), methyl thiazole blue (MTT), crystal violet all were purchased from Sigma Company. DMEM medium and Australian fetal bovine serum were purchased from Waltham Company. Annexin V-FITC Apoptosis Detection kit was purchased from BD Company. Polyclonal antibodies against Ki-67 purchased from Bioworld Technology Co. Ltd. (Nanjing, China).

Lewis lung cancer (LLC) cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> incubator.

C57BL/6J mice (female, 5 weeks old) were purchased from Chengdu Dashuo Biotechnology Co. Ltd. All animal experiments were implemented in accordance with the Institutional Animal Care and Use Guidelines, and approved by the Institutional Animal Southwest Medical Care and Use Committee (Chendu, China).

### **2.2. Cytotoxicity test**

They are treated with concentration gradients of and each group has 6 replicates. The phloretin powder was dissolved in cell culture medium. Incubate to the set time, then add 20 µL of MTT to each well, terminate the incubation after 4 hours, add 200 µL of DMSO to each well, and set it at 480 µL in a microplate reader.

### **2.3. Colony forming assay**

Ph were added to the LLC cells and the medium was replaced after 2h to remove the drug. One hour after adding the Ph, the cells were irradiated up to 10 Gy using a linear accelerator (varian) at the dose rate of 60 cGy/min. To assess colony formation, the variously treated Lewis cells were seeded into 6-well plates and cultured for 10-14 days. Plating efficiency (PE), surviving fraction (SF) and the sensitivity

enhancement ratio (SER) were calculated by the multi-target model fitting of the data using nonlinear regression.

#### **2.4. Establishment of lung cancer model and treatment protocol**

A lung cancer model was established by subcutaneously injecting each mouse with a 100 $\mu$ l suspension of LLC cells in the dorsal aspect of the right foot. After the tumors volumes were  $\sim$ 100-200 mm<sup>3</sup>, the tumor-bearing mice were randomized into the following four treatment groups (n = 12 each): control (0.9% normal saline), Phloretin, RT, RT + Phloretin. The phloretin solution was injected intraperitoneally at a dose of 20mg/kg based on the previous report[10], once every 2 days, for a total of 6 treatments, and radiotherapy was given after the third intraperitoneal injection. The truncal region of the mice harboring the tumor xenograft were irradiated before the third injection at the dose rate of 60 cGy/min and source–subject distance of 70 cm, to a total dose of 10 Gy. After treatment, half of the animals in each group were randomly euthanized, and the tumors were harvested for various analyses. A tumor growth curve was plotted based on tumor size against days after treatment. The survival time of each mouse was recorded. Tumor growth curve (TGC) was calculated with ( $T_{i5}$ - $T_5$ ) as the time taken for the treated tumors ( $T_{i5}$ ) and the control tumors ( $T_5$ ) to increase to 5 times their initial tumor volume.

#### **2.5. Micro <sup>18</sup>F-FDG PET/CT imaging**

The metabolic status of the tumors in response to different treatments were evaluated in terms of <sup>18</sup>F-FDG uptake by using Inveon micro PET/CT (Siemens, Munich, Germany). The mice were fasted for 12 hours, anesthetized with 1% pentobarbital at the dose of 5 ml/kg, and then injected with 100-200 mCi FDG into their tail veins. Tracer uptake values of the tumors were measured in attenuation-corrected lateral chromatographic sections by calculating standard uptake values (SUVs) measured by ROI.

#### **2.6. Apoptosis analysis**

The soy bean-sized tumors were digested into individual tumor cells. For apoptosis analysis, the cells were stained with PI and annexin V-FITC, and the apoptotic cells were detected by flow cytometry (BD FACSVerser, Piscatway, NJ).

#### **2.7. Immunohistochemistry (IHC)**

The tissue sections were immuno-stained using antibodies against Ki-67 according to the manufacturer's instructions, and observed under an optical microscope (Leica TE2000-S microscope, Tokyo, Japan). The Ki-67 positive and total numbers of cells were counted in 5 randomly selected regions in each tumor section under 400x magnification, and the percentage of positive cells was calculated.

#### **2.8. Statistical analysis**

All data are expressed as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare different groups, and survival curves were plotted according to the Kaplan–Meier

method. P values less than 0.05 and 0.01 were considered statistically significant. Data analysis was performed using SPSS statistics 17.0 software (SPSS Inc., Chicago, IL).

### 3. Results

#### 3.1. Cytotoxicity results

The OD480 value of Lewis cells decreased with the increase of phloretin concentration and the inhibitory effect of phloretin on Lewis cells is obviously dose- and time-dependent (Fig. 1). When the concentration of phloretin is lower than 50µg/ml, it has no obvious inhibitory effect on the proliferation of Lewis cells.

#### 3.2. Clone formation result

According to the cytotoxicity test to screen the drug concentration in the clone formation test. Cells treated with phloretin, then irradiated with 2, 4, 6, 8 and 10 Gy X-rays, the radiosensitizing ability of phloretin at a moderately toxic dose (50 µg/ml) was determined. Phloretin showed significant radiosensitization at the specified drug dose, with a SER of 1.645. Calculated by fitting a multi-target model, the Dq, D0 and SF2 of the combination of phloretin and RT were lower than those of the RT group (Table 1).

