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Article

# Piperine Induces Apoptosis and Cell Cycle Arrest via Multiple Oxidative Stress Mechanisms and Regulation of PI3K/Akt and MAPK Signaling in Colorectal Cancer Cells

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**Abstract:** Piperine, a phytochemical alkaloid, exhibits anticancer properties on several cancer cells. The present study explored the important oxidative stress and signaling mechanisms by which piperine provokes cell death and apoptosis in colorectal DLD-1 cancer cells. We investigated the cell viability, cell cycle, and apoptosis-inducing of piperine by performing MTT assay, flow cytometry, gene overexpression, and Western blot analysis. Piperine treatment of DLD-1 cells diminished cell viability, and stimulated cell cycle G1 arrest and apoptosis. Piperine induced intracellular reactive oxygen species (ROS) generation by regulating mitochondrial complex III, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and xanthine oxidase. Piperine inhibited the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway and activated the p-38 and p-extracellular signal-regulated kinase (ERK) pathways. Pretreatment with antimycin A (a mitochondrial complex III inhibitor), apocynin (an NADPH oxidase inhibitor), allopurinol (a xanthine oxidase inhibitor), and PD98059 (an ERK inhibitor) and the overexpression of p-Akt significantly recovered cell viability and reduced apoptosis. This study was the first to demonstrate a multiple oxidative stress mechanism and the critical regulating PI3K/Akt and ERK signaling pathways of piperine, inducing apoptosis on colorectal cancer cells.

**Keywords:** piperine; apoptosis; cell cycle arrest; oxidative stress; PI3K/Akt; MAPK; colorectal cancer

## 1. Introduction

Piperine is a phytochemical alkaloid that exhibits the potential to provide anticancer activities in colorectal cancer. In human HRT-18 rectal cancer cells, piperine-induced apoptosis and cytotoxicity are partially achieved through ROS [1]. Piperine prompts autophagy-related cell death in colorectal cancer cells by increasing ROS level and obstructing Akt/mTOR signaling [2]. Piperine lessens ADP-ribosylation factors, such as GTPase 3-intermediated endoplasmic reticulum stress reaction, disrupts cell cycle development, and inhibits epithelial-to-mesenchymal transition in colorectal adenocarcinoma cells [3]. In several colorectal cell lines, piperine can downregulate the Wnt/β-catenin pathway to suppress cellular migration and proliferation [4]. Moreover, piperine can be used in adjuvant radiotherapy and chemotherapy to enhance sensitivity to colorectal cancer. Piperine combined with γ-radiation radiotherapy enhances cell cycle G2/M phase arrest, increases



estrogen receptor beta expression, and induces HT-29 colorectal cancer cells to conduct apoptosis [5]. Piperine can be used as a chemosensitizer to 5-fluorouracil in chemoresistant colorectal cancer cells [6]. It can mediate Wnt/β-catenin signaling to synergistically suppress celecoxib-treated cell proliferation in colon cancer [7]. Hence, piperine exhibits a valuable potential against colorectal cancer.

ROS refer to the highly reactive substances including oxygen radicals. Major ROS specimens include superoxides, hydrogen peroxides, hydroxyl radicals, hypochlorous acid, singlet oxygen, and alpha-oxygen [8]. In mammalian cells, ROS are generated through various types of biochemical responses inside cellular organelles, such as mitochondria, peroxisome, and endoplasmic reticulum [8]. Mitochondria are known for generating about 90% of cellular ROS [9]. Many cellular enzymatic reactions can induce ROS production, such as cyclooxygenase, xanthine oxidase, and NADPH oxidase [10–12]. The increased amount of ROS can delay the growth of tumors by continuously augmenting during cell cycle inhibition. The augmented concentration of ROS can stimulate apoptosis through extrinsic and intrinsic pathways [8]. A high ROS level is believed to be a tumor-destroying mediator because the generation of intracellular ROS is used in most chemotherapeutic drugs to induce cancer cell death [8]. Piperine can stimulate cell cycle arrest and apoptosis in colorectal cancer by inducing ROS production. However, the detailed mechanisms by which piperine induces ROS production in colorectal cancer cells is currently unclear.

Many anticancer drugs can stimulate the apoptosis and inhibit the proliferation of colorectal cancer cells by stimulating ROS generation to regulate the PI3K/Akt and MAPK signaling pathways. Pyrvinium pamoate, an anthelmintic drug, can effectually prevent cell proliferation through the ROS-intervened Akt-reliant signaling pathway in colorectal cancer [13]. Antrodin C, a maleimide derivative compound extracted from *Antrodia cinnamomea* induces apoptosis via the ROS/Akt/ERK/p38 signaling pathway in colorectal cancer cells [14]. Metformin augments cisplatin to exhibit sensitivity in colorectal cancer cells via the ROS-related PI3K/Akt signaling pathway [15]. Lycorine reduces cell proliferation and cytostatic effects in colorectal cancer by triggering the ROS/p38 and Akt signaling pathways [16]. Delicaflavone prompts ROS-intervened apoptosis and impedes the PI3K/Akt/mTOR and Ras/MEK/Erk signaling pathways in colorectal cancer cells [17]. Fibulin-5 causes to apoptosis in colorectal cancer cell through the Akt and ROS/MAPK signaling routes [18]. Polyphyllin I stimulates apoptosis via the ROS-reduced Akt/mTOR signaling pathway in colon cancer cells [19]. Quinalizarin stimulates apoptosis via the ROS-intervened MAPK signaling pathway in colorectal cancer [20]. Manumycin and cocoa tea (*Camellia ptilophylla*) trigger mitochondrion-related apoptosis in HCT116 colorectal cells through the PI3K/Akt signaling pathway and ROS production [21,22]. The piperine-induced ROS mechanisms and the control of the MAPK and PI3K/Akt signaling pathways of colorectal cancers deserves further study.

