Biochemical and Biophysical Research Communications 601 (2022) 59–64

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anticancer activity of Neosetophomone B by targeting AKT/SKP2/MTH1 axis in leukemic cells

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ARTICLE INFO

Article history:
Received 16 February 2022
Received in revised form
17 February 2022
Accepted 18 February 2022
Available online 22 February 2022

Keywords:
Natural products
Meroterpenoids
Fungal secondary metabolites
Neosetophomone B
Apoptosis
Leukemia

ABSTRACT

Neosetophomone B (NSP–B), a meroterpenoid fungal secondary metabolite, was investigated for its anticancer potential in leukemic cell lines (K562 and U937). NSP-B treatment of leukemic cells suppressed cell viability by triggering apoptotic cell death. Apoptosis induced by NSP-B is triggered by mitochondrial signaling and caspase activation. Additionally, NSP-B treatment of leukemic cells causes AKT's inactivation accompanied by downregulation of SKP2 oncogene and MTH1 with a concomitant increase of p21Cip1and p27Kip1. Furthermore, NSP-B causes suppression of antiapoptotic proteins, including cIAP1, cIAP2, XIAP, survivin and BCl-XL. Overall, NSP-B reduces cell viability by mitochondrial and caspase-dependent apoptosis. The inhibition of AKT and SKP2 axis could be a promising therapeutic target for leukemia treatment.

1. Introduction

Meroterpenoids are natural products with mixed biosynthetic origins [1]. They are partially derived from terpenoid biosynthetic pathways [2]. They have been isolated from fungi, marine organisms, animals, and plants and display remarkable structural diversity [3]. Meroterpenoids exhibit various biological properties, including anticancer, anti-inflammatory, antioxidant, and antibacterial activities [1]. Their chemical structural diversity and complexity, potential bioactivities, and pharmacological effects make them attractive targets for chemists and pharmacologists [1–3]. Neosetophomone B (NSP–B), a meroterpenoid fungal secondary metabolite, was isolated along with five structurally related ones from an undescribed *Neosetophoma* sp. (strain MSX50044) [4]. The cytotoxic effect of NSP-B at micromolar concentrations has been reported in breast and ovarian cancer cell lines [4]. However, the mechanism of NSP-B mediated cytotoxicity is not known.

The proteasome is a multi-catalytic complex consisting of two major regulators, PA28 and PA700, forming 26S proteasome [5]. The 26 S proteasome is responsible for the degradation of many polypeptides critical for cell death signaling [6]. These include tumor suppressors (p53, p21), cyclins, nuclear factor kappa B (NF-kB), and mitosis-regulating proteins [7,8]. The oncogenic F-box protein, S-phase kinase protein-2 (SKP2), regulates the expression of cell cycle inhibitor protein, p27Kip1, via associating with ubiquitin E3 ligase complex [9,10]. A high level of SKP2 expression with a

Abbreviations: NSP-B, Neosetophomone B; SKP2, S-phase kinase protein-2; IAPs, inhibitors of apoptosis proteins; PARP, poly-ADP ribose polymerase.

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https://doi.org/10.1016/j.bbrc.2022.02.071

concomitant low level of p27Kip1 is observed in the G1-S phase during cell cycle progression [9]. Deregulated expression of SKP2 in many cancer cells has been associated with genetic alterations and oncogenic signaling pathways, including Jak/Stat and PI3K/AKT s [11,12]. For example, the PI3K/AKT/Sp1 pathway is involved in BCR-ABL induced expression of SKP2 [12]. PI3-kinase/mTORC2 signaling regulates the SKP2/P27Kip1 axis in many human cancer cells [13].

The study investigated whether NSP-B can inhibit cell proliferation due to the arrest of cell cycle progression or apoptosis in leukemic cells. Next, we determined whether NSP-B mediated apoptosis is associated with targeting the SKP2/AKT/MTH1 axis. In addition, we determined whether NSP-B induced apoptosis is mediated by caspase-cascade signaling.

2. Materials and methods

2.1. Isolation of NSP-B from fungi

NSP-B (Fig. 1A) was isolated from *Neosetophoma* sp. [strain MSX50044] as reported by El-Elimat et al. (2019) [4]. The purity of NSP-B was evaluated by UPLC and found to be >97% [4,14].

2.2. Reagents and antibodies

CCK-8, methanol, dimethylsulfoxide (DMSO), Syber green, and Agarose were obtained from Sigma-Aldrich (St. Louis, MO, USA). AllPrep DNA/RNA Mini Kit was purchased from Qiagen (Hilden, Germany). Antibodies against caspase-9, caspase-3, cleaved caspase-3, PARP, Bcl2, Bid, cleaved caspase-8, p-AKT, AKT, XIAP, CIAP1, CIAP2, Survivin, SKP2, p21Cip1, P27Kip1, MTH1, GAPDH were obtained from Cell Signaling Technologies (Beverly, MA, USA). p-H2AX, HSP60, BCL-XL, and BAX were obtained from Santa Cruz Biotechnology, Inc., (CA, USA). FITC Annexin V apoptosis detection kit I, Apo-Direct kit, Fixation/Permeabilization solution kit, BD MitoScreen (JC-1), BV421mouse anti-gH2AX (pS139), PE rabbit anti-active caspase-3, and Alexa Fluor 700 mouse anti-cleaved PARP (Asp214) antibodies were purchased from BD Biosciences (San Jose, USA). RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin, and live and dead assay kit (Life Technologies, Inc., Carlsbad, CA).