Table 1  
The cell survival rate was measured by colony forming assay in Lewis cell

	D0	Dq	SF2	SER
RT	2.394	2.310	0.775	
RT + Phloretin	1.455	1.511	0.561	1.645

#### 3.3. Phloretin and RT combination therapy delayed tumor growth

To evaluate the anti-tumor efficacy of Ph and RT combination, the tumor volumes were measured after each treatment (Fig. 3A), and the duration of survival was recorded (Fig. 3B). Tumor growth was respectively delayed for 3 and 4 days in mouse treated with Ph or RT alone, while the combination of RT + Ph resulted in a TGD of 9 days. In addition, the median survival time of the RT + Ph group was 60.5 days, which was significantly longer than the 25.1, 31 and 37.5 days seen in the control, Ph and RT groups respectively.

#### 3.4. Phloretin and RT combination therapy reduced <sup>18</sup>F-FDG uptake

Representative <sup>18</sup>F-FDG PET/CT images and the maximal standardized uptake value (SUVmax) of tumor-bearing mice treated with various drug regimens are shown in Fig. 4. Compared to the SUVmax value of the control group (6.73 ± 0.25), that of the phloretin (5.51 ± 0.26), RT (3.15 ± 0.17) and RT + phloretin (2.05

$\pm 0.21$ ) groups were significantly reduced ( $P < 0.01$ ). The lowest SUVmax value in RT + phloretin group indicates a superior anti-tumor response of the combination therapy compared to either monotherapy.

### 3.5. Phloretin and RT combination therapy increased apoptosis in tumor cells

To elucidate the mechanisms associated with Phloretin-mediated enhanced radio-sensitization. RT + Phloretin group had the highest percentage of apoptotic cells ( $39.36 \pm 1.52\%$ ), followed by RT ( $23.78 \pm 1.08\%$ ), Phloretin ( $14.63 \pm 0.24\%$ ), and control ( $6.94 \pm 1.68\%$ ) groups. The apoptosis rate of Lewis cells induced by RT + Phloretin was significantly higher than that of either monotherapy, which indicate the radio-sensitization effects of Phloretin (Fig. 5).

### 3.6. Phloretin and RT combination therapy decreased tumor cell proliferation index

Immunohistochemistry showed that Ki67 positive cells in the RT + Phloretin group were significantly reduced (Fig. 6A). The relative proportion of Ki-67 positive cells was significantly lower in the RT + Phloretin group ( $8.35 \pm 0.81\%$ ) compared to the RT ( $14.72 \pm 1.08\%$ ), Phloretin ( $23.10 \pm 1.24\%$ ) and control ( $45.29 \pm 2.42\%$ ) groups (all  $p < 0.05$ ).

## 4. Discussion

For more than a century, radiotherapy (RT) has been the mainstream of non-surgical cancer treatment. The problem of radiotoxicity has been constantly evolving, and there is no evidence that the increased toxicity of radiotherapy can increase the ability of local control. Therefore, we are committed to anti-cancer drugs combined with radiotherapy to improve the efficacy of radiotherapy[11, 12]. Extensive research has been conducted to explore anti-cancer phytochemicals in fruits and vegetables. Among these dietary sources, apples are a source of many antioxidants and inflammatory agents. There is a proverb that eating an apple a day can keep doctors away[5]. Among the natural dihydrochalcone found in apple, phloretin is one of the main active ingredients. Some evidence suggests that phloretin can treat cardiovascular disease, cancer, chemical perfusion injury, neurodegeneration, diabetes and ameliorates colon inflammation [13–15]. The four hydroxyl groups in the phloretin structure can inhibit the transmembrane transport of sugars in cells, block energy transport, and induce cancer cell apoptosis. Moreover, an attempt is made to delineate the direction of future studies that could lead to the development of apple components as a potent chemopreventive/chemotherapeutic agent against cancer[16].

After treating Lewis cells with phloretin, we found that the inhibitory effect of phloretin on the proliferation is concentration and time dependent. In order to clarify the synergistic inhibitory effects of phloretin and radiotherapy on Lewis lung cancer (LLC) cells, a clone formation experiment was carried out by combining appropriate toxic concentrations with different radiation doses. Compared with radiotherapy alone, the combination of phloretin and RT showed higher clonal formation inhibition and fewer surviving colonies, shown by calculations of increased SER and decreased  $D_0$  and  $D_q$  (Table 1). The potential

mechanism of phloretin-induced radiosensitization may be connected with the inhibition of proliferation and glucose transport, and promotion of apoptosis[16].

We demonstrated the radiosensitization effect by intraperitoneal injection of phloretin in Lewis tumor-bearing mouse. The results showed that phloretin inhibited tumor growth and prolonged survival in mouse. Similarly, there is a report phloretin inhibit tumor growth by increasing the penetration efficiency of HSP70 in melanoma xenograft models [17]. It has been reported to inhibit metastasis and angiogenesis in cervical cancer xenograft models[10]. In our study, phloretin combined with radiotherapy has the most obvious tumor inhibitory effect and the longest survival time, indicating that the combination of chlorophyll and radiotherapy has a synergistic inhibitory effect.