In the current work, we intend to characterize the anticancer effects of piperine. This study aims to fill the gap among detailed oxidative stress mechanisms, PI3K/Akt, and MAPK signaling, offering new opinions into potential role of piperine in colorectal cancer progression.

## 2. Materials and Methods

### 2.1. Reagents and Chemicals

Penicillin streptomycin–glutamine and fetal bovine serum (FBS) were bought from Gibco Inc. (Billings, MT, USA). RPMI 1640 medium was obtained from Hyclone (South Logan, UT, USA). Piperine, trypan blue, crystal violet, DMSO, propidium iodide (PI), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), MTT, and other chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA). X-tremeGENE™ HP DNA transfection reagent was purchased from Roche (Raleigh, NC, USA). The protein assay kit was acquired from Bio-Rad Laboratories (Richmond, CA, USA). Primary antibodies against p53, Bax, p27, poly (ADP-ribose) polymerase (PARP), cyclin E, p-Akt, Akt, p-ERK, p-p38, p-JNK, tubulin, and GAPDH were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The 1036 pcDNA3 Myr HA Akt1 vector was obtained from Addgene Company

(Watertown, MA, USA). The pcDNA 3.1<sup>(+)</sup> vector was bought from Thermo Fisher Scientific Company (Taiwan).

## 2.2. Cell Culture

Human colorectal DLD-1 cancer cells were cultured in RPMI 1640 medium. The medium was supplemented with 10% FBS, 2 mM of L-glutamine, 100 units/mL of penicillin G, and 100 µg/mL of streptomycin. All cells were retained at 37 °C in a 5% CO<sub>2</sub> incubator. Stock solutions of piperine were suspended in DMSO, and all treated concentrations were attuned in the culture medium. The concentration of DMSO did not exceed 0.05%.

## 2.3. MTT Assay for Cell Viability

MTT assay was performed to determine cell viability. Approximately  $4 \times 10^4$  cells/well in 0.5 mL of RPMI 1640 medium were incubated in 24-well plates. After growing overnight, the DLD-1 cells were treated with piperine (0, 62.5, 125, and 250 µM), or various compounds for various time points. The plates were then added with 0.5 mg/mL of MTT solution and maintained at 37 °C for additional 2 h. The supernatant was removed, and the formazan crystals were dispersed in 1 mL of DMSO. An aliquot of the DMSO lysed solution (200 µL) was collected from every well and transferred to 96-well reader plates. Optical density was measured with a microplate reader (Bio-Rad, Richmond, CA, USA) at 570 nm.

## 2.4. Colony Formation Assay

DLD-1 cells were placed in 24-well culture plates ( $5 \times 10^4$  cells/ well) and cultured with piperine (0, 62.5, 125, and 250 µM) for 48 h. The colonies were then washed with PBS, fixed with a fixing solution (methanol:glacial acetic acid; 3:1) for 10 min, cleaned again with PBS, and stained with 1% crystal violet solution for 30 min. Images were captured, and stained cell colony densities were determined using Image J software for analysis. Each well insert was added with 200 µL of the dissolving solution (33% acetic acid) to disperse the cells, and 100 µL of each dissolving solution was disperse and measured at 570 nm by using a microplate reader (Bio-Rad, Richmond, CA, USA).

## 2.5. Cell Cycle Analysis

DLD-1 cells ( $5 \times 10^5$ ) were incubated in six-well plates for overnight and then cultured with piperine (0, 62.5, 125 and 250 µM) for 48 h. Cells were collected and fixed in methanol solution (methanol:PBS = 2:1) at 4 °C for at least 24 h. After removing the methanol solution, cells were stained with a PI solution (40 µg/mL) that contained DNase-free RNase A (40 µg/mL) for 30 min at room temperature in the dark and later assessed via flow cytometry. In the resulting histogram, the X-axis denotes PI fluorescence intensity while the Y-axis denotes the number of cells.

## 2.6. Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) Analysis

Apoptosis was assessed via the TUNEL assay. After piperine incubation, DLD-1 cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were cleaned with PBS and then added with a TUNEL reaction kit for 1 h at 37°C. This procedure was followed by cleaning with PBS and PI addition for 30 min. TUNEL-positive cells were distinguished through flow cytometry.

## 2.7. Western Blot

Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to immobilon polyvinylidifluoride (PVDF) membranes. The PVDF membranes were blocked and probed using primary antibodies (1:1000) at 4°C overnight. The blots were then incubated with secondary antibodies at room temperature for 1 h. The antigen–antibody complexes signals were

evaluated via the enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by using a chemiluminescence analyzer.

#### 2.8. Determination of Intracellular ROS

Intracellular ROS production was assessed via flow cytometry by using DCFH-DA staining after treating DLD-1 cells ( $5 \times 10^5$  cells/6 cm dish) with piperine (250  $\mu$ M) for 1, 3, 6, and 24 h with 10  $\mu$ M of DCFH-DA at 37°C for 30 min in the dark and then stained with PI (4  $\mu$ g/mL) to eliminate dead cells. Intracellular ROS production was identified through the mean intensity of green fluorescence (2',7'-DCF) via flow cytometry.

