VNU Journal of Science: Natural Sciences and Technology, Vol. 38, No. 2 (2022) 10-16

VNU Journal of Science: Natural Sciences and Technology

Journal homepage: https://js.vnu.edu.vn/NST

Original Article

Naringin Inhibits Multiple Myeloma Cells Proliferation by Altering Mitochondrial Function

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Received 28 March 2022
Revised 19 May 2022; Accepted 20 May 2022

Abstract: This study was conducted to evaluate the effect of Naringin (NAR) on multiple myeloma cells through assessment of cell proliferation and mitochondrial function. Methods: Human myeloma cell line (KMS-20) was subjected to normal (control) and treatment conditions. The viability, toxicity, cardiolipin content and mitochondrial membrane potential of KMS-20 cells in all groups were analyzed by using suitable kits. Results: The obtained data showed that NAR is toxic and inhibits the growth of KMS-20 cells. Similar to Doxorubicin (DOX)-treated cell group, the KMS-20 cell group supplemented with NAR had a sharp decrease in cardiolipin content compared to those in control group. The cardiolipin contents of KMS-20 cells in the DOX- and NAR-treated groups were 38.78±3.74 and 47.23±4.65 (% of control, $p<0.05$), respectively. In contrast to DOX, NAR strongly elevated the mitochondrial membrane potential of the KMS-20 cells. Conclusion: This study shows that NAR has the ability to inhibit the growth of KMS-20 multiple myeloma celline by altering their mitochondrial structure and function.

Keywords: Naringin, KMS-20, mitochondria.

1. Introduction

Multiple myeloma is a blood cancer that affects plasma cells and accounts for about 10% of all blood cancers [1]. Although the understanding of the disease and the discovery of novel therapies have resulted in a significant increase in overall survival time, almost all patients relapse and eventually succumb to their condition [2]. In facts, many studies have shown that mitochondria play an important role in the development and the progression of multiple myeloma [1, 3]. Therefore, the search for mitochondrial-targeting drugs or/and natural compounds to inhibit the growth of cancer cells has paid attention to scientists.

Naringin (NAR) is a flavanone glycoside found in the peels of citrus fruits and has showed the biological and pharmacological activities [4]. NAR exerts a variety of pharmacological effects such as anti-inflammatory and antioxidant properties [5]. Moreover, NAR positively effects on hypertension [6], hyperlipidemia [7], hyperglycemia [8], obesity [9] and hypoxia-

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https://doi.org/10.25073/2588-1140/vnunst.5474

reoxygenation injury [10, 11]. Many reports have demonstrated that NAR exhibits anticancer activity against cervical cancer [12], stomach cancer [13], breast cancer [14], ect. Although it has been reported that cell cycle modulation, antiangiogenic effect, or apoptosis induction might be involved in the antitumor activity of NAR, the role of NAR on multiple myeloma has not been fully elucidated yet. Therefore, this study investigated the effect of NAR on KMS-20 cells by analyzing the cell viability, the cell toxicity as well as the mitochondrial function.

2. Materials and Methods

2.1. Materials

The main materials and equipment used in this study were: KMS-20 cell line (ATCC® - USA); Naringin ($C_{27}H_{32}O_{14}$; Molecular weight: 580.53g/mol, ≥ 95% HPLC, National Institute of Medical Materials, Viet Nam); RPMI 1640 (RBMI, Gibco, USA); Fetal bovine serum (FBS, Gibco, USA); Penicillin-Streptomycin (PS, Gibco, USA); Phosphate buffered saline (PBS, Gibco, USA); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; Sigma, USA); Doxorubicin (DOX, serves as positive control drug); Dimethyl sulfoxide (DMSO, Sigma, USA); Inverted microscopy Axiovert (S100, Carl Zeiss, Germany); CellTox™ Green (Promega, Madison, WI, USA); Tetramethyl rhodamine ethyl ester (TMRE, excitation/emission: 535/570 nm, Invitrogen, USA); 10-N-nonyl acridine orange (NAO, excitation/emission: 495/519 nm, Invitrogen, USA); Culture dishes 90x20 mm (SPL, Korea); 96-well black, glass bottom plates (CAT. 33196, SPL); Confocal dishes (CAT. 100350, SPL); CO_2 Incubator (Shellab, USA); ApoTome fluorescence microscope (Zeiss, Germany); and Microplate reader (Tristar, USA). The study was carried out at the Animal Cell Biotechnology Laboratory, Life Science Research Center, Faculty of Biology, VNU University of Science.

