1 Introduction

Cancer is the second leading cause of death worldwide [1] and the discovery and development of new anticancer drugs and effective therapeutic strategies are critically important [2]. Anticancer drugs employing various mechanisms inhibit the division of cancer cells effectively [3-4], but they show severe cytotoxic and side effects, particularly to normally proliferating tissues [3]. It has been reported that coumarin derivatives act on various tumor cells and show anti-proliferative activity through down regulating oncogene expression or caspase-9-mediated apoptosis [3]. Moreover, they can suppress cancer cell proliferation by arresting cell cycle in G0/G1 phase, G2/M phase and affecting the p-glycoprotein (p-gp) of the cancer cell, including A549 cells, ACHN cells, H727 cells, MCF7 cells and HL-60 cells [3].

Mitochondria are the energy generators that maintain cellular metabolism and essential cell functions, including multiple signaling cascades that regulate cell cycle control, development, and cell death [5]. Lipophilic cations can preferentially accumulate in tumor cell mitochondria driven by their higher transmembrane potential compared with normal cells [7]. Owing to this, mitochondria-targeted cationic agents show selective cytotoxic effects to tumor cells compared to normal cells [6]. In this study, we synthesized a mitochondria-targeted 6-(nicotinamide) methyl coumarin by coupling 6-methyl coumarin to nicotinamide. Our results demonstrate that 6-(nicotinamide) methyl coumarin preferentially kills A549 cells through inducing A549 cells apoptosis, mediated by increasing ROS level and causing mitochondrial depolarization. Strikingly, the viability of the A31 cells treated with 6-(nicotinamide) methyl coumarin did not decrease, indicating that 6-(nicotinamide) methyl coumarin preferentially accumulates in A549 cells and A549 cells are much more susceptible to 6-(nicotinamide) methyl coumarin treatment compared with A31 cells.

Keywords: 6-(nicotinamide) methyl coumarin, mitochondrial-targeted, A549 cells
that 6-(nicotinamide) methyl coumarin preferentially induces A549 cells apoptosis, compared with A31 cells, mediated by promoting ROS generation and initiating mitochondrial depolarization.

2 Materials and methods

6-methyl-coumarin, benzoyl peroxide, nicotinamide and Hoechst 33258 were purchased from the Aladdin (Shanghai). N-bromosuccinimide was obtained from the Macklin (Shanghai). Cyclohexane and acetone were purchased from Jiangtian Chemical Industry (Tianjin). 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) was obtained from Solarbio Biology. PBS and penicillin-streptomycin mixture were purchased from Solarbio Biology. 2, 7-Dichlorodihydrofluorescein-diacetate (DCFH-DA) was obtained from Solarbio Biology. Rhodamine 123 was purchased from Aladdin (Shanghai). A549 cells were stored in 80°C refrigerator. Fetal Bovine Serum (FBS) and trypsin were purchased from Gibco. DMEM Basal Medium was obtained from Hyclone.

2.1 The synthesis of 6-(nicotinamide) methyl coumarin

Studies have revealed that coumarin derivatives inhibit tumor cell proliferation [3-4], indicating that coumarin ring is required for maintaining biological activity. Furthermore, nicotinamide also exhibited antitumor potency [8]. 6-methyl coumarin is poorly soluble in water, but attaching the nicotinamide to 6-methyl coumarin causes a significant increase in hydrophilicity. Based on these results, we tried the synthesis of 6-(nicotinamide) methyl coumarin.

6-bromomethyl-coumarin was first synthesized through a reaction between 6-methyl-coumarin and NBS, followed by coupling 6-bromomethyl-coumarin to nicotinamide in acetone. Thus 6-(nicotinamide) methyl coumarin was first synthesized by the attachment of nicotinamide to 6-methyl-coumarin through alkyl linker, 6-bromomethyl-coumarin (Fig 1.).

3.2 g 6-methyl-coumarin, 80 ml cyclohexane and 100 mg benzoyl peroxide were added to 250 ml flask while heating under stirring. After the reaction mixture became clear, 3.6 g N-bromosuccinimide (NBS) was added in five batches and refluxed for 2 h under illumination, then cooled for 2 min and filtered. The solution was concentrated to dryness. 2.4 g 6-bromomethyl-coumarin was obtained. 2.4 g 6-bromomethyl-coumarin and 1.82 g nicotinamide were added to 20 ml acetone in 100 ml flask and refluxed for 1 h, then cooled and filtered. The white solid product was washed with petroleum ether and dried.