#### 2.9. Overexpression of Akt in Cancer Cells

Approximately 6  $\mu$ g of 1036 pcDNA3 Myr HA Akt1 vector (Addgene, Cambridge, MA, USA) or pcDNA 3.1<sup>(+)</sup> vector (an empty vector; Thermo-Fisher Scientific, Taiwan) was transfected to DLD-1 cells via an X-treme transfection reagent. Approximately  $4 \times 10^4$  DLD-1 colorectal cancer cells were cultured in a six-well plate and maintained in a 37 °C, 5% CO<sub>2</sub> incubator for 24 h. Thereafter, 2 mL of serum-free RPMI 1640 medium was substituted. Approximately 0.2 mL of the serum-free RPMI 1640 medium was added into an Eppendorf tube with 6  $\mu$ L of X-tremeGENE HP DNA transfection reagent and 2  $\mu$ g of vectors, gently pipetted and mixed smoothly. After reacting at room temperature for 15 min, approximately 400  $\mu$ L was collected and placed in a dish. The cells were mixed smoothly, returned to the incubator at 37 °C for 24 h, and then incubated with of 1  $\mu$ g/mL puromycin antibiotics for 14 days to create a steady Akt overexpression cell line. The stable DLD-1 cell line was cultured in RPMI 1640 medium with 10% FBS and then subjected to piperine for 48 h.

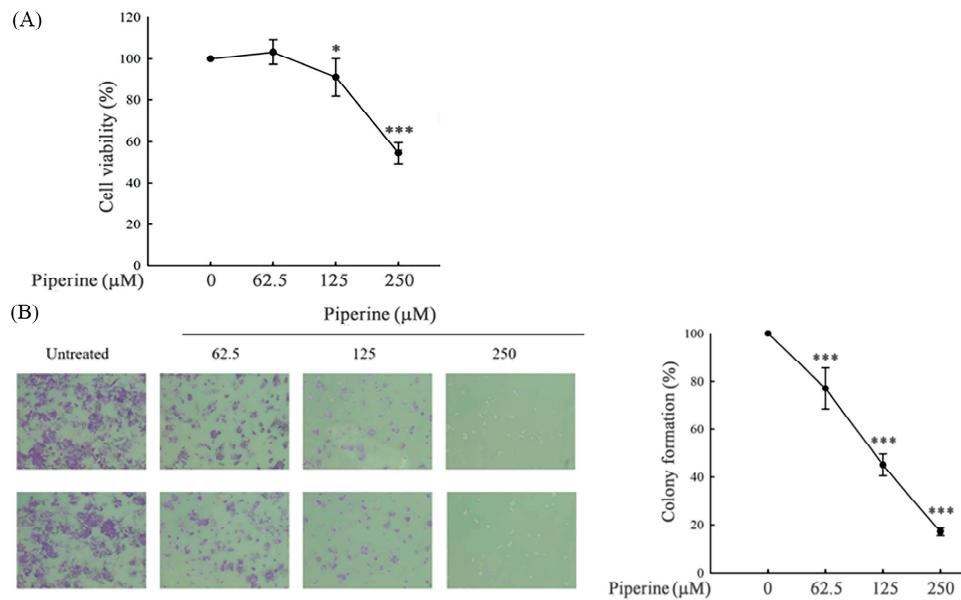
#### 2.10. Statistical Analysis

Statistical analysis was performed using Student's t-test with SigmaPlot 10.0 software. Data were presented as the mean  $\pm$  standard deviation from at least three independent experiments. A *P* value of  $< 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Piperine Inhibits Cell Viability and Colony Formation in DLD-1 Cells

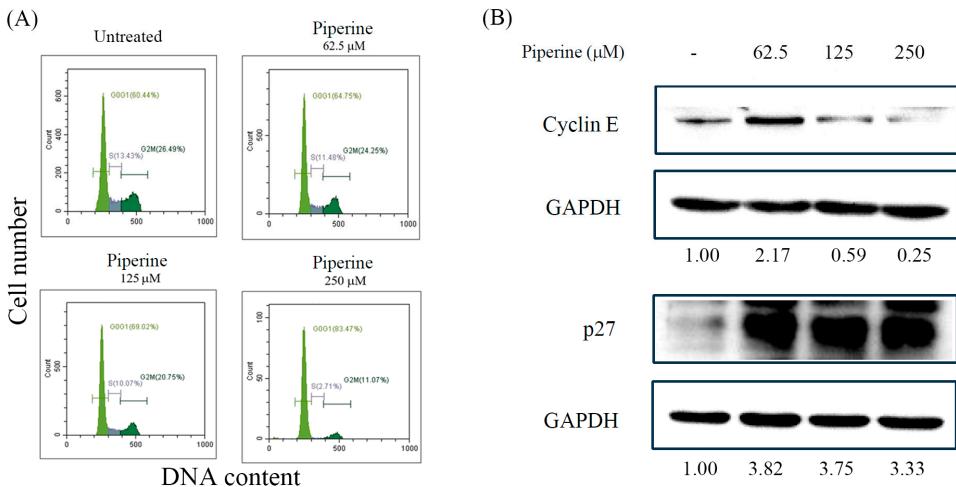
We analyzed the cell viability influence of piperine (0–250  $\mu$ M) on DLD-1 cells for 48 h. Our results showed that piperine treatment significantly diminished DLD-1 cell viability and inhibition concentrations were 125  $\mu$ M and 250  $\mu$ M (Figure 1A). To further evaluate the cell proliferation effect of piperine, we treated DLD-1 cells with various concentrations of piperine (0, 62.5, 125, and 250  $\mu$ M) for 48 h to assess colony formation. The colony formation capability of DLD-1 cells was inhibited significantly by incubation with piperine and inhibition was dose-dependent (Figure 1B).