2.2. Methods

2.2.1. Cell Cultured and Treatments

KMS-20 cells were maintained culture dishes (90x20 mm) containing RPMI, 10% FBS, and 100 µg/mL of PS at 37 °C with 5% CO_2. Culture medium was changed every 2-3 days. For drug treatment, KMS-20 cells were further transferred to 96-well at density of 2.10^4 cells per well and three cells per group at 37 °C, 5% CO_2. After 24 h, the old medium was removed, the cells were further divided into three groups:

+ The control group (RPMI, control): KMS-20 cells were continuously cultured in RPMI medium plus DMSO 0.1% for 24 h;

+ The DOX group (positive control drug): KMS-20 cells were continuously grown for 24 h in new media containing DMSO 0.1% and DOX 10 µM as previously discribed [15];

+ The NAR group (tested compound): KMS-20 cells were continuously grown for 24 h in new media containing DMSO 0.1% and NAR 16 µM - the optimal concentration is chosed following the obtained IC50 value and referring to previous reports [16, 17].

2.2.2. Measurement of Cell Viability

Cell viability was determined using the MTT assay. KMS-20 cells were seeded at a density of 2.10^4 cells per well in 96-well plates. After different treatments, cells were treated with MTT at 37 °C for 4 h. Then, the old medium was removed, and DMSO (150 µL) was added to each well to dissolve the formazan crystals. The optical density (OD) was determined at 570 nm using a microplate reader (Spectramax plus 384, Molecular devices, USA). The alive cell number in each well (3 wells per group) was expressed as a value relative to the normal control well. Experiments were repeated four times [15].

2.2.3. Measurement of Cell Toxicity

The cell toxicity was determined using CellTox™ Green, in which cell death is measured with a fluorescent dye that binds the DNA of cells with impaired membrane integrity as previously presented [15]. KMS-20 cells were seeded at a density of 2.10^4 cells per well

in 96-well black, glass bottom plates (CAT. 33196, SPL). Next, a mixture of CellTox™ Green Dye and cell medium (1:1000) was added to the 96-well plate. Subsequently, cells were treated with NAR (16 µM) and DOX (10 µM). Wells without cells were used as a background control. Cell death was determined after 1, 6 and 18h of treatment by measuring the fluorescence intensity (em/ex: 485/520 nm) using microplate reader (Tris-star, USA). Experiments were repeated four times and there were three wells per group for each repeat.

2.2.4. Measurement of Mitochondrial Cardiolipin and Mitochondrial Membrane Potential

The mitochondrial cardiolipin content and mitochondrial membrane potential were measured by NAO and TMRE fluorescence kits followed the previous reports [15, 18]. KMS-20 cells were seeded at a density of 2.10^4 cells per well in 96-well black, glass bottom plates (CAT. 33196, SPL). After treatments, the cells were stained with either 0.1 µM NAO (ex/em: 495/519 nm) or 0.1 µM TMRE (ex/em: 535/570 nm) at room temperature for 30 min. The cells were washed twice with PBS before measuring fluorescence intensity [4]. The NAO or TMRE intensity in each well was expressed as a percentage value relative to the normal control. There were three wells per group for each repeat. Experiments were repeated four times.

2.2.5. Statistical Analysis

Data are presented as means ± Standard error of the mean (SEM) by using Excel 2016, Origin 8.5. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test. $P<0.05$ was considered to be a statistically significant difference.

3. Results and Discussion

3.1. Naringin Suppresses KMS-20 Cell Proliferation

KMS-20 cells were normally cultured for 24 h before treated with NAR (0÷500 µM) for next 48 h. The effect of NAR on cell viability was determined by using the CCK-8 kit as mentioned in another report [19]. The 48h EC50 of NAR was determined to be 15.6 µM. The obtained results showed that NAR exhibited strong cytotoxicity on KMS-20 cells. This result is quite similar to other studies testing the effects of NAR on other cancer cells [17, 20]. On the basis of IC50 value and reference to previous studies [16, 17, 21], we selected the NAR concentration of 16 µM for the next evaluations.

