Int J Clin Exp Med 2015;8(7):10723-10730
www.ijcem.com /ISSN:1940-5901/IJCEM0009755

Original Article

Rapamycin, an mTOR inhibitor, induced apoptosis via independent mitochondrial and death receptor pathway in retinoblastoma Y79 cell

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Received April 30, 2015; Accepted June 23, 2015; Epub July 15, 2015; Published July 30, 2015

Abstract: Rapamycin is helpful in the treatment of certain cancers by inhibiting mTOR (mammalian target of rapamycin) pathway. Here, rapamycin mediated apoptosis were investigated in human retinoblastoma Y79 cells. The MTT assay showed that the IC_{50} value of rapamycin against Y79 cells was 0.136 ± 0.032 μmol/L. Flow cytometry analysis indicated that the percentage of apoptotic cells was increased from 2.16 ± 0.41% to 12.24 ± 3.10%, 20.16 ± 4.22%, and 31.32 ± 5.78% after 0.1, 0.2, and 0.4 μmol/L rapamycin or without rapamycin treatment for 48 hours. Flow cytometry analysis showed that rapamycin induced mitochondrial membrane potential (ΔΨm) collapse in Y79 cells in a concentration-dependent manner. Western blot assay showed that rapamycin led to release of cytochrome c from mitochondrial membranes to cytosol. Further Western blot assays showed that rapamycin induced activation of caspase-9 and caspase-8 and the cleavage of caspase-3. Rapamycin induced cleavages of caspase-3 and apoptosis was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment. Together, all these results illustrated that rapamycin induced apoptosis in human retinoblastoma Y79 cells involvement of both intrinsic and extrinsic pathways.

Keywords: Retinoblastoma, rapamycin, intrinsic apoptosis signaling pathway, extrinsic apoptosis signaling pathway

Introduction

Retinoblastoma (Rb) is the most common intraocular malignancy in children [1]. Chemotherapy has become an integral part of the current management of retinoblastoma [2]. However, present chemotherapy treatments result in noteworthy complications including second malignancies (e.g., acute myeloid leukemia) [3]. Therefore, there is an urgent need to identify new therapeutic strategies to improve the clinical outcome of patients with retinoblastoma [4].

The mammalian target of rapamycin (mTOR) has emerged as a critical effector in cell growth, proliferation, survival, angiogenesis, and autophagy [5]. Rapamycin is a macrolide produced by the bacteria *Streptomyces hygroscopicus* [6]. Which was originally developed as an antifungal agent [7]. However, this use was abandoned when it was identified as have potent immunosuppressive and antiproliferative properties [8, 9]. Now rapamycin is helpful in the treatment of certain cancers by inhibiting mTOR (mammalian target of rapamycin) pathway [10, 11]. Over the years, apoptosis has turned out to be the major mechanism to eliminate cancer cells. Which involves both intrinsic or mitochondrial pathways and extrinsic or death receptor pathways [12]. In this study, we investigated rapamycin mediated toxicity and apoptosis in human retinoblastoma Y79 cells.

Materials and methods

Chemicals and reagents

RPMI 1640 media were purchased from Gibco BRL. Fetal bovine serum (FBS) was purchased from Life Technologies Corporation. Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit was a product from Beyotime Corporation. Rapamycin, penicillin, streptomycin, MTT, DiOC6, Z-LETD-FMK, Z-IETD-FMK and other chemicals were purchased from Sigma