**Figure 1.** Piperine inhibits cell viability and colony formation in DLD-1 cells. (A) Cell viability of DLD-1 cells incubated for 48 h with different concentrations of piperine assessed via MTT assays. (B) Colony formation in DLD-1 cells incubated with piperine (0, 62.5, 125, and 250  $\mu$ M) for 48 h. Significant differences in the 0  $\mu$ M-treated group are presented  $P < 0.05$  (\*) and  $P < 0.001$  (\*\*).

### 3.2. Piperine Induces Cell Cycle Arrest in DLD-1 Cells

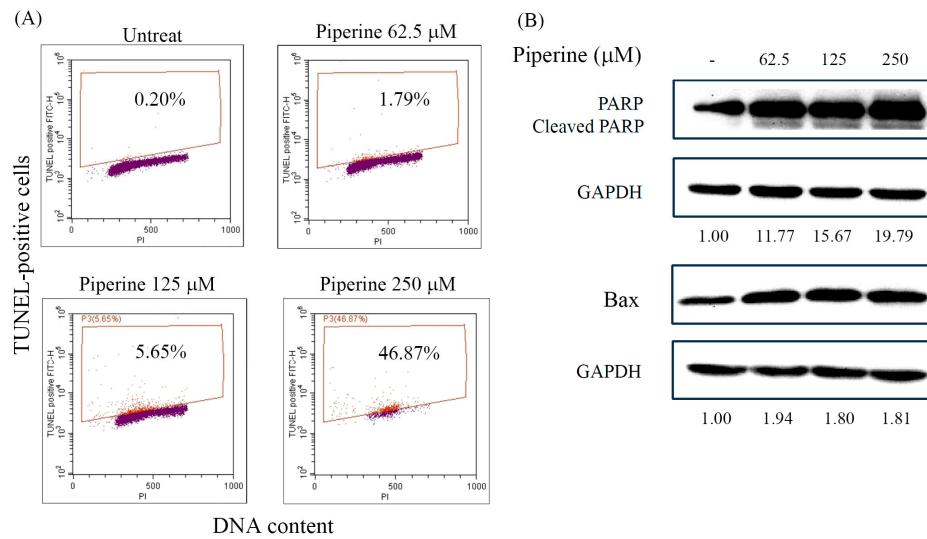
To investigate whether the piperine-induced colony formation inhibition was a consequence of cell cycle arrest, we operated a cell cycle assay via flow cytometry. In DLD-1 cells, piperine treatment for 48 h exhibited a time-dependent decrease in S phase populations (Figure 2A). Moreover, piperine at 250  $\mu$ M evidently induced cell cycle G1 arrest (Figure 2A). The cell cycle G1 arrest was further verified via Western blot assay for cell cycle-regulated proteins, such as cyclin E, which demonstrated downregulation, and p27, which exhibited upregulation upon piperine treatment (Figure 2B). These results indicate that cell cycle arrest is responsible for the anticancer effect of piperine on human colorectal DLD-1 cancer cells.



**Figure 2.** Piperine induces cell cycle G1 arrest in DLD-1 cells. (A) Cell cycle of DLD-1 cells cultured for 48 h with different concentrations of piperine assessed via PI staining and flow cytometry. (B) Expression of cell cycle-regulated proteins, cyclin E, and p27 in DLD-1 cells incubated with piperine (0, 62.5, 125, and 250  $\mu$ M) for 48 h.

### 3.3. Piperine Induces Apoptosis in DLD-1 Cells

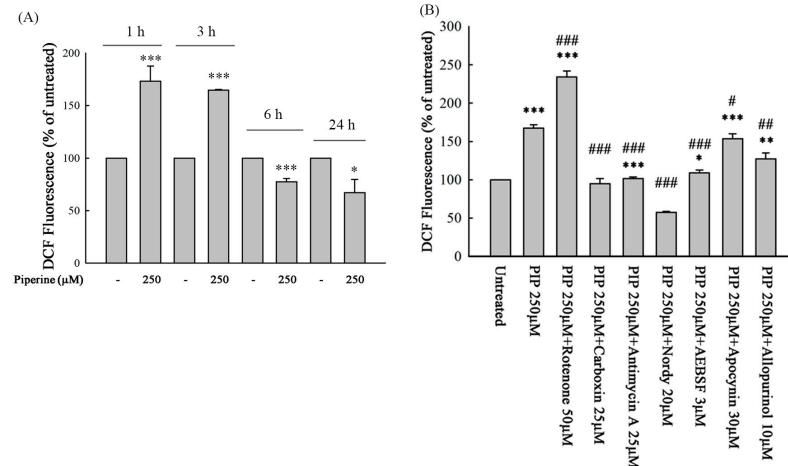
Next, we verified the cell death mode by which piperine intermediates its cytotoxic influences on DLD-1 cells. To examine whether piperine induces apoptosis in DLD-1 cells, we treated DLD-1 cells with piperine for 48 h, followed by quantified TUNEL assay by using flow cytometry. A trend toward dose-dependent increase in TUNEL-positive cells was detected in piperine treated DLD-1 cells, representing the occurrence of apoptosis in DLD-1 cells (Figure 3A). These results were further verified through apoptosis regulated proteins, cleaved PARP, and Bax, via Western blot assay. The expression of cleaved PARP and Bax exhibited upregulation upon piperine treatment (Figure 3B).



**Figure 3.** Piperine induces apoptosis in DLD-1 cells. (A) Percentages of apoptosis in DLD-1 cells incubated for 48 h with piperine (0, 62.5, 125, and 250 μM) assessed via TUNEL assay and flow cytometry. (B) Expression of apoptosis marker proteins, cleaved PARP, and Bax in DLD-1 cells incubated with piperine (0, 62.5, 125, and 250 μM) for 48 h.

