Activation of JNK/p38 Pathway is Responsible for α-Methyl-n-butylshikonin Induced Mitochondria-Dependent Apoptosis in SW620 Human Colorectal Cancer Cells

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Abstract

α-Methyl-n-butylshikonin (MBS), one of the active components in the root extracts of Lithospermum erythrorhizon, possesses antitumor activity. In this study, we assessed the molecular mechanisms of MBS in causing apoptosis of SW620 cells. MBS reduced the cell viability of SW620 cells in a dose- and time-dependent manner and induced cell apoptosis. Treatment of SW620 cells with MBS down-regulated the expression of Bcl-2 and up-regulated the expression of Bak and caused the loss of mitochondrial membrane potential. Additionally, MBS treatment led to activation of caspase-9, caspase-8 and caspase-3, and cleavage of PARP, which was abolished by pretreatment with the pan-caspase inhibitor Z-VAD-FMK. MBS also induced significant elevation in the phosphorylation of JNK and p38. Pretreatment of SW620 cells with specific inhibitors of JNK (SP600125) and p38 (SB203580) abrogated MBS-induced apoptosis. Our results demonstrated that MBS inhibited growth of colorectal cancer SW620 cells by inducing JNK and p38 signaling pathway, and provided a clue for preclinical and clinical evaluation of MBS for colorectal cancer therapy.

Keywords: α-Methyl-n-butylshikonin - apoptosis - colorectal cancer - JNK - p38

Introduction

Colorectal cancer is one of the aggressive malignant tumors threatening human survival world-wide (Siegel et al., 2013). Although with the development of chemotherapy and radiotherapy, the potential complications are unavoidable. These problems highlight the imperative and urgent requirement for identifying and developing novel anticancer agents.

α-Methyl-n-butylshikonin (MBS), one of the shikonin derivatives, is a naphthoquinone pigment isolated from the traditional medical herbs Lithospernum erythrorhizon (Figure 1A) (Chen et al., 2011). Previous studies have demonstrated that shikonin and its derivatives possess multiple biological functions such as anti-microbial, anti-inflammatory, anti-HIV and anti-platelet effects (Tanaka et al., 1986; Ko et al., 1995; Shen et al., 2002; Chen et al., 2003). In addition, accumulating lines of evidence have shown that shikonin and its derivatives exhibit antitumor activity in a variety of human cancers. Huang C et al reported that shikonin induced necroptosis via activation of RIP-1 in glioma cells (Huang et al., 2013). Recently, we reported that β,β-dimethylacrylshikonin induced mitochondria-dependent apoptosis via ERK1/2 pathway in human gastric cancer SGC-7901 cells (Shen et al., 2012).

However, the effects of MBS on human colorectal cancer cells still remain elusive. The present study was performed to investigate the role and mechanism by which MBS elicits its biological effects on colorectal cancer cells.

Materials and Methods

Materials

SW620 cells were purchased from Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). MBS was purchased from Tokyo Chemical Industry (Tokyo, Japan). MTT, DAPI, SP600125, and SB203580 were purchased from Calbiochem (San Diego, CA, USA). Pan-caspase inhibitor (Z-VAD-FMK) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Antibodies against JNK, phospho-JNK, p38, phospho-p38, Bcl-2, Bak, cleaved caspase-9, cleaved caspase-8, cleaved PARP and β-actin were purchased from Cell Signaling (Boston, MA, USA). FITC-Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (San Jose, CA, USA).
purchased from Becton Dickinson (San Diego, CA, USA).

**Cell culture and cell proliferation assay**

SW620 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (Hyclone, UT), and maintained at 37°C in a humidified atmosphere of 5% CO2. Then cells were seeded in a 96-well dish to a final concentration of 5x10³ cells/well and incubated in RPMI 1640 medium containing 10% FBS for 24h, after that, cells were treated with the indicated concentration of MBS for 24h and 48h. Medium was removed and fresh medium was added to each well along with 20µl of MTT solution (5mg/ml). After 4h incubation, 150µl of DMSO was added to each well. The plates were read at wavelength of 570 nm using Varioskan Flash Multimode Reader (Thermo Scientific, USA). Four reduplicate wells were used for each treatment, and experiments were repeated three times.