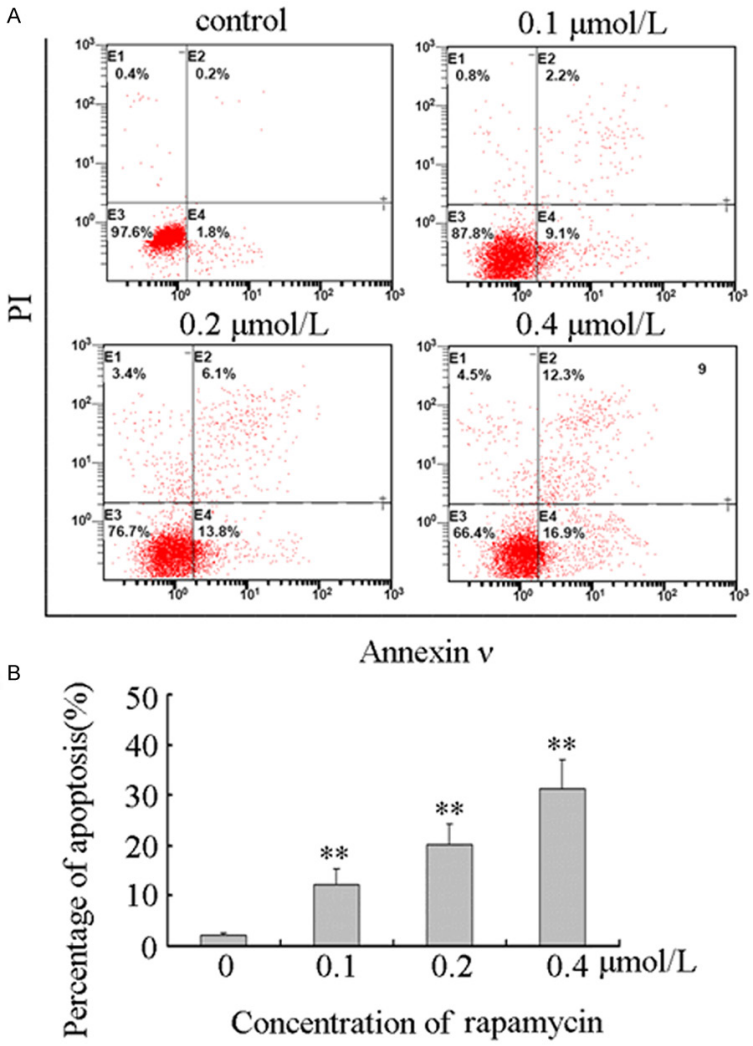

Figure 1. Rapamycin induced apoptosis in Y79 cells. A. Rapamycin induced apoptosis in Y79 cells. Y79 cells were plated in six-well culture plates and treated for 48 hours with vehicle control or 0.1, 0.2, and 0.4 μmol/L rapamycin, respectively. Cells were harvested and stained with Annexin V-FITC and PI as described in "Materials and methods". Stained apoptotic cells were classified by flow cytometry. All these experiments were replicated at least thrice, and a representative example of apoptotic histograms was shown. B. The number of apoptotic cells was quantified by CellQuest software. The percentage of apoptotic cells was increased from 2.16 ± 0.41% to 12.24 ± 3.10%, 20.16 ± 4.22%, and 31.32 ± 5.78%. Columns, means of triplicate determinations; bars, SDs; **, $P < 0.01$ as compared with respective controls.

Chemical Co. All antibodies were obtained from Cell Signaling Technology Inc.

Cell lines and cell culture

The human retinoblastoma cell line Y79 was obtained from American Type Culture Collection (ATCC). Y79 cells were incubated in RPMI 1640 media supplemented with 10% FBS, 1% penicillin, and streptomycin. Cells were cultured at 37°C in saturating humidity of 5% CO_2 and 95% air.

Cell proliferation assays

The effect of rapamycin on proliferation of Y79 cells was determined by MTT assay. Briefly, cells were seeded in 96-well plates at 3000 cells per well and grown overnight. Then, various concentrations of rapamycin were added to the wells for 72 hours. Cell number was determined later 10 μL MTT (1 mg/ml) was added to each well and incubated at 37°C for an additional four hours. After media were removed, DMSO was added to dissolve purple crystals of formazan. Optical Density (OD) value was read at 490 nm by Thermo Scientific Fluoroskan Ascent FL (Thermo Fisher Scientific Inc.). Finally, the half inhibitory concentration (IC50) was calculated as the relative viability against untreated control cells [13]. All experiments were replicated at least three times.

Cell apoptosis analyses

Y79 cells were harvested at 48 hours after incubation with rapamycin as indicated concentration. Collected cells were stained with Annexin V-FITC and PI in the dark, according to the manufacturer's protocol. Then, cells were classified by flow cytometry (Beckman Coulter, Inc). Finally, number of apoptotic cells was quantified by CellQuest software. All experiments were replicated at least three times.