### 3.4. Piperine Induces ROS in DLD-1 Cells

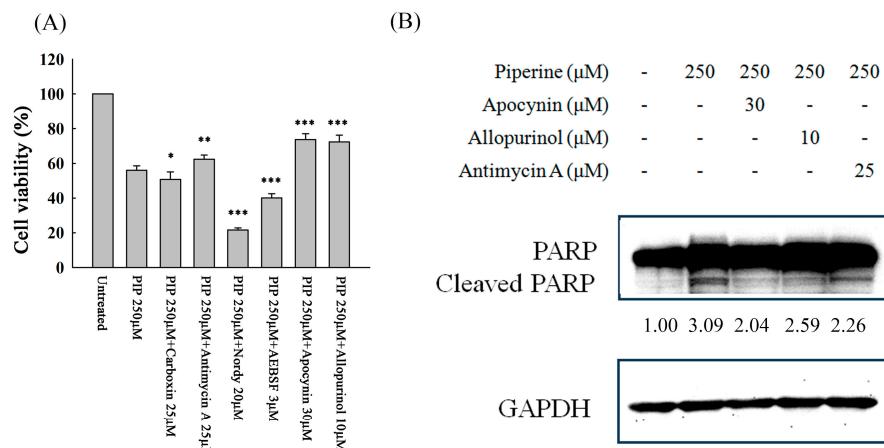
To measure the probable participation of the intracellular ROS level of cells in the apoptotic effect of piperine, we determined intracellular ROS generation in piperine-treated cells. Piperine treatment significantly increased intracellular ROS generation at 1 h and 3 h (Figure 4A). To investigate the possible sources of ROS generation induced by piperine, DLD-1 cells were pretreated with special inhibitors of mitochondrial complex I, II or III, NADPH oxidase, lipoxygenase, and xanthine oxidase for 1 h and then incubated with piperine for 1 h. Cells were estimated for intracellular ROS generation. Carboxin (a mitochondrial complex II inhibitor), antimycin A (a mitochondrial complex III inhibitor), Nordy (a lipoxygenase inhibitor), AEBSF and apocynin (NADPH oxidase inhibitors), and allopurinol (a xanthine oxidase) reduced the piperine-induced ROS production (Figure 4B). These results indicate that sources of ROS generation stimulated by piperine appears via mitochondrial respiratory chain complexes II and III, lipoxygenase, NADPH oxidase, and xanthine oxidase.



**Figure 4.** Piperine induces ROS in DLD-1 cells. (A) Intracellular ROS of DLD-1 cells incubated for 1, 3, 6, and 24 h with 250  $\mu$ M of piperine assessed via DCFH-DA staining and flow cytometry. (B) Intracellular ROS of DLD-1 cells pre-treated for various ROS-generated inhibitors for 1 h and then incubated with 250  $\mu$ M of piperine for 1 h. After treatment, intracellular ROS was assessed via DCFH-DA staining and flow cytometry. Significant differences in the untreated group are exhibited  $P<0.05$  (\*) and  $P<0.001$  (\*\*). Significant differences in the piperine-incubated group are presented  $P<0.01$  (##) and  $P<0.001$  (###).

### 3.5. Piperine-Mediated ROS Induces Cell Death and Apoptosis in DLD-1 Cells

To investigate whether piperine-induced ROS sources were the critical events that caused DLD-1 cell death, we pretreated the inhibitors of mitochondrial complexes II and III, lipoxygenase, NADPH oxidase, and xanthine oxidase, treated them with piperine, and then evaluated cell viability via MTT assay. The inhibition of cell viability by piperine was significantly recovered by antimycin A (mitochondrial complex III inhibitor), apocynin (NADPH oxidase inhibitor), and allopurinol (xanthine oxidase) (Figure 5A). Moreover, the piperine-induced cleaved PARP was inhibited by pretreatment with antimycin A (mitochondrial complex III inhibitor), apocynin (NADPH oxidase inhibitor), and allopurinol (xanthine oxidase) (Figure 5B). These results indicate that ROS overgeneration from mitochondrial complex III, NADPH oxidase, and xanthine oxidase induced by piperine is a critical factor for DLD-1 cell death and apoptosis.

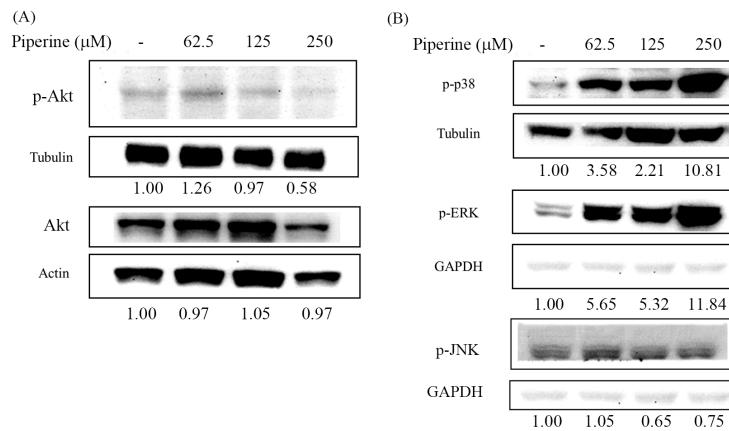


**Figure 5.** Piperine-mediated ROS induces cell death and apoptosis in DLD-1 cells. DLD-1 cells were incubated with various ROS-generated inhibitors for 1 h and then with 250  $\mu$ M of piperine for 48 h. After treatment, cell

viability and cleaved PARP were analyzed via (A) MTT assay and (B) Western blot. Significant differences in the piperine-treated group are exhibited  $P<0.05$  (\*),  $P<0.01$  (\*\*), and  $P<0.001$  (\*\*\*)

