2-Methoxy-1,4-Naphthoquinone (MNQ) Induces Authophagy in Triple Negative Breast Cancer Cells

Syukriyah Mat Daud[1], Nik Soriani Yaacob[1], Zulkifli Mustafa[2], Agustine Nengsih Fauzi[1]*

[1]Department of Chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

[2]Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

Abstract

Autophagy may occur in cancer cells as a survival mechanism in the process to restrict tumor development, besides attributed to the programmed cell death. The dual function of autophagy has contributed to anticancer research by focusing on autophagy in cancer cells. This study was conducted to investigate the ability of 2-methoxy-1,4-naphthoquinone (MNQ) to induce autophagy in triple negative breast cancer model (MDA-MB-231) *in vitro*. The cells were tested with MNQ at 29 µM for determination of autophagy by confocal microscopy. The expression levels of autophagy-related molecules were measured using gene analysis real-time PCR. The results showed that MNQ induced autophagy in MDA-MB-231 cells, indicated by the formation of green-colored autophagosomes and the upregulation of autophagy-related molecules, Beclin 1 and LC3. The autophagy activity of MDA-MB-231 cells that may lead to cancer cell death indicated the potential of MNQ to be further developed as an effective agent/adjuvant against breast cancer.

Keywords: 2-methoxy-1,4-naphthoquinone (MNQ), autophagy, cell death, triple negative breast cancer cells, MDA-MB-231

Article Info

Received 18th October 2020
Accepted 16th March 2021
Published 1st April 2021
*Corresponding author: Agustine Nengsih Fauzi; email: agustine@usm.my

ISSN: 1823-7010, eISSN: 2600-7444

Introduction

Metabolic stress, such as nutrient deprivation and cytotoxic drugs, can lead to autophagy. The term 'autophagy' is derived from Greek, which means 'self-eating' [1,2]. It is a cellular catabolic degradation reaction to nutrient deprivation or stress, in which cellular proteins, organelles, and cytoplasm are engulfed, digested, and recycled to sustain cellular metabolism [3,4]. Autophagy is regulated by the mammalian target of rapamycin (mTOR) kinase, a downstream component of the P13K/Akt signaling pathway. The inhibition of mTOR will occur upon the initiation of stress in the cells, while the AMP-activated protein kinase (AMPK) will be activated. Consequently, the formation of an autophagy-related gene (Atg) complex will induce autophagy [5,6,7]. Cancer cells initiate autophagy through the degradation of intracellular contents and damaged organelles to ensure cellular energy homeostasis**.** Through intracellular degradation, autophagy provides substrates that enable tumor cells to survive in the tumor microenvironment and promotes tumor progression [8]. Autophagy is also a pathway used to eliminate and degrade pathogenic or apoptotic cells [9]. Vera-Ramirez *et al.* (2018) showed that breast cancer cells in a dormant state are highly dependent on autophagy for metastasis process and survival [10].

It is known that autophagy might be required for cell death, but little is known about how autophagy kills cells. One possibility is that autophagy causes metabolic exhaustion by depleting mitochondria of metabolic substrates. Prolonged nutrient starvation will cause the cells to continue degrading their intracellular organelles, ultimately causing autophagic cell death [11]. Moreover, the continuous autophagic activity of the cells leads to the accumulation of ROS species, which is toxic to the cells. Without a proper counter mechanism to balance the ROS, cells are susceptible to severe oxidative stress, leading to cell death [12,13]. Studies have shown that autophagy inducer therapy in cancer cells could inhibit the growth of cancer cells by inhibiting angiogenesis [14,15]. The unique ability of autophagy to either support cell survival or promote cell death has led to the exploration of anticancer treatment by the induction of autophagy [16]. Several studies have focused on the induction of autophagy in cancer cells as an alternative to eliminate cancer cells by targeting the autophagy-related molecules/enzymes/signaling pathway [17]. Additionally, studies have also been conducted around the world on natural compounds mainly from plants for the development of therapeutic agents against various cancers, and several chemotherapeutic drugs have been studied for autophagy [18,19].