Mitochondrial membrane potential (ΔΨm) assays

To observe the changes in mitochondrial membrane potential (ΔΨm), Y79 cells were stained

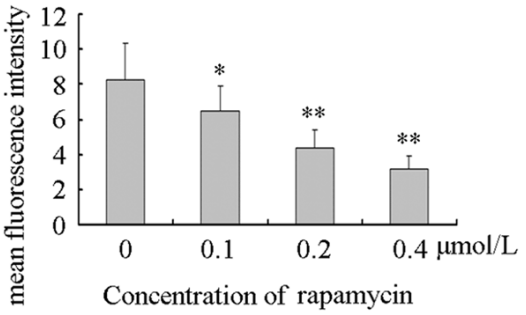

Figure 2. Rapamycin induced mitochondrial membrane potential (ΔΨm) collapse in Y79 cells. Rapamycin induced ΔΨm collapse in Y79 cells. Y79 cells were plated in six-well culture plates and treated for 48 hours with vehicle control or 0.1, 0.2, and 0.4 μmol/L rapamycin, respectively. Cells were harvested and stained with DiOC6 as described in "Materials and methods". The mean fluorescence intensity (MFI) of DiOC6 was obtained by flow cytometry analysis. The MFI was decreased from 8.24 ± 2.13 to 6.51 ± 1.42, 4.36 ± 1.05, and 3.18 ± 0.73. Data were shown as means ± SD of triplicate determinations. Columns, means of triplicate determinations; bars, SDs; *, $P < 0.05$, **, $P < 0.01$ as compared with respective controls.

by mitochondrial tracking fluorescent dye DiOC6 and measured by flow cytometry. Briefly, Y79 cells were treated with rapamycin as indicated concentration for 48 hours. Then, cells were harvested and incubated with 40 nmol/L DiOC6 at 37°C in the dark for 20 min. Finally, mean fluorescence intensity (MFI) was obtained by flow cytometry analysis (Beckman Coulter, Inc). All experiments were replicated at least three times.

Western blot analyses

Y79 cells were harvested at 48 hours after incubation with rapamycin as indicated concentration. Cells were lysed and equal amounts of protein lysate was separated on 8-12% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore. USA). Membranes were blocked with 5% nonfat milk powder (w/v) for 2 h, and then incubated with primary antibodies at 4°C overnight. Thereafter, HRP-conjugated secondary antibody was incubated for one hour. Finally, the immunoblotted proteins were visualized by Western Blot Detection System (Millipore, USA). GAPDH was used as an endogenous control. All experiments were replicated at least three times.

Western blot analysis of cytosolic cytochrome c

Y79 cells were harvested at 48 hours after incubation with rapamycin as indicated concentration. The pellets were suspended with 5-fold volume cytosol extraction buffers containing DTT and protease inhibitors (Abcam plc. USA). After incubated on ice for 10 minutes, the pellets were homogenized in an ice-cold Dounce tissue grinder. Then, the cells were centrifuged at 700 g for 10 minutes at 4°C. The supernatant was collected into a fresh 1.5 ml tube, and centrifuged at 10,000 g for 30 minutes at 4°C. The final supernatant was used as cytosolic fraction. Finally, cytosolic cytochrome c was identified by Western blot analysis as described before.

Statistical analysis

Data were statistically analyzed by SPSS Statistics 16.0 software. Independent t-test was used between two groups, and the comparisons of multiple groups were performed with a one-way analysis of variance (ANOVA) followed by LSD-t (for equal variances assumed) or Dunnett's (for equal variances not assumed) test. The significance determined at $P < 0.05$.

Results

Effects of rapamycin on Y79 cells viability

The sensitivity of human retinoblastoma Y79 cells to rapamycin was determined by performing cell proliferation assay. The MTT assay showed that rapamycin inhibited Y79 cell proliferation in a concentration-dependent manner. The IC_{50} value was 0.136 ± 0.032 μmol/L. The results indicated that rapamycin was effective in inhibiting the growth of human retinoblastoma Y79 cells.