The effects of NAR at the chosen concentration on the viability of KMS-20 cells were presented in Figure 1 and Figure 2.

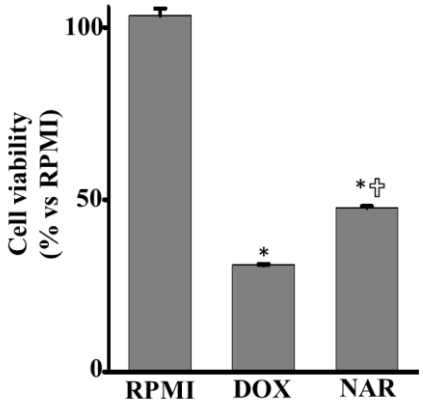

Figure 1. Cell viability of KMS-20 cells under different conditions. RPMI (control): KMS-20 cells cultured in normal condition; DOX (positive control drug): KMS-20 cells cultured in medium containing DOX at 10 µM; NAR (tested compound): KMS-20 cells cultured in medium containing NAR at 16 µM; media containing DMSO 0,1%; *$p<0.05$ vs. RPMI, ✞$p<0.05$ vs. DOX, n=4.

The results showed that DOX and NAR both exhibit cytotoxicity and inhibit the growth of KMS-20 cells. The cell viabilities of RPMI, DOX and NAR groups were 103.49±2.02%, 31.00±0.18% and 47.72±0.17%, respectively (Figure 1). The survival rates of KMS-20 cells in the DOX and NAR groups were significantly lower than those in the control group (RPMI group, $p<0.05$). This result is quite consisted to previous studies on the effect of NAR on other cancer cells [12-14, 17]. Treatment with NAR for 24h resulted in growth inhibition, apoptosis

and cell cycle arrest of breast, cervical and gastric cancer cells [12-14]. The effects of NAR in this study resemble a NAR derivative, Naringenin. Previous research had proved that after 24 h incubated with Naringenin, the cell viabilities of multiple myeloma cell lines, such as NCl-H929 and U266, were significantly reduced [22].

To further confirm the impact of NAR on cancer cells, we continued to analyze the toxicity of NAR on KMS-20 cells at 1h, 6h and 18h by using CellTox™ Green kit.

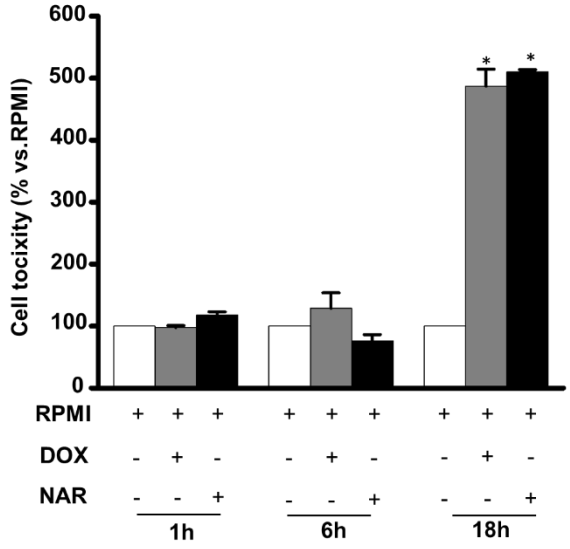

Figure 2. The toxicity of NAR on KMS-20 cells. RPMI (control): KMS-20 cells cultured in normal condition; DOX (positive control drug): KMS-20 cells cultured in medium containing DOX (10 µM); NAR (tested compound): KMS-20 cells cultured in medium containing NAR (16 µM); media containing DMSO 0,1%; (+) present; (-): absent; *p<0.05 vs. RPMI, n=4.

The obtained results showed that there was no difference in toxicity of DOX and NAR after either 1h or 6h of treatment (Figure 2). However, the toxicity of DOX and NAR to KMS-20 cells all spiked with statistically significant values (Figure 2). Specifically, at 18h of treatment, the cellular toxicity in DOX and NAR groups were significantly elevated, up to 486.97±27.51% and 510.00±3.93% (% of control), respectively. This result is consistent with the previous studies [12, 23]. Thus, NAR might suppress the proliferation of KMS-20 cells in time dependence.