It is known that malignant cells have accelerated metabolism, high glucose demand and increased glucose uptake. The transport of glucose across the plasma membrane of mammalian cells is the first rate-limiting step of glucose metabolism and is mediated by the facilitating glucose transporter (GLUT). In human studies, high levels of GLUT expression in tumors are associated with poor survival rates. Disruption of glucose uptake by glucose transporters may alter the metabolism of malignant cells, leading to reduced tumor growth[18]. Tracking  $^{18}\text{F}$ -FDG uptake by PET/CT is widely used in tumor detection and monitoring treatment outcome. Normal cells and cancer cells have different glucose metabolism states. Most solid tumor cells rely heavily on aerobic glycolysis to produce energy and adapt to their heterogeneous microenvironment, which allows them to sustain a high rate of proliferation and resist signals of apoptosis. This phenomenon is called the Warburg effect, which is a hallmark of cancer[19, 20]. The therapeutic importance of the Warburg effect is increasingly recognized, and blocking glucose transporters has become a common anti-cancer strategy. Previous reports have identified Ph as a new type of small compound that inhibits glucose transport in liver and colon cancer cells and suppresses the growth of cancer cells through glucose deprivation[9, 21, 22]. A microPET study in mouse bearing HepG2 tumors showed that compared with the control group, the uptake of  $^{18}\text{F}$ -FDG in Ph-treated tumors was reduced by 10 times. This result indicate that Ph-induced HepG2 cell apoptosis involves the mechanism of inhibition of GLUT2 glucose transport [23]. A higher FDG uptake in tumor indicates higher glucose metabolism, suggesting poor response and prognosis, while tumors with lower FDG uptake may have a better response to treatment. Based on this index, RT + Phloretin combination therapy was most effective in inhibiting tumor metabolism and growth (Fig. 4). Taken together, Phloretin inhibits the transport of glucose and enhances the killing effect of radiotherapy on tumor cells.

Phloretin has a strong ability to induce tumor cell apoptosis. Previous researchs has shown that phloretin markedly augment TRAIL mediated apoptosis in LNCaP cells[24]. Ph can inhibit the growth of A549 cells by inducing apoptosis through P38 MAPK and JNK1/2 pathways. After Ph treatment, the expression level of BAX, cleaved caspase-3 and - 9, and degraded form of PARP was increased and Bcl-2 was decreased. [25]. Another, phloretin markedly increased caspase-3 activity as well as JNK and p38 mitogen-activated protein kinase signaling in H-Ras MCF10A Human Breast Tumor Cells[26]. Phloretin induces apoptosis of liver cancer cells possibly by inhibiting the production of ATP, leading to a decrease in mitochondrial membrane potential, and then mitochondria decompose and activate downstream apoptotic

pathways[23]. It has been reported that the inhibition of glucose transport is related to the earliest steps of cell apoptosis. Glucose deprivation may induce three main pathways, including the mitochondrial death pathway induced by ATP depletion, the cell death pathway related to oxidative stress, and finally the expression of HIF-1 $\alpha$  regulated by hypoglycemia[23, 27]. Apoptosis is of great significance in the occurrence and development of tumors. Disregulation of apoptosis is considered to be one of the hallmarks of cancer, and promoting tumor apoptosis is an important treatment[28]. Compared with other groups, radiotherapy combined with phloretin significantly increased Lewis cell apoptosis ( $P < 0.05$ ), which supports the above view.

The synergistic effect of phloretin and RT on tumor cell proliferation was also analyzed by detecting the expression levels of Ki67. Ki67 is a nuclear antigen associated with proliferation, and RT + phloretin caused a significant reduction in the percentage of Ki67 positive cells in the tumor xenografts (Fig. 6). Phloretin may inhibit cell proliferation by blocking the cell cycle[29, 30]. The combination of radiotherapy and phloretin significantly reduced the expression of Ki-67 compared with other groups ( $P < 0.05$ ), which was consistent with the MTT results, indicating that intraperitoneal injection can also exert anti-tumor effects in vivo.

## 5. Conclusions

In general, the combination of phloretin and radiotherapy has a good anti-tumor effect on lung cancer, which was proven to significantly inhibit tumor growth and prolong the survival time of tumor-bearing mouse. This indicates that phloretin is a promising radiosensitizer for lung adenocarcinoma by intraperitoneal injection, and the mechanism involved may be related to the inhibition of glucose transport, promotion of cell apoptosis, and tumor cell proliferation inhibition.

## Abbreviations

phloretin (Ph)

radiotherapy (RT)

Lewis lung cancer (LLC)

radiotherapy sensitization ratio (SER)

tumor growth delay (TGD)

proliferation index (Ki67)

Dimethyl sulfoxide (DMSO)

methyl thiazole blue (MTT)

Plating efficiency (PE)

surviving fraction (SF)

sensitivity enhancement ratio (SER)

Tumor growth curve (TGC)

standard uptake values (SUVs)

mean±standard deviation (SD)

One-way analysis of variance (ANOVA)

## **Declarations**

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### **Authors' contributions**

Juan Tang: project development, data collection, data analysis, manuscript writing; Youling Gong: project development, manuscript editing.