**DAPI (4’,6-diamidino-2-phenylindole) staining**

SW620 cells were placed in the well of a six-well plate. After 24h cell culture, they were treated with MBS for the indicated time periods, then cells were fixed in cold acetone for 30 min, and incubated with DAPI (1mg/ml) for 30 min. The apoptotic nuclei characterized as intensively stained were detected using fluorescent microscopy (model IX71; Olympus, Tokyo, Japan).

**Annexin V/PI assays for apoptosis**

For Annexin V/PI assays, cells were stained with AnnexinV-FITC and PI, and evaluated for apoptosis by flow cytometry according to the manufacturer’s protocol (BD PharMingen, San Diego, CA, USA). Briefly, 1x10⁴ cells were washed twice with PBS, and stained with 5µl of AnnexinV-FITC and 5µl of PI in 500µl binding buffer for 15 min at room temperature in the dark. The apoptotic cells were determined using BD FACS Diva software (BD Biosciences, Franklin Lakes, NJ). Annexin V staining serves as a measure of phosphatidylserine externalization and cells that are Annexin V+/PI- represent early apoptotic cells.

**Measurement of mitochondrial membrane potential**

Cells (5x10⁴ cells/well) were treated with or without MBS (10µmol/L) for 24h and stained with JC-1 (BD MitoScreen JC-1 Kit, Becton Dickinson, USA) for 15 min at 37°C. Mitochondrial membrane potential was detected by flow cytometry (FACS Canto II, Becton Dickinson, USA).

**Western-blot analysis**

Cells were treated with the indicated concentration of MBS for the indicated time in RPMI 1640 medium with 10% FCS. The cells were collected in ice-cold PBS, and the cell extracts were prepared in RIPA buffer with protease inhibitor cocktail from Calbiochem (San Diego, CA). The protein concentrations of the cell lysates were determined and boiled with gel-loading buffer for 10 min at 100°C. Samples containing 30 µg of total protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to PVDF membrane (Millipore, Temecula, CA). Following transfer, the membrane were blocked in TBST (TBS containing 0.1% Tween 20) containing 5% skimmed milk for 2h, followed by incubation overnight at 4°C with appropriate primary antibodies. After washing three times in TBST, the membranes were incubated for 2h at 37°C with 1:1000 horseradish peroxidase-conjugated appropriate secondary antibodies. Finally, the membranes were visualized using the enhanced chemiluminescence detection system (Immun-Star Western C Kit, Bio-Rad, USA).

**Statistical analysis**

Data reported are the mean±standard deviation (SD) of three independent experiments. They were evaluated by one-way analysis of variance (ANOVA). Significant differences were established at p<0.05.

**Results**

**MBS inhibits viability of SW620 cells**

The cytotoxic effects of MBS on colorectal cancer cell lines were determined by using the MTT assay. SW620 cells were treated with different concentrations of MBS (5, 7.5, 10, and 15µM) for 24h or 48h, and the cell viability was examined by MTT assay. As shown in (Figure 1B), after 24h incubation with MBS at the concentration of 5µM, 7.5µM, 10µM, and 15µM, the viability of SW620 cells reduced to 87.88±0.80%, 77.39±1.90%, 64.80±0.62% and 40.22±0.79% when compared with the vehicle-treated cells. Treatment of SW620 cells with MBS for 48h, cell viability reduced to 77.53±1.32%, 58.92±1.58%, 35.42±0.97% and 13.05±0.67% at the MBS concentration of 5µM, 7.5µM, 10µM, and 15µM, respectively (Figure 1B). The half maximal inhibitory concentration (IC₅₀) of MBS for SW620 cells was 13.07µM and 8.39µM at 24h and 48h, respectively.

![Figure 1. MBS Inhibits SW620 Cells Viability. A) The chemical structure of MBS (MW.372.41). B) Effects of MBS on cell viability inhibition of SW620 cells. Cells were treated with different concentrations of MBS for 24 h and 48 h. The related cell viability was determined by MTT assay. The viability of the control group (0.1% DMSO) was set to 100%. Data represent the mean±SD obtained from three independent experiments. *indicated p<0.05 compared to control group respectively](image)
MBS induces SW620 cell apoptosis

SW620 cells were first treated with MBS, results showed the cells underwent marked morphological changes upon treatment with 15 μM MBS compared with the untreated control for 24h. MBS induced nuclear condensation with intensive DAPI staining compared to the nuclear of control SW620 cells for 24h (Figure 2A).