### 3.6. Piperine Inhibits p-Akt and Regulates MAPKs in DLD-1 Cells

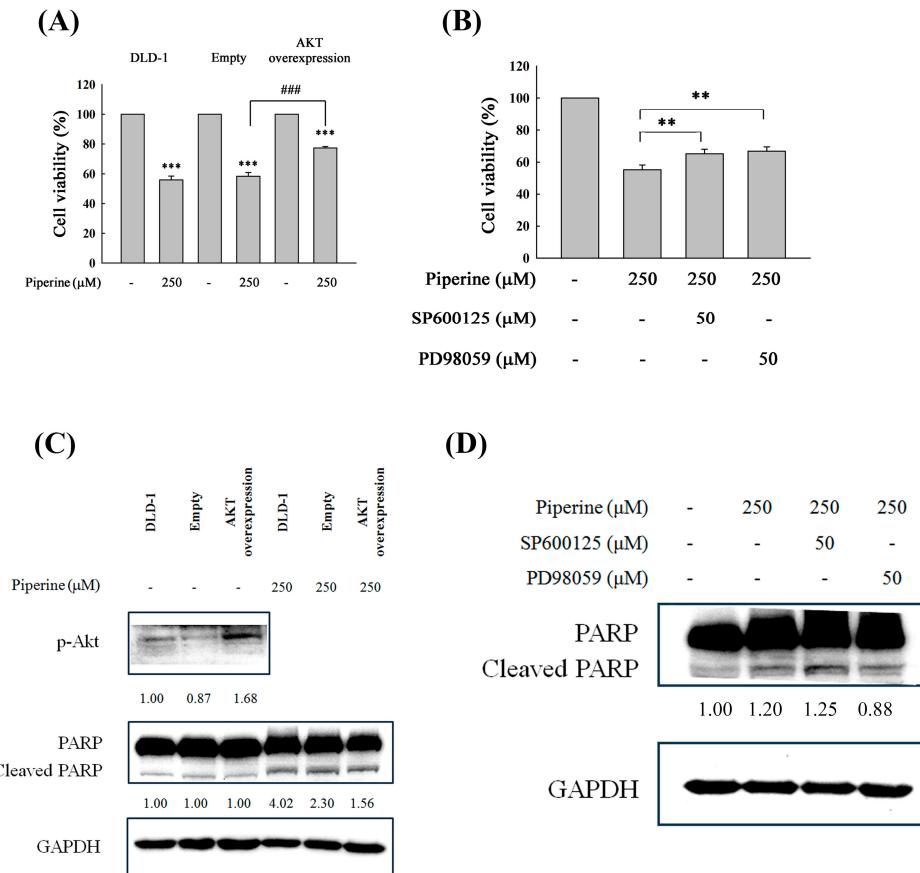
ROS overproduction can mediate the PI3K/Akt and MAPK signaling pathways, followed by inducing apoptosis. To investigate whether piperine treatment can affect the PI3K/Akt and MAPK signaling pathways, we evaluated the expression of p-Akt, p-38, p-ERK, and p-JNK via Western blot. The expression of p-Akt was repressed by piperine treatment, and inhibition was concentration-dependent (Figure 6A). By contrast, the expression of p-p38 and p-ERK was enhanced by piperine treatment (Figure 6B). However, the expression of p-JNK was repressed by piperine treatment (Figure 6B).



**Figure 6.** Piperine inhibits p-Akt and regulates MAPKs in DLD-1 cells. Expression of (A) p-Akt and Akt and (B) MAPK proteins in DLD-1 cells incubated with piperine (0, 62.5, 125, and 250  $\mu$ M) for 48 h.

### 3.7. Piperine Induces Apoptosis through Akt and ERK Signaling Regulation

To investigate whether the PI3K/Akt, p38 and ERK signaling pathways are included in piperine-induced cell death and apoptosis, we utilized the p-Akt overexpression plasmid to perform exogenous p-Akt expression, pretreated with SB203580 (a p38 inhibitor) and PD98059 (an ERK inhibitor), treated it with piperine for 48 h, and then assessed cell viability and cleaved PARP. Cell viability was significantly recovered in p-Akt overexpression and SB203580 and PD98059 pretreatment (Figure 7A,B). Meanwhile, cleaved PARP level was decreased by p-Akt overexpression (Figure 7C) and PD98059 pretreatment (Figure 7D), suggesting that the regulation of the PI3K/Akt and ERK signaling pathways by piperine was involved in inducing cell death and apoptosis.



**Figure 7.** Piperine induces cell death and apoptosis through the Akt, p38, and ERK signaling pathways. (A) DLD-1 cells were transfected with empty plasmid or pAkt-overexpressed plasmid and then treated with 250 μM of piperine for 48 h. After treatment, cell viability was assessed via MTT assay. Significant differences in the untreated and piperine-treated empty plasmid groups are presented  $P<0.001$  (\*\*\*) and  $P<0.001$  (###), respectively. (B) Pretreatment of DLD-1 cells with 50 μM SP600125 or 50 μM PD98059 for 1 h, then incubation with 250 μM of piperine for 48 h. Cell viability was analyzed via MTT assay. Significant differences in the piperine-treated group are presented  $P<0.01$  (\*\*). (C) p-Akt expression was detected in the DLD-1, empty plasmid-, or pAkt-overexpressed plasmid transfected DLD-1 cells. Cells were incubated with 250 μM of piperine for 48 h. Cleaved PARP was measured via Western blot. (D) Pretreatment of DLD-1 cells with 50 μM SP600125 or 50 μM PD98059 for 1 h, then incubation with 250 μM of piperine for 48 h. Cleaved PARP was measured via Western blot.