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### **Conflict of interest**

The authors declare that they have no conflict of interest.

### **Code availability**

Not applicable

### **Ethical approval**

This article does not contain any studies with human participants performed by any of the authors. I regret not being able to provide informed consent. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal experiments were implemented in accordance with the Institutional Animal Care and Use Guidelines, and approved by the Institutional Animal Southwest Medical Care and Use Committee (Chendu, China).

## Consent for publication

All authors have given their consent for the publishing of this manuscript.

## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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30. Phloretin flavonoid exhibits selective antiproliferative activity in doxorubicin-resistant gastric cancer cells by inducing autophagy, inhibiting cell migration and invasion, cell cycle arrest and targeting ERK1/2 MAP pathway. *J*

## Figures

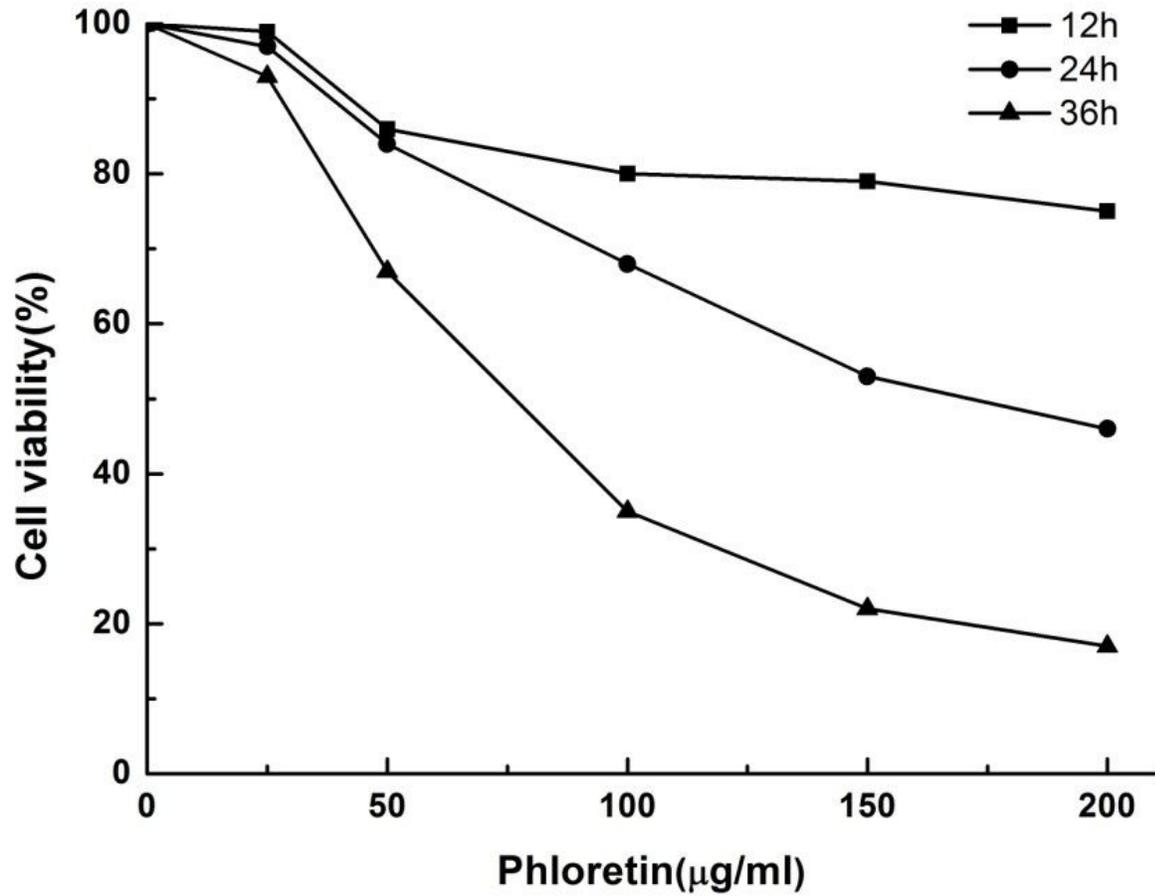
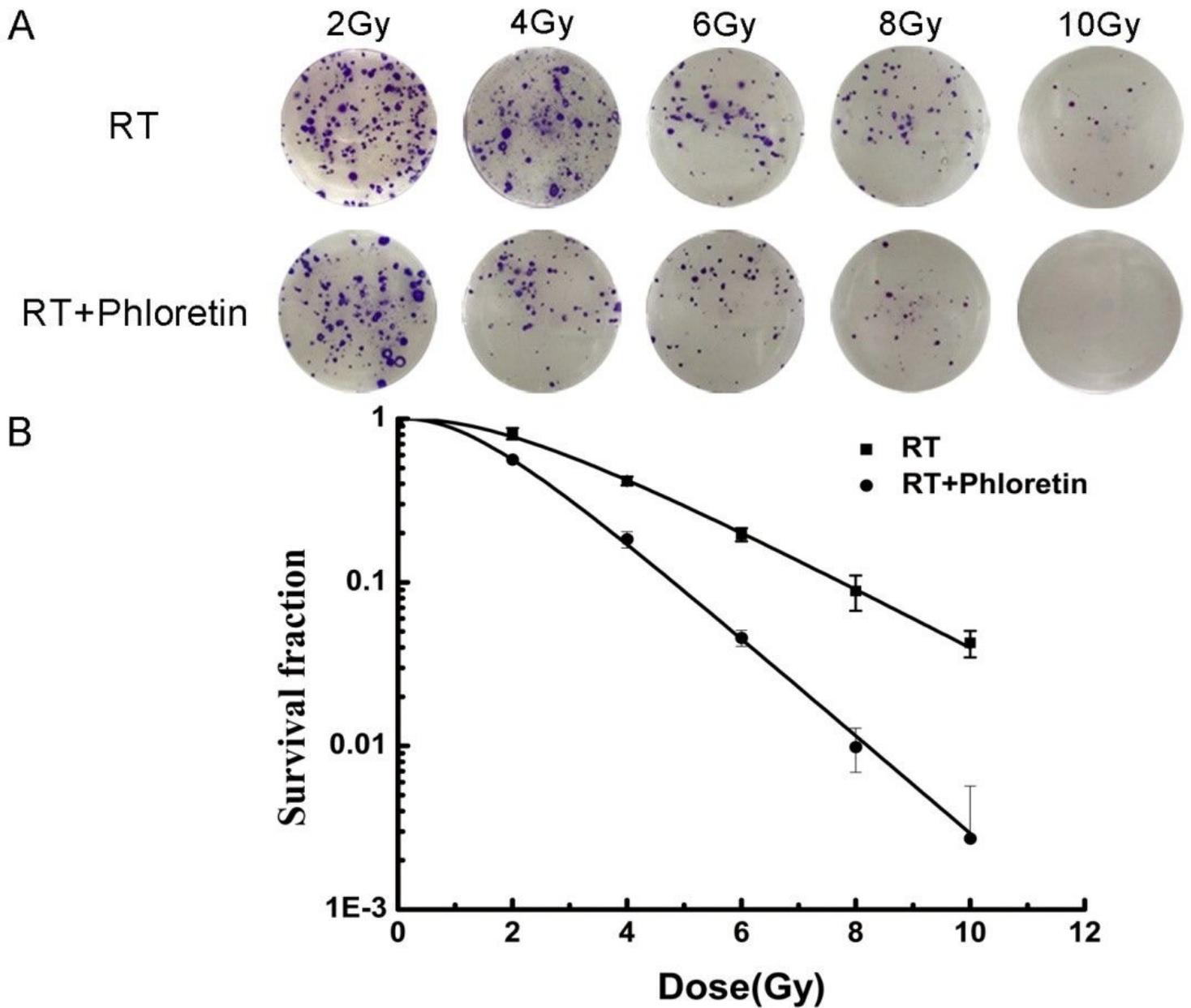