3.2. Naringin Alters Mitochondrial Function of KMS-20 cells

As mitochondria are involved in multiple cellular biological process [24], in this study, the effects of NAR on mitochondria of KMS-20 cells were also assessed via mitochondrial cardiolipin contents and mitochondrial membrane potential indexes.

3.2.1. Naringin Reduces the Mitochondrial Cardiolipin Content of KMS-20 Cells

Cardiolipin is a mitochondrial membrane phospholipid and plays an important role in the respiration [25]. Cardiolipin was reported to be involved in the apoptosis of animal cells through interaction with lethal proteins [26]. In this study, the changes in cardiolipin content among cell groups were determined using NAO fluorescence assay (Figure 3).

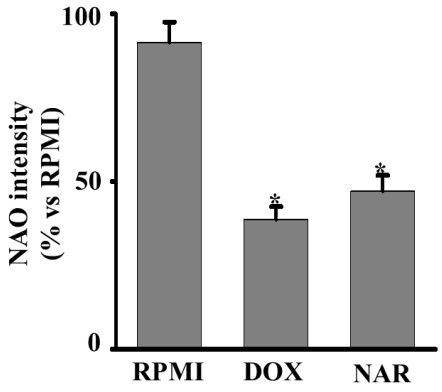

Figure 3. NAO intensity in KMS-20 cells. RPMI (control): KMS-20 cells cultured in normal condition; DOX (positive control drug): KMS-20 cells cultured in medium containing DOX (10 µM); NAR (tested compound): KMS-20 cells cultured in medium containing NAR (16 µM); media containing DMSO 0,1%; *p<0.05 vs. RPMI, n=4.

The mitochondrial inner membrane and cardiolipin contents of KMS-20 cells were significantly reduced in the experimental groups compared to the control group (Figure 3). The percentage of NAO intensity in DOX and NAR groups were 38.78±3.74% and 47.23±4.65%,

respectively. It was reported that a sharp decrease in the cardiolipin component leads to mitochondrial dysfunction and may lead to cell death [25]. In this study, the cardiolipin content is quite consistent with the change in survival rate (Figure 1) and the cytotoxicity (Figure 2) shown above.

3.2.2. Naringin Elevates the Mitochondrial Membrane Potential of KMS-20 Cells

Mitochondrial membrane potential is an important parameter for assessing mitochondrial function. In this study, mitochondrial membrane potential index was indirectly assessed by measuring TMRE fluorescence intensity.

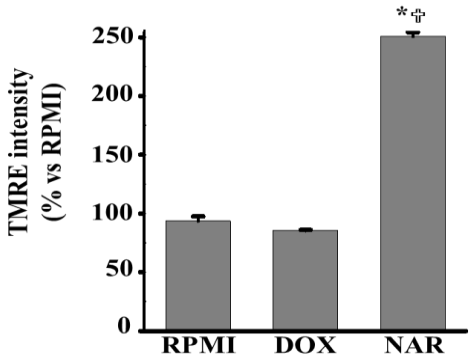

Figure 4. TMRE intensity in KMS-20 cells. RPMI (control): KMS-20 cells cultured in normal condition; DOX (positive control drug): KMS-20 cells cultured in medium containing DOX (10 µM); NAR (tested compound): KMS-20 cells cultured in medium containing NAR (16 µM); media containing DMSO 0,1%; *p<0.05 vs. RPMI, ✞p<0.05 vs. DOX, n=4.

In contrast to the DOX, a positive control drug, the mitochondrial membrane potential value of KMS-20 cells treated with NAR increased sharply 251±3.12% compared to the control group (93.8±3.58%) (Figure 4, $p<0.05$). The increase in mitochondrial membrane potential or mitochondrial hyperpolarization was reported in some types of cancer cells and causes cell death [27, 28]. Last study also showed that mitochondria are likely to involve in the drug resistance of KMS-20 myeloma cells to bortezomib [3]. This might result in the membrane potential of KMS-20 being different when tested with different anticancer agents.

4. Conclusion

The current findings indicate that NAR is potential to inhibit the growth of multiple myeloma cell by altering mitochondria. This might be the basis for further studies on the mechanism of action of NAR on multiple myeloma.

Aknowledgements

We thank Dr. P. T. Bich, Dr. N. H. Ly, Associate Professor P. T. Thuong, Associate Professor K. H. Kyu and Professor H. Jin and MSc N. T. Chuc for their helps and supports.

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