2.2 The MTT assay

A549 cells (cancer cells) and A31 cells (normal cells) were seeded at 5 × 10^3 cells and 3 × 10^3 cells per well, respectively, in 96-well plates and incubated overnight. The following day, media was replaced with various concentrations of 6-(nicotinamide) methyl coumarin and the cells were incubated for 24, 48, and 72 h, respectively. Blank medium was used as control. Each experiment was repeated three times. Cell viability was measured by MTT assay.

Figure 1. Synthesis and structure of 6-(nicotinamide) methyl coumarin by the bromination of 6-methyl coumarin and attachment to nicotinamide.
MTT was first prepared as a 5 mg/ml stock solution in PBS and filtered. At the end of the treatment, 10 μl MTT solutions were added to each well and the plates were incubated for 4 h at 37°C, the medium and solvent were removed from each well and replaced with 150 μl DMSO. Then the absorbance was measured at 490 nm using a microplate reader.

2.3 Colonies Formation Assay

A549 cells were seeded at 3 × 10^5 cells per well in 96-well plates and allowed overnight to adhere. The next day, media was replaced with various concentrations of 6-(nicotinamide) methyl coumarin and incubated for 48 h. Blank medium was used as control. A549 cells were further incubated for 10 days to allow colonies formation. Colonies were stained with Giemsa, fixed by methanol, rinsed and then photographed. The assay was performed in triplicate.

2.4 Measurement of Reactive Oxygen Species

Reactive oxygen species (ROS) was measured using DCFH-DA (2, 7-Dichlorodihydrofluorescein diacetate). DCFH-DA is hydrolyzed by intracellular esterase to produce a non-fluorescent DCFH product. It can then be oxidized by ROS and other oxidizing species to produce a highly fluorescent DCF product. A549 cells were seeded at 2 × 10^4 cells per well in black-bottomed 96-well plates and incubated overnight. The following day, A549 cells were washed with DMEM medium without FBS (Fetal Bovine Serum) two times, and then A549 cells were stained with 10 μM DCFH-DA (final concentration) and allowed to incubate for 30 minutes in a humidified atmosphere containing 5% CO2 at 37°C. At the end of incubation, A549 cells were treated for 1 h with the desired concentration of 6-(nicotinamide) methyl coumarin in PBS. Following drug treatment, A549 cells were collected and washed with PBS. ROS was detected using a microplate reader at 488/525 ex/em wavelengths. The assay was performed in triplicate.

2.5 Measurement of Mitochondrial Membrane Depolarization by Rhodamine 123 staining

Mitochondrial membrane potential (ΔΨm) was detected qualitatively using lipophilic fluorescent probe Rhodamine 123 (2-(6-Amino-3-imino-3H-xanthen-9-yl) benzoic acid methyl ester). A total of 2000 A549 cells were seeded in 24-well plates. After 24 h incubation, A549 cells were treated with 6-(nicotinamide) methyl coumarin in PBS. Six hours after treatment, cells were washed with PBS, and then A549 cells were incubated with 20 μg/ml Rhodamine 123 (final concentration) in fresh medium without FBS (Fetal bovine serum) and allowed to incubate for 15 minutes in a humidified atmosphere containing 5% CO2 at 37°C. Following the incubation, A549 cells were collected in fresh medium without FBS. The Rhodamine 123 fluorescence was measured using a microplate reader in black-bottomed 96-well plates. The excitation wavelength was 505 nm and emission wavelength was 529 nm. The assay was performed in triplicate.

2.6 Apoptosis Assay

A549 cells were seeded at 2 × 10^6 cells per well in 6-well plates and allowed overnight to adhere. The next day, media was replaced with various concentrations of 6-(nicotinamide) methyl coumarin and incubated for 6 h. Blank medium was used as control. Six hours after treatment, cells were washed with PBS, and then A549 cells were stained with 10 μg/ml Hoechst 33258 in PBS and allowed to incubate for 30 minutes in a humidified atmosphere containing 5% CO2 at 37°C. Following the incubation, A549 cells washed with PBS. Images were obtained using fluorescence microscopy.