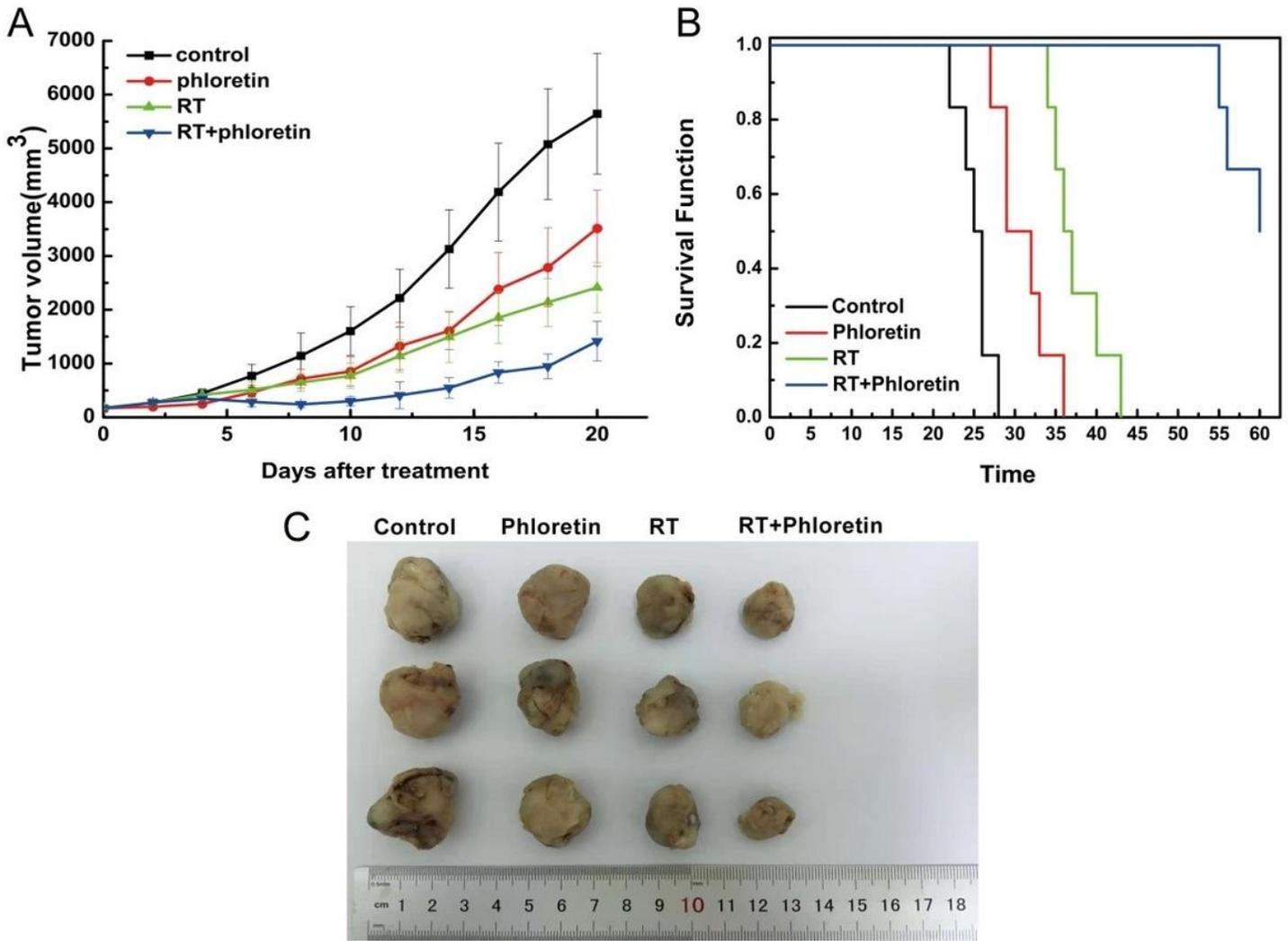
Figure 1

Phloretin inhibits the proliferation of Lewis cells. Cell viability of Lewis cells treated with different concentrations for 12, 24, and 36 hours.



**Figure 2**

Clone formation result. Colony formation capacity of LLC cells exposed to different radiation doses alone (square symbols) or in combination with 2h phloretin treatment (round symbols). Irradiation was performed 1h after the phloretin treatment. Data points represent the mean and standard deviation from at least three independent experiments. The solid curve is obtained by fitting a multi-target model to the data.



**Figure 3**

RT + Phloretin combination therapy inhibited Lewis tumor growth in vivo. (A) Suppression of subcutaneous tumor growth by RT + Phloretin. (B) Survival curve of mouse in each group. (C) Comparison of the size of the tumor after the treatment is completed.