To examine whether the cytotoxic effect of MBS involved an induction of apoptosis, we evaluated the apoptotic effect of MBS in SW620 cells by flow cytometry with Annexin V and propidium iodide (PI) double staining. As Figure 2B showed, after 24h incubation, the early apoptotic (Annexin V+/PI-) rates of SW620 cells were 1.2%, 5.2%, 21.1%, 26.5% and 28.8% in response to the vehicle, 5, 7.5, 10, and 15μM MBS, respectively. Interestingly, the late apoptotic cells (Annexin V+/PI+) were increased in SW620 cells (11.1% with 15μM MBS treatment) (Figure 2B).

MBS induces mitochondrial events associated with apoptosis in SW620 cells

The Bcl-2 family proteins play an important role in the regulation of apoptosis, which include both pro- and anti-apoptotic proteins. They can activate or inhibit the release of various apoptotic mediators which lead to the activation of caspases in the execution of apoptosis (Ola et al., 2011). In order to detect the expression of apoptosis-related proteins, pro-apoptotic proteins (Bax, Bak) and anti-apoptotic proteins (Bcl-2) were assayed by western blotting after SW620 cells were treated with varying concentrations of MBS (0, 5, 7.5, 10, and 15μM) for 24h. The results indicated that MBS decreased the expression of Bcl-2 and increased the expression of Bak in a dose-dependent manner (Figure 3A). We also found that MBS had no influence on expression levels of Bax (Figure 3A).

Mitochondrial dysfunction has even been shown to be central to the apoptotic pathway (Ly et al., 2003). Apoptotic stimuli often perturb mitochondrial function by decreasing the mitochondrial membrane potential. Loss of mitochondrial membrane potential has been linked to the initiation and activation of some apoptotic cascades (Mantena et al., 2006). Therefore, we determined whether MBS induced the loss or disruption of mitochondrial membrane potential in SW620 cells. For this purpose, the mitochondrial membrane potential was measured by flow cytometry with JC-1 staining. The SW620 cells were treated with or without MBS (15μM) for 24h, results showed mitochondrial membrane potential was decreased from 93.7% to 43.9% after treatment of SW620 cells with MBS (Figure 3B).

Figure 3. MBS Induced Mitochondrial Events Associated with Apoptosis in SW620 Cells. Western blotting analysis for anti- and pro-apoptotic proteins: Bcl-2, Bax, Bak A), and cleaved PARP, cleaved caspase-9, cleaved caspase-8, cleaved caspase-3 B) in whole cell extracts after the SW620 cells were treated with MBS for 24 h. Protein levels of actin were also measured as controls. C) Detection of mitochondrial membrane potential by flow cytometry. SW620 cells were treated with or without MBS (15μM) for 24 h and were stained with JC-1 for 15 min at 37℃ and subjected to flow cytometry. D) SW620 cells were treated with MBS in the presence or absence of Z-VAD-FMK (10μM) for 24 h. Protein extracts were prepared and subjected to western blot assay using antibody against cleaved caspase-3, cleaved PARP. Protein levels of actin were also measured as controls. E) SW620 cells were treated with MBS in the presence or absence of Z-VAD-FMK (10μM) for 24 h. Protein levels of actin were also measured as controls. F) SW620 cells were treated with MBS in the presence or absence of Z-VAD-FMK (10μM) for 24 h. Protein levels of actin were also measured as controls.
to measure levels of phosphorylated JNK. Protein levels extracts were prepared and subjected to western blot assay to measure levels of phosphorylated forms of ERK A), JNK B), p38 C). Membranes were reprobed with antibodies against total ERK, JNK and p38 for normalization.

Effects of MBS on MAPK Pathways. SW620 cells were treated with the indicated MBS concentration or the indicated interval, total cellular extracts were prepared and subjected to western blot assay to measure levels of phosphorylated JNK B). Protein levels of total p38 were also measure as controls.