2.7 Statistical Analysis

Unless otherwise stated, the results were presented as mean ± SD. Statistical analysis was performed by two-tailed t test using Prism software (version 5, GraphPad). P < 0.05 was accepted as being statistically significantly different.

3 Results

3.1 The synthesis of 6-(nicotinamide) methyl coumarin

The 6-(nicotinamide) methyl coumarin was confirmed by LC-MS (liquid phase-mass spectrometry) and further characterized by 1H NMR (hydrogen-1 nuclear magnetic resonance spectroscopy) and 13C NMR (carbon-13 nuclear magnetic resonance spectroscopy).
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This compound had significant inhibitory effect on A549 cells. It is noteworthy that there were apparent dose and time responses when A549 cells were treated with 6-(nicotinamide) methyl coumarin. Toxicity from chemotherapy is frequently credited to its lack of tumor specificity, making tumor-targeted delivery of anticancer drugs, especially mitochondria-targeted anticancer drugs, one of the most important strategies for the development of chemotherapeutic agents. Following the assumption that 6-(nicotinamide) methyl coumarin would selectively accumulate in cancer cells driven by the higher transmembrane potential, we aimed to test its tumor selectivity by using normal cells, A31 cells. As was shown in Figure 6, the cells viability treated with 6-(nicotinamide) methyl coumarin did not decrease compared to control group. Even the cells viability treated with 100 μM and 200 μM 6-(nicotinamide) methyl coumarin increased significantly, which indicated a good selectivity and low toxicity of the compound. These results suggest that

3.2 6-(nicotinamide) methyl coumarin preferentially kills A549 cells

To test the efficacy of 6-(nicotinamide) methyl coumarin in A549 cells, the inhibition rate of this compound was determined through the MTT assay. As depicted in Figure 5, this compound had significant inhibitory effect on A549 cells. It is noteworthy that there were apparent dose and time responses when A549 cells were treated with 6-(nicotinamide) methyl coumarin. Toxicity from chemotherapy is frequently credited to its lack of tumor specificity, making tumor-targeted delivery of anticancer drugs, especially mitochondria-targeted anticancer drugs, one of the most important strategies for the development of chemotherapeutic agents. Following the assumption that 6-(nicotinamide) methyl coumarin would selectively accumulate in cancer cells driven by the higher transmembrane potential, we aimed to test its tumor selectivity by using normal cells, A31 cells. As was shown in Figure 6, the cells viability treated with 6-(nicotinamide) methyl coumarin did not decrease compared to control group. Even the cells viability treated with 100 μM and 200 μM 6-(nicotinamide) methyl coumarin increased significantly, which indicated a good selectivity and low toxicity of the compound. These results suggest that

Figure 2. Liquid phase-mass spectrometry results for 6-(nicotinamide) methyl coumarin. The theoretical molecular weight is 281.29; the calculated molecular weight is 281.1.

Figure 3. The 1H NMR (hydrogen-1 nuclear magnetic resonance spectroscopy) spectra of 6-(nicotinamide) methyl coumarin (500 Hz, DMSO-D6) 8.6(1H,s,-NH); 8.2(1H,s,-NH); 6.00(2H,d,-CH2); 6.54(1H,d,-CH=CH-); 7.5(1H,d,-CH=CH-); 9.64(1H,s,-Py); 9.30(1H,d,-Py); 8.98(1H,l,-Py); 8.29(1H,dd,-Py); 8.04(1H,d,-Ph); 7.93(1H,d,-Ph); 7.83(1H,dd,-Ph).
6-(nicotinamide) methyl coumarin should be chosen as a lead compound for further studies.

3.3 6-(nicotinamide) methyl coumarin significantly inhibits colony formation of A549 cells

We used a clonogenic assay to monitor the antiproliferative effects of 6-(nicotinamide) methyl coumarin. As was shown in Figure 7A, there was a significant decrease in colony formation in A549 cells, as was compared to control group, when treated with 6-(nicotinamide) methyl coumarin (50 μM, 500 μM) for 48 h. Figure 7B showed the calculated survival fractions of A549 cells. 6-(nicotinamide) methyl coumarin significantly decreased the survival fraction in A549 cells compared with control group. Notably, the colony formation data indicated that 48 h treatments with 50 μM and 500 μM 6-(nicotinamide) methyl coumarin were sufficient to induce significant
The antitumor effects of mitochondria-targeted 6-(nicotinamide) methyl coumarin (6-NMC) have been extensively studied due to its potential in cancer therapy. Recent studies have demonstrated that 6-NMC induces reactive oxygen species (ROS) generation, which plays a crucial role in mediating antitumor effects. The toxicity of 6-NMC is selectively exerted on cancer cells, making it a promising candidate for targeted cancer therapy.