A

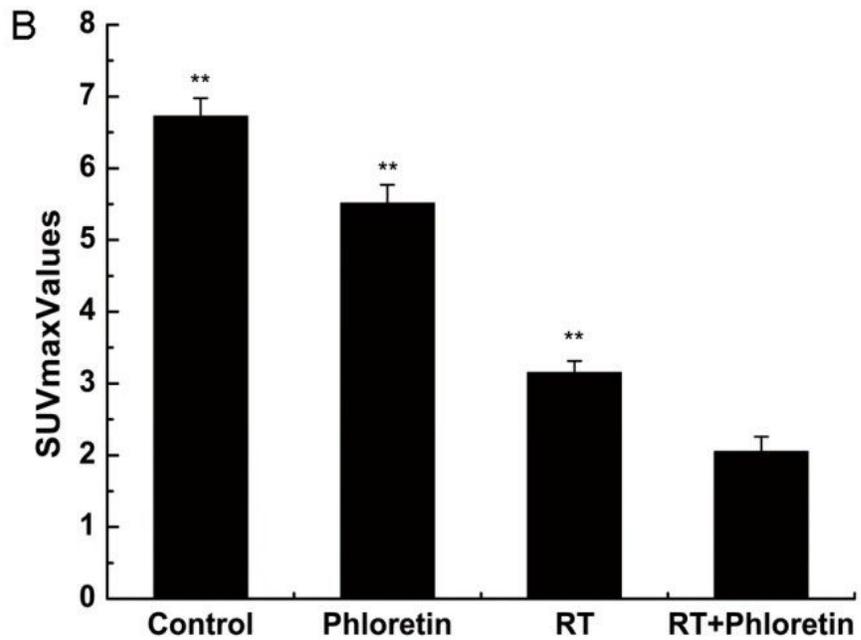
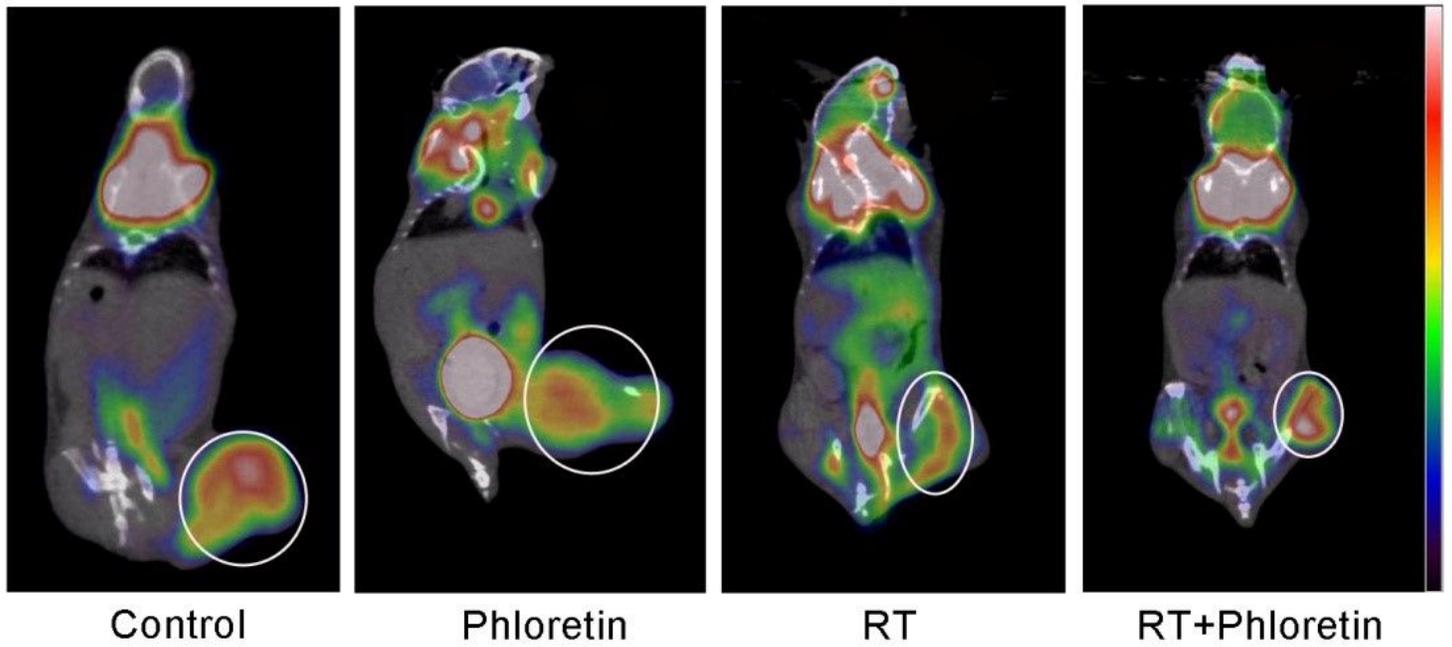