Rapamycin induced apoptosis in Y79 cells

To determine the apoptotic effect of rapamycin on human retinoblastoma Y79 cells, we treated cells with 0.1, 0.2, and 0.4 μmol/L rapamycin or without rapamycin for 48 hours. Apoptosis was assessed by Annexin V/PI double staining, and apoptotic cell populations were quantified by flow cytometry as showed in **Figure 1A**. The results showed that the percentage of apoptotic cells was increased from 2.16 ± 0.41% to 12.24 ± 3.10%, 20.16 ± 4.22%, and 31.32 ±

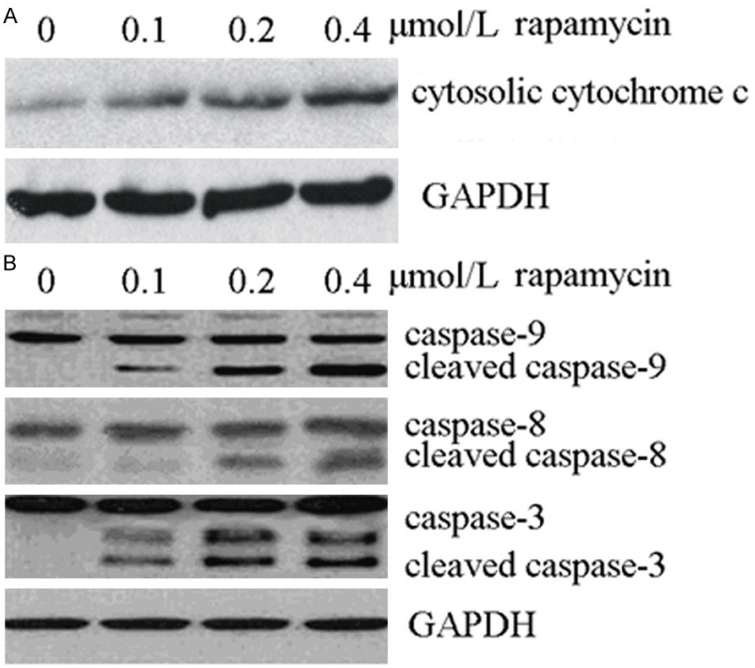

Figure 3. Rapamycin induced apoptosis through intrinsic and extrinsic signaling pathways in Y79 cells. A. Rapamycin induced release of cytochrome c from mitochondrial membrane to cytosol. Y79 cells were plated in six-well culture plates and treated for 48 hours with vehicle control or 0.1, 0.2, and 0.4 μmol/L rapamycin, respectively. Cells were harvested and cytosolic protein was extracted as described in "Materials and methods". Cytosolic cytochrome c was identified by Western blot analysis. GAPDH protein levels were used as a cytosolic control. All these experiments were replicated at least thrice, and a representative experiment was shown in each panel. B. Rapamycin induced activation of caspase-9 and caspase-8 and the cleavage of caspase-3. Y79 cells were plated in six-well culture plates and treated for 48 hours with vehicle control or 0.1, 0.2, and 0.4 μmol/L rapamycin, respectively. Cells were harvested and total protein was extracted as described in "Materials and methods". The whole-cell lysate was assayed by Western blot and corresponding antibodies. GAPDH protein levels were used as a loading control. All these experiments were replicated at least thrice, and a representative experiment was presented in each panel.

5.78% (**Figure 1B**). The results showed that rapamycin induced apoptosis in human retinoblastoma Y79 cells in a dose-dependent manner.

Effects of rapamycin on mitochondrial membrane potential (ΔΨm) in human retinoblastoma Y79 cells

DiOC6 is a lipophilic cationic dye that specifically accumulates into mitochondrial matrix depending on ΔΨm [14]. In apoptotic cells, the Δψm collapses. So the mean fluorescence intensity (MFI) of DiOC6 was reduced by flow cytometry detection [15]. To observe the effects of rapamycin on ΔΨm, Y79 cells were treated with 0.1, 0.2, and 0.4 μmol/L rapamycin or without rapamycin for 48 hours. As showed in **Figure 2**, the MFI of DiOC6 was decreased from 8.24 ± 2.13 to 6.51 ± 1.42, 4.36 ± 1.05, and 3.18 ± 0.73. The results indicated that rapamycin can lead to dissipation of ΔΨm in human retinoblastoma Y79 cells.

Rapamycin induced apoptosis through intrinsic pathways in human retinoblastoma Y79 cells

ΔΨm is critical for proper cellular functions. Disruption of ΔΨm might alter the membrane dynamics of mitochondria leading to release of cytochrome c, formation of apoptosome complex, and activation of caspase-9. Which initiated a cascade of caspase activation leading to apoptosis [16]. To observe the release of cytochrome c and activation of caspase-9, Y79 cells were treated with 0.1, 0.2, and 0.4 μmol/L rapamycin or without rapamycin for 48 hours. Cytosolic cytochrome c was identified by Western blot analysis. As showed in **Figure 3A**, cytosolic cytochrome c was increased after rapamycin treatment. Released cytochrome C triggered caspase-9 and caspase-3 activation (**Figure 3B**).