MNQ is a type of quinone that can be extracted from garden balsam (*Impatiens balsamina*). In traditional Chinese medicine, the entire garden balsam plant has been used to treat various diseases, such as superficial infections, rheumatism, isthmus, and fingernail inflammation [20,21]. MNQ is one of the natural compounds that have been observed to exert an anticancer effect against cancer cells. MNQ has previously been shown to be cytotoxic and suppressed the invasion and migration of several cancer cell lines, including highly metastatic, triple negative breast cancer (TNBC) cells, MDA-MB-231 [22,23,24]. MNQ was also reported to induce apoptotic cell death in lung adenocarcinoma cells by stimulating reactive oxygen species (ROS) production, which led to oxidative DNA damage and subsequent activation of JNK and p38 MAPK signaling pathways [25]. A recent finding by Wang *et al*. (2019) showed that 1,4-Naphthoquinone derivatives inhibit cell proliferation, induce apoptosis, arrest cell cycle at the G2/M phase, and increase ROS generation via the inhibition of the Akt signaling pathway in human gastric cancer cells [26]. Thus, the present study aimed to determine whether MNQ could induce autophagy in MDA-MB-231 cells and be further developed as an effective agent/adjuvant against TNBC.

Materials And Methods

Cell lines and cell culture

Human breast adenocarcinoma, MDA-MB-231 cell line, were purchased from the American Type Cell Culture Collection (ATCC, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1 unit/ml penicillin/streptomycin (all purchased from Thermo Scientific, USA). The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere. MNQ was purchased from Sigma-Aldrich (USA) and dissolved in dimethyl sulfoxide (DMSO; Amresco, Canada) to make a 10 mM stock solution.

Autophagy detection

Cells were seeded on a coverslip at the concentration of 1×10^5 cells/ml and treated with MNQ at 29 μM [24] for 24 hours. The autophagic activity was detected using the Autophagy Detection Kit (Abcam, UK) according to the manufacturer's instruction. The cells were stained with 100 μL Dual Detection Reagent (Green Detection Reagent and Nuclear Stain) for 30 minutes at 37 °C and protected from light. The stained cells were analyzed by confocal microscopy using a fluorescein isothiocyanate (FITC) filter (autophagic signal) and 4′,6-diamidino-2-phenylindole (DAPI) filter (nuclear signal) at a wavelength of 488 nm.

Gene determination

MDA-MB-231 cells were treated with MNQ for 1, 4, 8, and 24 hours. The cells were extracted, and the RNA was collected using the RNeasy Mini kit (Qiagen, USA). The RNA was then converted into cDNA using the Revert Aid H Minus First Strand cDNA synthesis kit (Fermentas, USA) in the thermal cycler GeneAmp PCR system 9700 (Applied Biosystems, USA). The cDNA template was added to the master mix (Bioline Reagents Ltd, UK) before the addition of the gene primers: forward (Beclin 1: 5′-AGC TGC CGT TAT ACT GTT CTG-3′; LC3: 5′-GAT GTC CGA CTT ATT CGA GAG C-3′) and reverse (Beclin 1: 5′-ACT GCC TCC TGT GTC TTC AAT CTT-3′; LC3: 5′-TTG AGC TGT AAG CGC CTT CTA-3′; Integrated DNA Technologies, Singapore). The reaction mixture was run using the real-time PCR machine (Stratagene, California). The gene expression was conducted via the double delta Ct analysis method, with the value of more than 1 indicating an upregulated, and the value of less than 1 indicating a downregulated gene expression.

Statistical analysis

Data were obtained from at least three independent experiments. The values were expressed as mean ± standard error measurement (SEM). Statistical evaluations were performed using One-way and Two-way ANOVA on GraphPad Prism 6.0, and $P<0.05$ was considered significant.