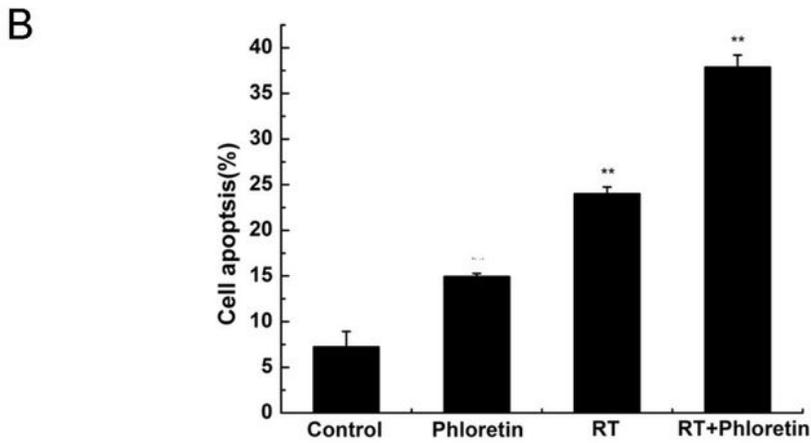
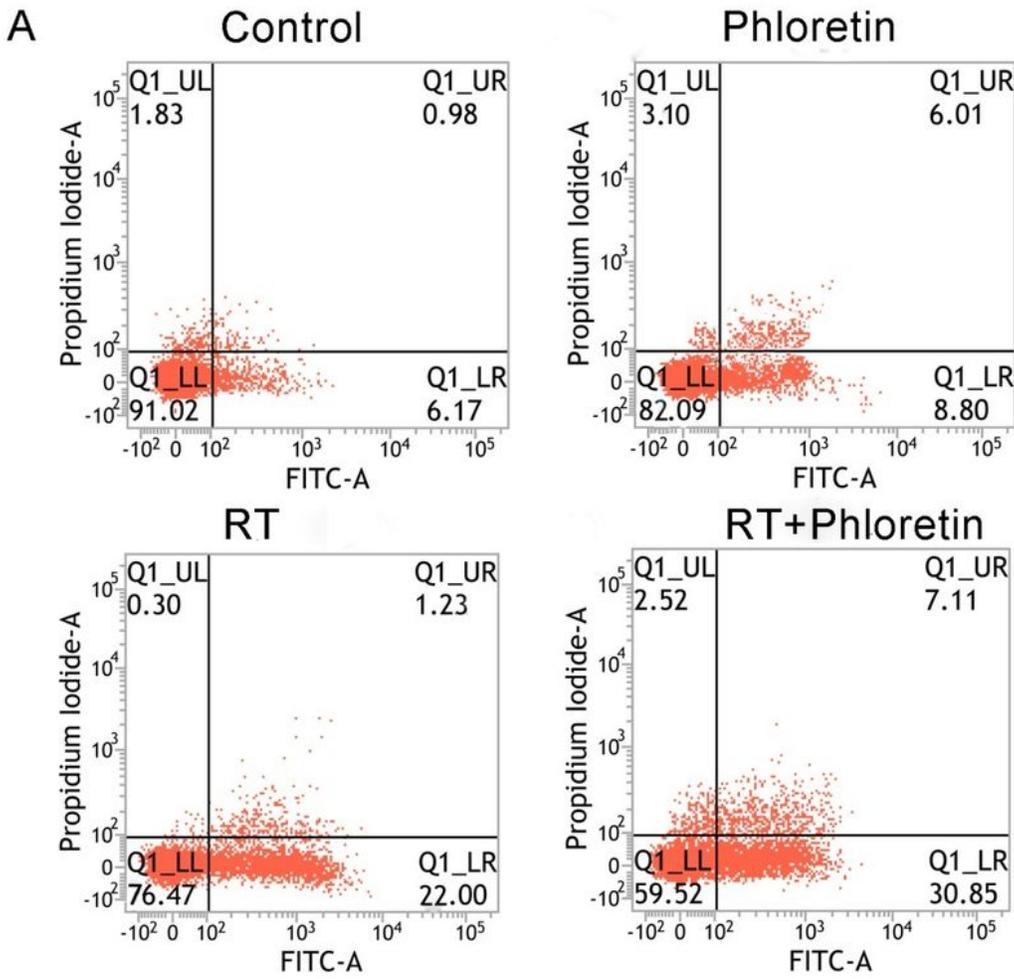