**3.4 6-(nicotinamide) methyl coumarin induces ROS generation significantly**

To investigate the role of ROS in mediating antitumor effects of 6-(nicotinamide) methyl coumarin, we measured the ROS level in A549 cells treated with various doses of 6-(nicotinamide) methyl coumarin. As shown in Figure 8, 6-(nicotinamide) methyl coumarin increased significantly ROS level in A549 cells, even the ROS level had been increased 50% by 500 μM 6-(nicotinamide) methyl coumarin. Furthermore, the ROS level of compound-treated cells increased in a dose-dependent manner. These results indicate that 6-(nicotinamide) methyl coumarin induces ROS generation and ROS production mediates its selectivity toxicity and anti-proliferative effects to A549 cells. Moreover, excessive ROS will damage cellular lipids, proteins and DNA. Particularly, oxidation of the permeability transition pores by ROS will disrupt the mitochondrial membrane potential, which makes tumor cells vulnerable to antitumor drugs [11]. On basis of these theories and the result that 6-(nicotinamide) methyl coumarin induces ROS generation, we will identify whether ROS could cause the disruption of the mitochondrial membrane potential.

**3.5 6-(nicotinamide) methyl coumarin causes mitochondrial depolarization**

In our study, [6-(nicotinamide) methyl coumarin]-treated cells exhibited a dramatic dose-dependent increase in green fluorescence, particularly, 500 μM [6-(nicotinamide) methyl coumarin]-treated cells exhibited more than four times than control group, Rhodamine 123 exhibits potential-dependent accumulation in mitochondria of living cells, leading to the quench of green fluorescence.
mitochondria are depolarized, there is a formation in green fluorescence. Therefore, our results strongly suggest that 6-(nicotinamide) methyl coumarin causes the reduction in their mitochondrial membrane potential in A549 cells (Figure 9), indicating mitochondrial depolarization. In addition, the decrease in mitochondrial membrane potential indicates that early apoptotic processes occur. Therefore, the results imply that 6-(nicotinamide) methyl coumarin induces A549 cells apoptosis. Furthermore, the reduction in the mitochondrial membrane potential is related to mitochondria ROS-induced apoptosis through contributing to cytochrome c release. We have identified that 6-(nicotinamide) methyl coumarin promotes ROS generation in A549 cells, thus we conclude that 6-(nicotinamide) methyl coumarin disrupts the mitochondrial membrane potential, which indicates early apoptosis occur through elevating ROS levels in A549 cells.

3.6 Apoptosis Assay

To confirm whether 6-(nicotinamide) methyl coumarin could induce A549 cells apoptosis furtherly, we performed apoptosis assay using Hoechst 33258. As was shown in Figure 10, 6-(nicotinamide) methyl coumarin increased significantly the fluorescence intensity in A549 cells. Furthermore, the fluorescence intensity of compound-treated cells increased in a dose-dependent manner. The apoptotic cells can be stained by Hoechst 33258 to emit strong blue fluorescence owing to their membrane permeability increase. However, the normal cells emit weak blue fluorescence. Based on these theories, these results suggest that 6-(nicotinamide) methyl coumarin induces A549 cells apoptosis in a dose-dependent manner.

4 Discussion

The drug-resistance and rapidly progressing nature of lung cancer determine the needs for new therapies. Combining the standard therapy with bevacizumab cannot improve the progression-free survival (PFS), overall survival (OS), 1-year survival coefficients for lung cancer patients [12-13]. However, drug-related adverse events increase dramatically, thus reducing overall quality of life, a critically important parameter for patients undergoing palliative treatment of advanced cancer.