#### 4. Discussion

ROS play principal roles in cell death, growth, and differentiation by modifying several signaling molecules [23–25]. Compared with their normal counterparts, cancer cells exhibit integrally increased ROS intensities. Augmented ROS levels are strictly related to drug resistance, metastasis, and cancer initiation [26,27]. Although moderate increases in ROS levels can be favorable to cancer cells, extreme amounts of ROS can trigger cell death [28,29]. Hence, anticancer substances may effectively destroy cancer cells by regulating ROS levels. In fact, several small compounds, such as furanodienone, have been found to destroy cancer cells by increasing ROS levels in these cells [30]. In the current study, we determined that piperine exposure significantly increased intracellular ROS during early periods (1 h and 3 h) (Figure 4A), and then reduced it after treatment with carboxin (a mitochondrial complex II inhibitor), antimycin A (a mitochondrial complex III inhibitor), Nordy (a lipoxygenase inhibitor), AEBSF and apocynin (NADPH oxidase inhibitors), and allopurinol (a xanthine oxidase) (Figure 4B). These results indicates that piperine induces multiple sites of intracellular ROS generation. Among these ROS inhibitors, only antimycin A, apocynin, and

allopurinol can recover cell viability (Figure 5A) and apoptosis (Figure 5B), indicating that ROS generated from mitochondrial complex III, NADPH oxidase, and xanthine oxidase are the major factors for piperine-induced cell death.

Under normal physiological conditions, the mitochondrial electron transfer chain provides 90% the intracellular ROS content in cells [31]. Some electrons are directly transferred to O<sub>2</sub> to generate ROS in the electron transfer chain [32]. Many natural products and phytochemicals can kill cancer cells by inducing intracellular ROS through mitochondrial dysfunction in cancer cells. Azoxystrobin inhibits the development of oral cancer via the specific inhibition of mitochondrial complex III activity, resulting in ROS increase, and finally inducing apoptosis [33]. Ginsenoside Rh2, a monomeric compound from ginseng, is highly likely to cause mitochondrial ROS generation and stimulate apoptosis of cervical cancer cells by targeting the electron transfer chain complex, particularly electron transfer chain complex III [34]. Taxodione diminishes the activities of mitochondrial respiratory chain complexes III and V, generating ROS in leukemia cells [35]. Miltirone results in a dose-dependent decrease in complex III activity that involves mitochondrial dysfunction and increased ROS production in leukemia cells [36]. Kari et al. determined that mitochondrial electron transfer chain complex III bypasses electron transfer chain complex I to increase intracellular ROS in guanine nucleotide binding protein coupled receptor 17, signaling activation in glioblastoma multiforme and providing new opportunities to develop developing targeted therapy for glioblastoma multiforme [37]. These results agree with our finding that pretreatment with antimycin A prevent the repressive effects of piperine on DLD-1 cell death and apoptosis, confirming that the induction of oxidative stress by piperine from mitochondrial complex III is the mechanism behind its action in colorectal cancer cells.

One significant endogenous ROS source in tumorigenesis is NADPH oxidase [38]. As stated earlier, the subunit Nox1 of NADPH oxidase is expressed in human colon cancer cells [39]. Various stimuli can activate NADPH oxidase in cancer cells [40]. At present, numerous studies have indicated that many anticancer compounds can affect NADPH oxidase activity, inducing intracellular ROS overproduction. For example, lutein-stimulated ROS generation is reliant on NADPH oxidase, which mediates apoptosis and NF-κB activation in gastric cancer AGS cells [41]. Imidazo [1,2-a]pyridine-based derivatives evidently stimulate cytotoxicity by notably augmenting NADPH oxidase activity, which leading the stimulation of ROS-mediated apoptosis in A549 lung cancer cells [42]. Furanodienone-induced apoptosis in colorectal cancer cells is introduced by mitochondrial ROS derived from NADPH oxidase 4 [43]. Glycyrrhetic acid can promote the productions of ROS by activating NADPH oxidases, subsequently triggering ferroptosis in triple-negative breast cancer cells [44]. The present study found that pretreatment with the NADPH oxidase inhibitor apocynin, combined with piperine treatment, effectively decreased intracellular ROS level (Figure 4B). Moreover, cell viability exhibited significant restoration under pretreatment with apocynin (Figure 5A), while cleaved PARP was demonstrated less in this group than in the piperine only group (Figure 5B). This finding proved that the repression of cell viability and the enhancement of apoptosis in DLD-1 cells by piperine partially resulted from the generation of intracellular ROS derived from NADPH oxidase. Thus, considering that piperine's anticancer efficiency may be partly attributed to its capacity to elevate intracellular ROS derived from an NADPH oxidase, initiating a sequence of mechanism cascade that leads to cellular apoptosis, is reasonable.

Xanthine oxidase is a metabolic enzyme for purine catalysis and xenobiotic substrate oxidation, producing ROS species [45]. Under hypoxia or ischemia-reperfusion circumstances, xanthine oxidase activation has been associated with ROS-dependent tissue damage [46]. A previous report indicated that alternol, a fermented extract of the mutant fungus *Alternaria alternata* var. *monosporus*, works as a unique and novel xanthine dehydrogenase/xanthine oxidase activator in malignant cells to stimulate ROS accumulation and apoptosis [11]. Incubation of cells with allopurinol (an inhibitor of xanthine oxidase) prevents berberine-stimulated oxidative stress in human prostate PC-3 cancer cells [47]. In our study, we determined that pretreatment with the xanthine oxidase inhibitor allopurinol significantly increased cell viability (Figure 5A) and inhibited apoptosis (Figure 5B) compared with