Figure 4. Effects of MBS on MAPK Pathways. SW620 cells were treated or not pretreated with SP600125 (15μM) for 24 h. Protein extracts were prepared and subjected to western blot assay to measure levels of phosphorylated p38. Protein levels of total p38 were also measure as controls.

Figure 5. MBS Induced SW620 Cells Apoptosis was Mediated Through JNK Activation. A) SW620 cells were pretreated or not pretreated with SP600125 (25μM) then added with DMSO (vehicle) or MBS (15μM) for 24 h. Protein extracts were prepared and subjected to western blot assay using antibody against cleaved caspase-3, cleaved PARP. Protein levels of actin were also measured as controls. B) SW620 cells were pretreated or not pretreated with SB203580 (10μM) then added with DMSO (vehicle) or MBS (15μM) for 24 h. Protein extracts were prepared and subjected to western blot assay to measure levels of phosphorylated p38. Protein levels of total p38 were also measure as controls.

Caspase family proteins are the critical enzymes responsible for the execution of apoptosis. Among caspase family members, caspase-3 is the dominant executor for apoptosis in mammalian cells (Brentnall et al., 2013). It can be cleaved and activated through either the extrinsic (death receptor-mediated) or intrinsic (mitochondria dependent) pathway (Igney et al., 2002; Hu et al., 2003).

In order to further understand the molecular mechanism of MBS-induced apoptosis in SW620 cells, the expression change of caspase-3, caspase-8 and caspase-9 were detected by western blotting analysis. Treatment of SW620 cells with MBS (0, 5, 7.5, 10, and 15μM) for 24h resulted in a dose-dependent elevation of cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 when compared with the cells which were not treated with MBS (Figure 3C). Cleaved PARP, another well-known characteristic of apoptosis, was also increased with the increasing doses of MBS (Figure 3C).

To further determine the role of caspase activation in MBS-induced apoptosis, we pre-treated SW620 with
pan-caspase inhibitor Z-VAD-FMK (10μM) before MBS treatment. Z-VAD-FMK pretreatment significantly decreased the expression of cleaved caspase-3, cleaved PARP and reduced MBS-induced apoptosis (Figure 3D, E). These data indicated that mitochondria-mediated caspase cascade pathway plays a key role in MBS-induced apoptosis.

**MBS induces sustained activation of JNK and p38 in SW620 cells**

Mitogen-activated protein kinases (MAPK) signaling pathway was involved in several events of cellular stress-induced cell apoptosis (Xia et al., 1995; Tournier et al., 2000). Hence, we examined the activation of ERK, JNK, p38 after MBS treatment. As shown in (Figure 4), the phosphorylation levels of ERK, JNK and p38 were apparently increased in response to the MBS treatment for 2h, and the phosphorylation levels showed a dose-dependent manner. Besides, the phosphorylation levels of JNK and p38 lasted for 24h. These results demonstrated that sustained activation of JNK and p38 may be involved in MBS-induced apoptosis in SW620 cells.

**The JNK and p38 signaling pathway are involved in MBS-induced apoptosis in SW620 cells**

In order to verify whether the MBS-induced sustained activation of JNK and p38 signaling pathways play a critical role in apoptosis, we have pretreated SW620 cells for 1h individually with specific inhibitors for JNK (SP600125) and p38 (SB203580) followed by MBS for 24h. As shown in (Figure 5A, B), western blot assay revealed that SP600125 (a specific inhibitor of JNK) inhibited JNK activation and cleavage of caspase-3, PARP in MBS treatment. Western blot analysis also demonstrated that SB203580 (a specific inhibitor of p38) inhibited cleavage of caspase-3 and PARP, but did not decrease the phosphorylation level of p38 (Figure 6A, B). In addition, JNK and p38 inhibitors (SP600125 and SB203580) reduced MBS-induced apoptosis in SW620 cells (Figure 5C, 6C). We also found that the pan-caspase inhibitor Z-VAD-FMK had no effect on MBS-induced activation of JNK and p38 (Figure 5D, 6D). These results showed that MBS-induced apoptosis in SW620 cells is mediated by the sustained activation of JNK and p38 signaling pathway.