Results and Discussion

Autophagy activation upon MNQ treatment occuring in MDA-MB-231 cells

As presented in Figure 1, we detected the autophagy activity in MDA-MB-231 cells treated with MNQ by confocal microscopy. The cells treated with MNQ and rapamycin (autophagy-inducer) emitted bright green and blue fluorescence, compared to the untreated cells, which only displayed blue nuclear fluorescence. As the green detection reagent is incorporated in the cells, the accumulation of the fluorescence probe is observed in the form of spherical vacuoles distributed throughout the cytoplasm.

Autophagy can be induced by various stimulants, e.g., nutrient deprivation, hypoxia, reactive oxygen species (ROS) as well as exposure to cytotoxic drugs [3,4]. The activation of autophagy could be due to the cytotoxic effects of MNQ, as shown in previous study [22,23,24]. This was observed through the formation of green-fluorescent autophagosomes analyzed by confocal microscopy. These green fluorescent-labeled vesicles co-localized with LC3, a specific autophagosome marker in the cytoplasm. The cells that undergo autophagy will recruit a complex of autophagy-related genes (Atgs), including Atg8/LC3, to form autophagosomes [2]. The Green Detection Reagent used in this study contains a fluorescent probe known as monodansylcadaverine (MDC) that served as a useful fluorescent marker for lysosomal/ autophagic vacuoles.