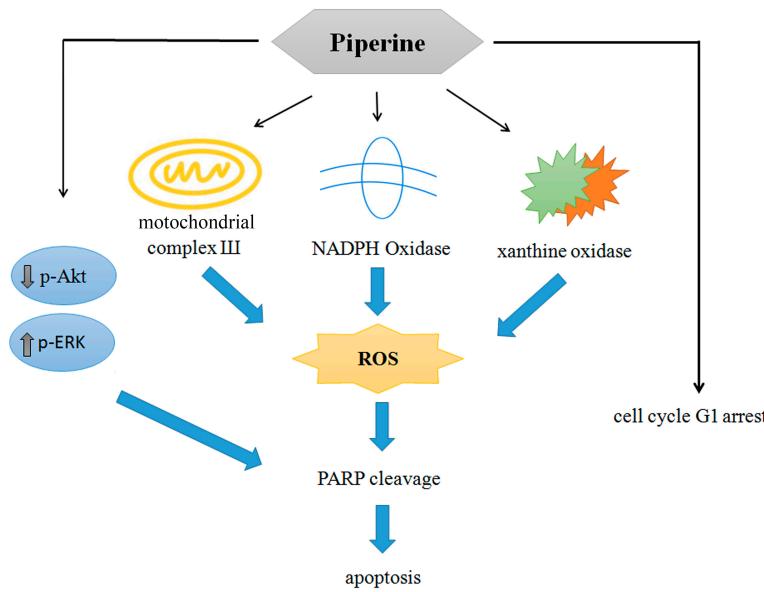
the piperine-treated group. These results indicate a potential mechanism of xanthine oxidase being involved in the ROS generation of piperine to induce cell death.

The PI3K/Akt pathway plays important role in drug resistance, metastasis, and cancer cell proliferation [48]. This pathway is typically regarded as a highly potential molecular target for cancer treatments. Previous investigations have shown that ROS suppress the PI3K/Akt/mTOR signaling pathways to induce apoptosis in colorectal cancer [14,17,18,49]. Delicaflavone triggers ROS-mediated cell cycle arrest and apoptosis via the mitochondrial pathway and endoplasmic reticulum stress accompanied by the suppression of the Ras/MEK/Erk and PI3K/Akt/mTOR signaling cascades [17]. Antrodin C extracted from *A. Cinnamomea* induces apoptosis via the ROS/Akt /ERK/p38 signaling pathway in colorectal cancer cells [14]. Fibulin-5 incites apoptosis via the Akt signal and ROS/MAPK pathways through the downregulation of the transient receptor potential cation channel subfamily V member 1 [18]. Hwang-Heuk-San-induced ROS generation is necessary for caspase-dependent apoptosis, including the suppression of the PI3K/Akt signaling pathway in HCT116 cells [49]. Our Western blot results confirmed that piperine deactivates the PI3K/Akt pathway in DLD-1 cells (Figure 6A). Piperine-induced cell death can be partly regained by the overexpression of continuously active Akt (Figure 7A). Moreover, our results revealed that the expression of cleaved PARP was reduced in Akt-overexpressed DLD-1 cells upon piperine treatment compared with the empty plasmid-transfected cells (Figure 7C). The overexpression of Akt can only partly remedy cell death and apoptosis, and thus, other pathways that are included in piperine-induced cell death probably exist.

The MAPK signaling pathway is downstream of ROS and plays a significant role in the stimulation of apoptosis [50]. Several investigations have proven that ROS increase can induce cell death via MAPK activation [51–53]. The p38 MAPK pathway is one of the stress response pathways triggered by increased ROS levels. The stimulation of the p38 pathway can induce apoptosis [54,55]. Moreover, the stress-triggered p38 pathway performs a crucial role in apoptosis control, cell cycle arrest, and cell growth inhibition [56]. The MAPK signaling pathway can be stimulated in response to oxidative stress in the endoplasmic reticulum [57], and the stimulated p38 MAPK induces either apoptosis or cell cycle arrest [59]. Moreover, increased ROS level in cells is related to the phosphorylation of p38 [60]. In general, the ERK pathway stimulated by K-ras and growth factors has been identified to prompt cell proliferation in cancer [61]. Nevertheless, several studies have demonstrated that ROS-dependent ERK activation induces apoptosis and cell cycle arrest in cancer cells [62]. Anticancer agents such as etoposide or cisplatin, need prolonged ERK activation to stimulate apoptosis in several transformed or immortalized cells [63]. In our study, the Western blot results revealed evident increases in the phosphorylation levels of ERK and p38, but not of JNK (Figure 6B). Pre-treatment with PD98059 (an inhibitor of ERK) and SB203580 (an inhibitor of p38) partially reversed cell death (Figure 7B) by piperine in DLD-1 colon cancer cells. However, only PD98059 pretreatment can decrease cleaved PARP compared with piperine-treated cells, suggesting that the activation of the ERK pathway is a signaling mechanism involved in piperine-induced cell death and apoptosis.

## 5. Conclusions

The aforementioned evidence first verifies the probability that piperine is a prospective anticancer natural compound, with the pharmaceutical capacity to inhibit colorectal cancer cell viability; induce cell cycle G1 arrest by decreasing cyclin E and increasing p27 expression; provoke apoptosis via multiple pathways, particularly by inducing multiple oxidative stress mechanisms from mitochondrial complex III, NADPH oxidase, and xanthine oxidase; and suppress the PI3K/Akt pathway and activate the ERK signaling pathway (Figure 8). However, systematic preclinical studies are still necessary to contribute scientific proof for further clinical investigations.



**Figure 8.** Proposed model of piperine induces apoptosis in colorectal DLD-1 cancer cells.

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