Rapamycin induced apoptosis via the extrinsic signaling pathways in human retinoblastoma Y79 cells

In order to investigate whether rapamycin would induce apoptosis through extrinsic pathways, we revealed the activation of caspase-8. As showed in **Figure 3B**, cleaved caspase-8 was detected after rapamycin treatment. These results suggested that extrinsic pathways involved in rapamycin mediated apoptosis in human retinoblastoma Y79 cells.

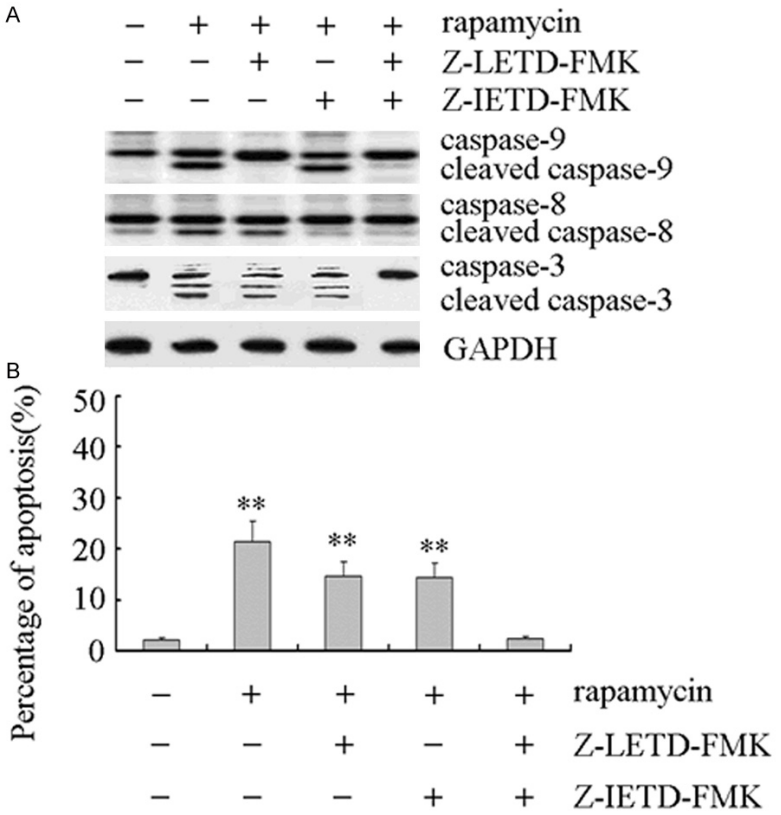

Figure 4. Rapamycin induced apoptosis involvement of both intrinsic and extrinsic signaling in Y79 cells. A. The cleavage of caspase-3 was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment. Y79 cells were plated in six-well culture plates. After preincubation with 20 μmol/L Z-IETDFMK or Z-LETD-FMK for 12 h, Y79 cells were treated with 0.2 μmol/L rapamycin for 48 h, respectively. Cells were harvested and total protein was extracted as described in "Materials and methods". The whole-cell lysates were assayed by Western blot and corresponding antibodies. GAPDH protein levels were used as a loading control. All these experiments were replicated at least thrice, and a representative experiment was presented in each panel. B. Rapamycin induced apoptosis was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment. Y79 cells were plated in six-well culture plates. After pre-incubation with 20 μmol/L Z-IETDFMK or Z-LETD-FMK for 12 h, Y79 cells were treated with 0.2 μmol/L rapamycin for 48 h, respectively. Cells were harvested and stained with Annexin V-FITC and PI as described in "Materials and methods". Stained apoptotic cells were classified by flow cytometry. The number of apoptotic cells was quantified by CellQuest software. Columns, means of triplicate determinations; bars, SDs; **, $P < 0.01$ as compared with respective controls.