Recent studies have demonstrated that coumarin derivatives act on various tumors cells through inhibiting the telomerase enzyme and protein kinase activity and down regulating oncogene expression or inducing the caspase-9-mediated apoptosis [3]. Furthermore, they can suppress cancer cell proliferation by arresting cell cycle in G0/G1 phase, G2/M phase and affecting the p-gp of the cancer cell [3]. Despite the potential antitumor activity exhibited in vitro, treating patients with coumarin derivatives has presented major challenges: poor water solubility and lack of tumor-targeted delivery. So far,
Figure 10. 6-(nicotinamide) methyl coumarin (0, 50, 100, 200, 400 μM) induce A549 cells apoptosis. Images were obtained using fluorescence microscopy, after incubation with Hoechst 33258.
antitumor activity of 6-methyl coumarin has not been investigated yet.

6-(nicotinamide) methyl coumarin with cationic pyridinium moiety targets more negatively charged organelles, most predominantly mitochondria in tumor cells due to their higher membrane potential than in normal cells, leading to 10-fold greater accumulation of lipophilic cations in mitochondria [14-17]. Moreover, positive charge of 6-(nicotinamide) methyl coumarin ensures greater water solubility. In one word, synthetized 6-(nicotinamide) methyl coumarin has better cell-membrane permeability and tumor-targeted delivery compared with 6-methyl coumarin.

Based on these theory and design, we compared toxicity to A549 cells with toxicity to A31 cells through the MTT assay. Results showed that 6-(nicotinamide) methyl coumarin has significant inhibitory effect on A549 cells, suggesting that 6-(nicotinamide) methyl coumarin preferentially accumulates in A549 cells and A549 cells are much more susceptible to 6-(nicotinamide) methyl coumarin treatment compared with A31 cells. These are consistent with theory and our design. Moreover, 48 h treatments with 50 μM and 500 μM 6-(nicotinamide) methyl coumarin were effective to inhibit A549 cells growth, suggesting that 6-(nicotinamide) methyl coumarin has great antitumor potential.

ROS is mainly produced in mitochondria and involved in signal pathway associated with apoptosis [18-19], indicating that ROS acts as upstream signaling molecules involved in activation of mitochondrial pathways. It has been reported that ROS generation can trigger cells apoptosis through activating both mitochondrial and ER stress pathways [9-10]. This is consistent with the fact that ROS generation can activate of JNK-mitochondrial and ER stress apoptotic pathways [20]. Particularly, oxidation of the mitochondria permeability transition pores by ROS can lead to cytochrome c release, which can trigger apoptosis. In addition, ROS has destructive effect both on DNA and proteins [11]. Therefore, we were interested to investigate the role of ROS in mediating anti-tumor effects of 6-(nicotinamide) methyl coumarin. Consistent with previous study [21], 6-(nicotinamide) methyl coumarin induced ROS generation, implying that its selectivity toxicity and anti-proliferative effect to A549 cells is related to ROS generation.

Mitochondrial depolarization is a hallmark of apoptosis, reflecting that increasing membrane permeability leads to cytochrome c release and activation of the downstream caspase cascade. We used Rhodamine 123, a cationic dye that forms green fluorescence when mitochondria are depolarized, to analyze the impact of 6-(nicotinamide) methyl coumarin on mitochondrial membrane depolarization. 6-(nicotinamide) methyl coumarin promoted an increase in green florescence, suggesting that 6-(nicotinamide) methyl coumarin causes mitochondrial depolarization in A549 cells.

6-(nicotinamide) methyl coumarin caused mitochondrial depolarization in A549 cells, which indicates that early apoptosis occurs. To confirm whether 6-(nicotinamide) methyl coumarin could induce A549 cells apoptosis furtherly, we performed apoptosis assay using Hoechst 33258. Consistent with mitochondrial depolarization assay, 6-(nicotinamide) methyl coumarin induced A549 cells apoptosis in a dose-dependent manner.

Here, we have confirmed that 6-(nicotinamide) methyl coumarin preferentially kills A549 cells through inducing A549 cells apoptosis, mediated by promoting ROS generation and initiating mitochondrial depolarization. Our studies provided strong evidence that 6-(nicotinamide) methyl coumarin is a potential agent which effectively kills A549 cells with no toxicity to A31 cells.

5 Conclusion

We succeed in obtaining 6-(nicotinamide) methyl coumarin through a reaction between 6-methyl-coumarin and NBS, followed by coupling 6-bromomethyl-coumarin to nicotinamide and 6-(nicotinamide) methyl coumarin preferentially killed A549 cells through inducing A549 cells apoptosis, mediated by increasing ROS level and causing mitochondrial depolarization.

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References