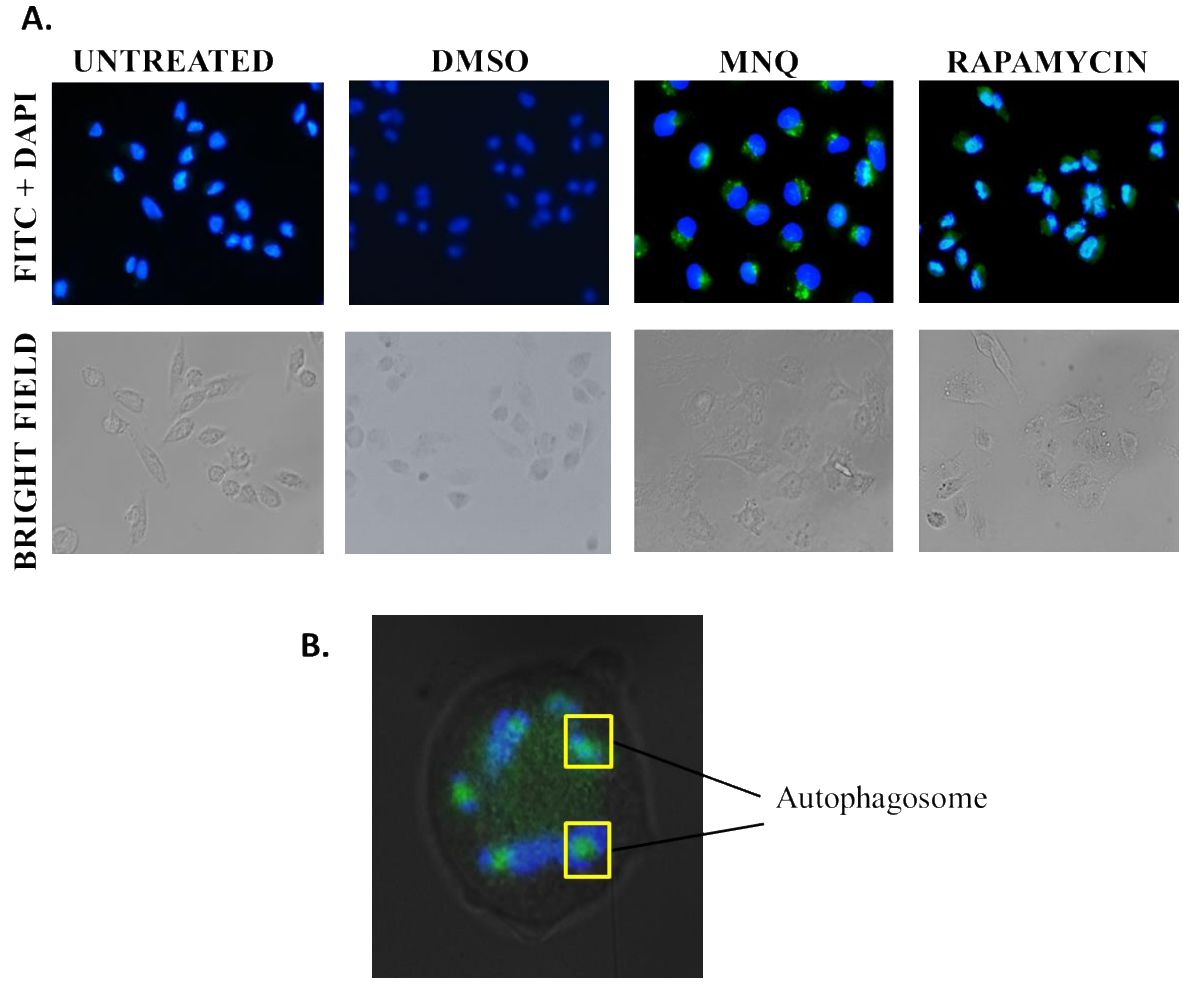

Figure 1: The autophagic activity in MDA-MB-231 cells treated with MNQ for 24 hours.

The cells treated with MNQ (29 μM), rapamycin (100 nM), and DMSO (1 % v/v) were subjected to the determination of autophagic activity. (A) The cells treated with MNQ and rapamycin display the formation of autophagosomes in bright green fluorescence compared to untreated cells. All the cells are visualized using FITC, DAPI, and bright field filters. The cell images are captured at 40× magnification. (B) The representative image of autophagosome formation in MNQ-treated MDA-MB-231 cells. The cell image is captured at 60× magnification.

Upregulation of autophagy-related molecules in MDA-MB-231 cells treated with MNQ

The expression of autophagy-related molecules Beclin 1 and LC3 genes were carried out using real-time PCR to study the autophagic activity at the gene level. Beclin 1 is a regulatory protein in the autophagic pathway, whereas LC3 is a specific marker of autophagosomes, whereby the level of LC3 expression directly reflects the level of autophagy [27]. Results obtained in Figure 2 show that the expression level of Beclin 1 and LC3 genes increased in a time-dependent manner after treatment with MNQ compared to untreated cells. The upregulation of these genes indicated that the autophagy process had occurred within the cells treated with MNQ.

Autophagy involves more than 38 Atg genes, and each of these Atgs performs different functions. In the first step of autophagy, the complex of Atg1, Atg13, Atg17, Atg29, and Atg31 translocate to form a pre-autophagosomal structure (PAS). This complex formation leads to the recruitment of other Atgs, including Atg6/Beclin 1 and Atg14. PAS became elongated at the end of autophagosome formation, and the membrane encloses a small portion of cytoplasmic material to form a double-membrane structure called an autophagosome via Atg12, Atg5, Atg16 complex, and the Atg8/LC3 phosphatidylethanolamine (PE) complex. The autophagosomes are subsequently fused with lysosomes to become autolysosomes, where the cytoplasmic materials will be degraded by lysosomal enzymes [2].

Mechanisms that regulate the mutually opposed survival-supporting and death-promoting roles for autophagy are still unclear. Probably the effect of autophagy in cancer metabolism is different depending on cancer itself, treatment, and types of autophagy inducer [28]. However, studies showed that prolonged nutrient starvation due to autophagy would cause the cancer cells to continue degrading intracellular organelles, and ultimately undergoing autophagic cell death. Our previous findings demonstrated the potential of MNQ to inhibit glycolysis in MDA-MB-231 cells through inhibition of glucose uptake and lactate production. Glucose is a main source of energy in cancer cells. Prolonged inhibition of glucose uptake into the cells may elicit autophagic responses.