Rapamycin induced apoptosis through both intrinsic and extrinsic signaling pathways in human retinoblastoma Y79 cells

The cross-talk was found between intrinsic and extrinsic apoptosis signaling pathways [17]. In order to investigate which apoptotic signaling pathways rapamycin involved, Z-LETD-FMK (a selective caspase-9 inhibitor) and Z-IETD-FMK (a selective caspase-8 inhibitor) were used to block the intrinsic or extrinsic apoptosis signaling pathway. As showed in **Figure 4A**, Z-LETD-FMK only inhibited rapamycin-mediated activation of caspase-9, and Z-IETD-FMK only inhibited rapamycin-mediated activation of caspase-8. When both Z-LETD-FMK and Z-IETD-FMK were used, rapamycin-mediated activation of caspase-9 and caspase-8 was inhibited, and followed inhibition of caspase-3 cleavage. The apoptotic analysis showed that Z-LETD-FMK or Z-IETD-FMK partially inhibited rapamycin-mediated apoptosis (**Figure 4B**). Rapamycin-mediated apoptosis was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment (**Figure 4B**).

Discussion

Retinoblastoma is the most common primary intracellular malignancy in childhood with an incidence of 1/15,000 to 1/20,000 births [18]. Untreated retinoblastoma is always fatal and the patients die of intracranial extension and disseminated disease within two years [19]. Primary management of retinoblastoma consists of chemoreduction with local consolidation, although newer techniques include local delivery via intra-arterial chemotherapy, periocular, or intravitreal injection [20, 21]. In developing countries, treatment is limited. Long-term survival rates are weak and current chemotherapy causes significant morbidity to pediatric patients and significantly limits dosing [3]. Now, the novel targeted therapeutic strategies are intended to effectively control cancer [22].

Serine-threonine kinase, mTOR, plays a key role in cell growth and angiogenesis and may be dysregulated in tumors [23, 24]. For these reasons, mTOR has recently promoted to the rank

as a potential target for anti-cancer therapy [25, 26]. Rapamycin was recognized as an inhibitor of mTOR pathway [27]. In this study, we found that rapamycin induced apoptosis in human retinoblastoma Y79 cell (**Figure 1**). Moreover, our further investigation elucidated that both intrinsic and extrinsic apoptosis signaling pathway involved in its action mechanisms.

Apoptosis can be triggered by internal and external signals. The loss of mitochondrial membrane potential (ΔΨm) has been suggested to causing the cytochrome C release [28]. The released cytochrome c was essential to activate caspase-9. Which results in internal damage to cells [29]. Death activator transmitted a signal to the cytoplasm that leads to activation of caspase-8. Which initiated extrinsic pathway leading to phagocytosis of the cells [30]. In this study, we observed cleaved caspase-8 and caspase-9, followed a cascade of caspase activation (**Figure 3**). It was increasingly believed that crosstalk existed between intrinsic and extrinsic apoptosis signaling pathway [31]. For example, the cleavage of Bid caused by caspase-8 can lead to the release of cytochrome c from mitochondria, which subsequently activated caspase-9 mediated intrinsic apoptosis pathway [32]. Next, we investigated the involved pathways. The result showed that Z-LETD-FMK or Z-IETD-FMK only inhibited rapamycin-mediated caspase-9 or caspase-8 activation and partial apoptosis (**Figure 4**). However, combined Z-LETD-FMK and Z-IETD-FMK can inhibit rapamycin-mediated cascade of caspase activation and apoptosis (**Figure 4**). The results suggested that rapamycin can trigger both intrinsic and extrinsic apoptosis pathways in human retinoblastoma Y79 cell.

mTOR assumes a key regulatory role in cell growth and homeostasis. Inhibition of mTOR now uses as a novel treatment strategy for several malignancies, either alone or in combination with strategies [33]. In this study, we concisely evaluate the best ability of rapamycin (an mTOR inhibitor) to induce apoptosis in human retinoblastoma Y79 cells. Our studies showed that rapamycin disturbed mitochondrial membrane potential and subsequently helped releasing cytochrome c from mitochondria to cytosol and activated caspase-8. In addition, we observed that selective caspase-9 or caspase-8 inhibitor cannot inhibit rapamycin-mediated activation of caspases-3 and block apoptosis. Combining all, results suggested that rapamycin induced apoptosis in human retinoblastoma Y79 cells involvement of both intrinsic and extrinsic pathways.

Acknowledgements

This work was supported by grants from Guangdong Province special science and technology (new drug discovery) project (2012-A080201002), Guangdong Province Foshan City industry-university-research special project of Gaoming District in 2012 (201211).

Disclosure of conflict of interest

None.

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