**Discussion**

Shikonin and its derivatives are the active pigments extracted from the traditional Chinese medicine Lithospermum erythrorhizon (Wang et al., 2012). These compounds are promising anticancer agents as they have been reported to possess multiple anticancer effects in vivo and in vitro (Andújar et al., 2013). Among the shikonin derivatives, MBS shows a strong anticancer effect compared to other compounds (Xuan et al., 2009). In this study, we investigated the effects of MBS on human colorectal cancer SW620 cells. Our results indicated that treatment of SW620 cells with MBS showed a significant cytotoxic effect against SW620 cells in a dose and time dependent manner, and MBS induced apoptosis in SW620 cells was evidenced by an increased early apoptotic cells (Annexin V+/PI-).

The ratio of anti- and pro-apoptotic members is crucial for the induction of apoptosis (Cory et al., 2002). Mitochondria plays a critical role in signal transduction of apoptosis (Tait et al., 2010). The translocalization of apoptotic proteins from the cytoplasm to the mitochondria membrane leads to cytochrome c release and second mitochondria-derived activator of caspases by a loss of mitochondrial membrane potential (Shimizu et al., 1999). Our previous findings have demonstrated that β,β-dimethylacrylshikonin induces apoptosis via mitochondria pathway in SGC-7901 cells (Shen et al., 2012). In the present study, we showed that MBS down-regulated the expression of Bel-2 and up-regulated the expression of Bak, and caused the loss of mitochondrial membrane potential in SW620 cells, consistent with activation of the mitochondria-dependent apoptosis. The observation of MBS mediated activation of caspase-9, caspase-3, and subsequent cleavage of PARP, as well as the result that the pan-caspase inhibitor Z-VAD-FMK reduced MBS-induced apoptosis in SW620 cells, suggesting that MBS-mediated caspase cascade pathway plays an essential role in MBS-induced apoptosis. Taken together, our results indicated that MBS regulated expression of apoptosis-related proteins, caused the loss of mitochondrial membrane potential and triggered caspase-dependent cell apoptotic death.

MAPK has been shown to participate in a diverse array of cellular programs, including embryogenesis, proliferation, differentiation and apoptosis (Raman et al., 2007). The MAPK family members consist of three protein kinases: ERK1/2, JNK and p38 (Baines et al., 2005). In most cases, p38 is simultaneously activated with these earlier reports, we found that the sustained activation of JNK and p38 contributes to apoptosis. Hu et al showed that bufalin induced growth inhibition and apoptosis in hepatocellular carcinoma cells via JNK activation (Hu et al., 2014). Li et al also found that glaucocalyxin A induced apoptosis through activation of the JNK pathway in human breast cancer cells (Li et al., 2013). Avisetti et al demonstrated that activation of p38/ JNK pathway played a critical role in embelin-induced apoptosis of lung cancer cells (Avisetti et al., 2014). Recently, Hsieh et al reported that arctigenin induced apoptosis of breast cancer MDA-MB-231 cells through the ROS/p38 pathway (Hsieh et al., 2014). In accordance with these earlier reports, we found that the sustained activation of JNK and p38 were involved in MBS-induced growth inhibition and apoptosis in SW620 cells. Both of JNK and p38 inhibitors (SP600125 and SB203580) could attenuate MBS-induced apoptosis. The JNK and p38 inhibitors (SP600125 and SB203580) specifically inhibit JNK and p38 catalytic activity respectively by binding to the ATP-binding pocket preventing the activation of downstream factors, but p38 inhibitor SB203580 does not result in the decreased phosphorylation levels of p38 (Kayali et al., 2000; Bennett et al., 2001). Here, we also found that SP600125 (a specific inhibitor of JNK) inhibited JNK activation, but SB203580 (a specific inhibitor of p38) had no effect on p38 activation. The above results indicated
that the pro-apoptotic effect of MBS in SW620 cells was mediated by the activation of JNK and p38 signaling pathway.

In conclusion, we presented experimental evidence that strongly supports the antitumor effects of MBS on colorectal cancer cells. Thus, we believed that MBS could be developed as a potential antitumor agent for the activation of JNK/p38 signaling pathway, resulting in the inhibition of colorectal cancer cell growth and induction of apoptosis. It is possible to provide a new therapeutic strategy for the treatment of colorectal cancer in the foreseeable future. However, further studies are warranted to fully uncover the molecular mechanism of action of MBS in colorectal cancer models in vitro and in vivo.

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References