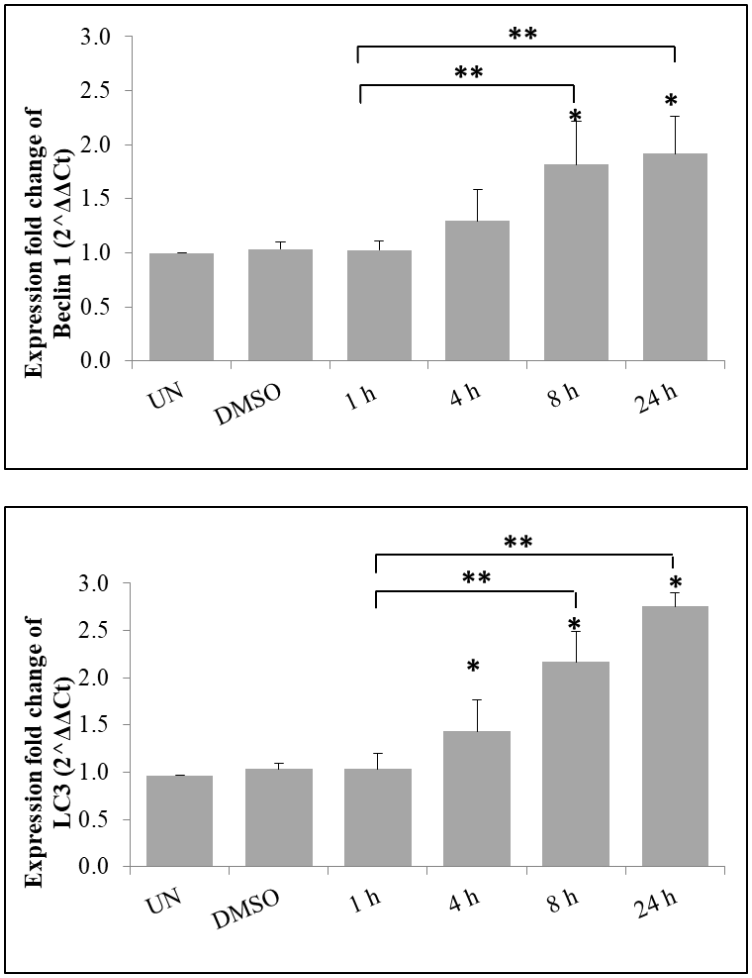

Figure 2: The expression fold change of Beclin 1 and LC3 genes in MNQ-treated compared to untreated cells.

The cells treated with MNQ (29 μM) and DMSO (1 % v/v) were subjected to the measurement of gene expression. The expression fold change of the genes is obtained using the double delta Ct analysis method. The expression levels of Beclin 1 and LC3 are upregulated compared to untreated (UN) cells. Data are expressed as mean ± SEM from three independent experiments. **P*<0.05 indicates the significant difference of treated cells from the untreated cells, whereas ***P*<0.05 indicates the significant difference within the time points of treatment.

Conclusion

The present study focused on determining the effect of MNQ on the formation of autophagy in triple negative breast cancer cell line, MDA-MB-231. The results obtained from the present study proved that MNQ induces autophagy in MDA-MB-231 cells, indicated by the formation of autophagosome and increased expression of autophagy-related genes (Beclin 1 and LC3). Although this study was performed *in vitro*, these results initiate the potential of MNQ to further develop as a chemotherapeutic drug in breast cancer patients.

Acknowledgement

This research was funded by the Universiti Sains Malaysia (USM) Short Term grant (304.PPSP.61313154). SM Daud was supported by USM Fellowship Scheme and Mybrain15 from the Ministry of Higher Education Malaysia.

Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

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