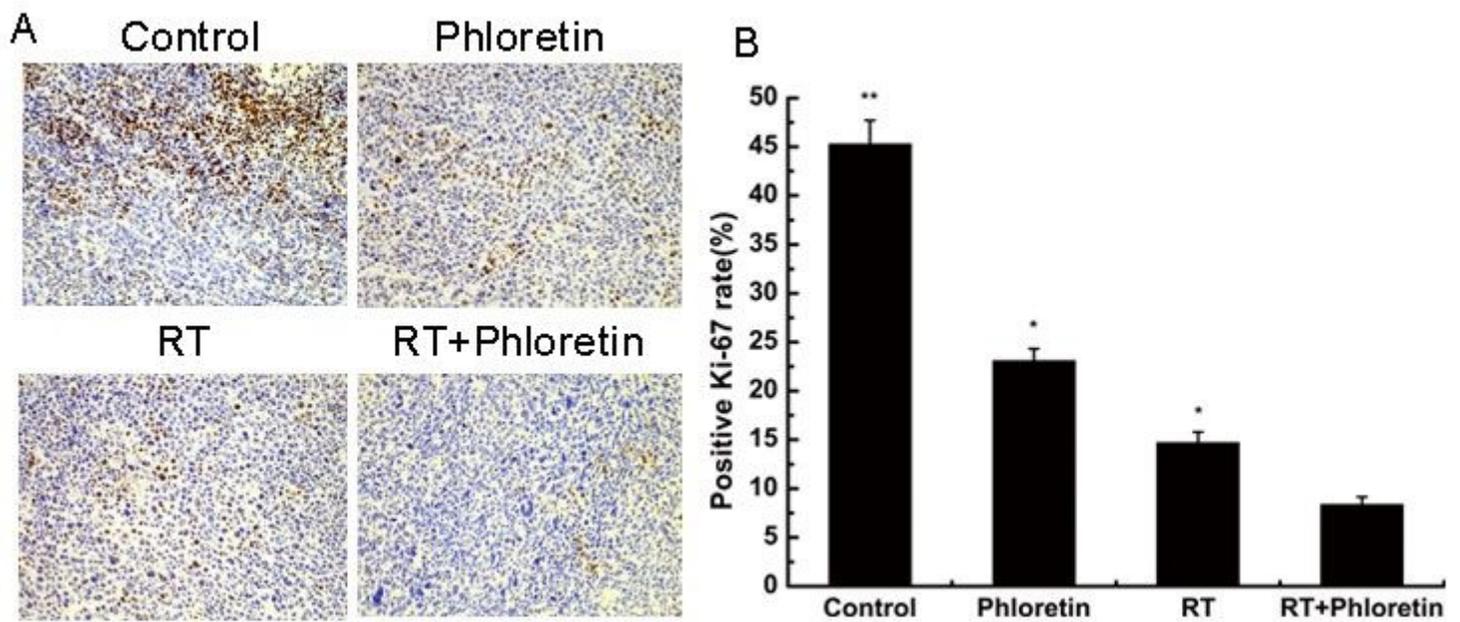
Figure 4

Representative  $^{18}\text{F}$ -FDG PET/CT images of the mice after the last treatment. \*\*  $p < 0.01$ , Control, Phloretin and RT compared to RT + Phloretin.



**Figure 5**

Analysis of the percentage of apoptotic cells in each group were quantitatively analyzed. \*\*  $p < 0.01$ , Phloretin, RT and RT + Phloretin compared to control.



**Figure 6**

Ki-67 expression in transplanted tumors from different groups. (A) Representative IHC images showing Ki-67 expression in tumor tissues, scale bars = 50  $\mu$ m (original magnification,  $\times$ 400). (B) Histogram showing percentage of Ki-67 positive cells in each group. Data are expressed as mean  $\pm$  SD. Control, Phloretin and RT compared to RT +Phloretin; \* $P$ <0.05, \*\*  $p$ <0.01.