2.3. Cell culture

K562 and U937 were procured from the American Type Culture Collection (Manassas, Virginia, US). Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100U/ml penicillin, 100U/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 as described previously [15]. Peripheral blood mononuclear cells (PBMCs) were extracted from fresh peripheral blood samples of healthy volunteers with written informed consent and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 incubator.

2.4. Cell viability assay

Cell viability was measured using a cell counting kit-8 reagent, a

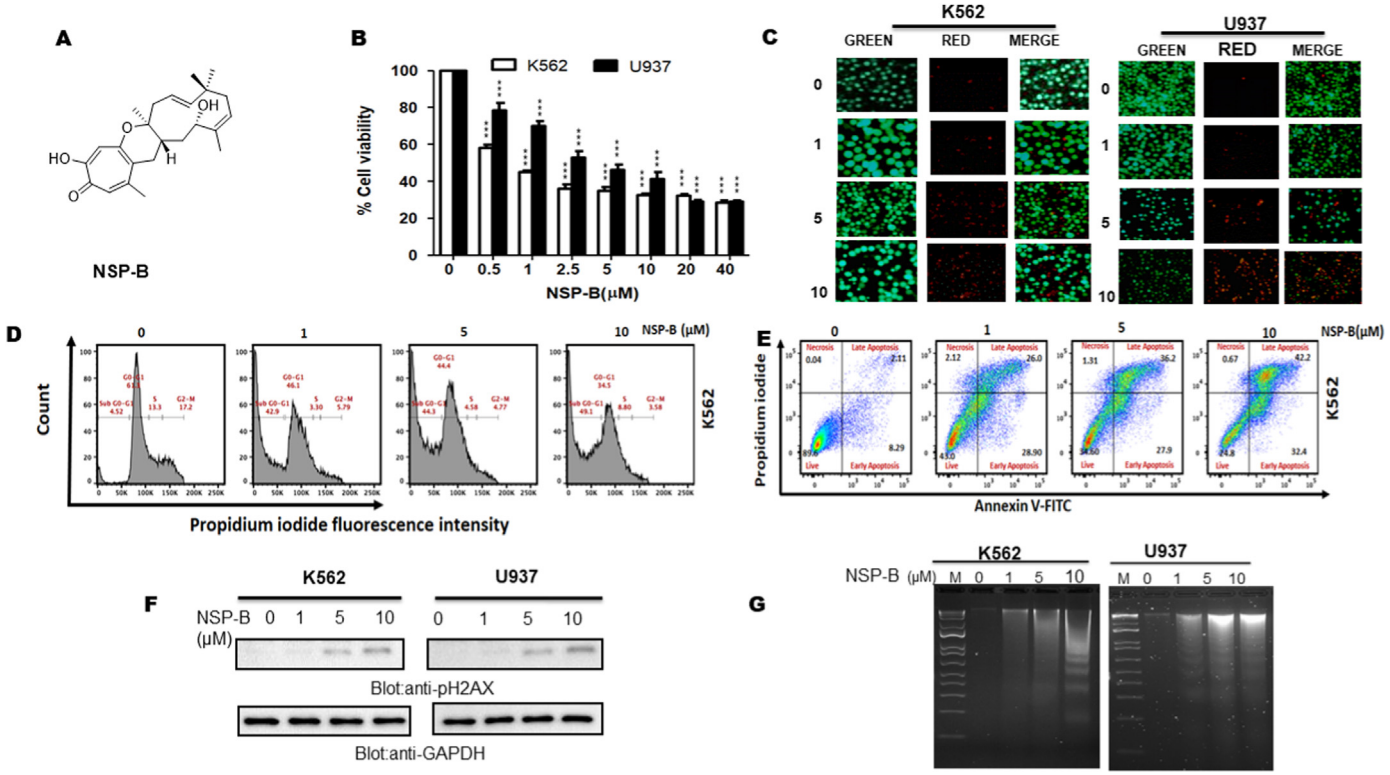

Fig. 1. Effect of NSP-B on the cell viability of Leukemic cell lines. (A) Chemical structure of NSP-B. **(B)** NSP-B inhibits the cell viability of leukemic cells. K562 and U937 cells were incubated with the indicated concentrations of NSP-B for 48 h. Cell viability assays were performed using CCK-8 as described in Materials and Methods. The graph displays the mean ± SD (standard deviation) of three independent experiments. *p < 0.05, **p < 0.01,***p < 0.001. (**C**) NSP-B induces cell death in Leukemic cell lines. K562 and U937 cells were treated with 1, 5, and 10 μM of NSP-B for 48 h The cells were subsequently stained with live and dead reagent and visualized under a fluorescent microscope. (**D**) Effect of NSP-B on cell cycle progression. K562 cells were treated with NSP-B, and cell cycle fractions were determined with flow cytometry as described in the method and materials. (**E**) NSP-B mediated apoptosis in leukemic cells. K562 cells were treated with NSP-B followed by staining with fluorescein-conjugated annexin-V/PI, and apoptotic cells were determined by flow cytometry. (**F**) NSP-B mediated phosphorylation of H2AX and DNA fragmentation in leukemic cells. K562 and U937 cells were treated with NSP-B, and pH2AX level was determined by Western blotting using antibodies against p-H2AX and HSP60. (**G**) NSP-B mediated DNA fragmentation in leukemic cells. K562 and U937 cell lines were treated with increasing doses of NSP-B for 48 h, and DNA was extracted and separated by electrophoresis.

colorimetric assay described earlier [16].

2.5. Cell cycle

K562 and U937 were treated with NSP-B for 48 h. After incubation, cells were stained with Hoechst 33342 and data acquired on flow cytometer BD LSRFortessa cell analyzer as described previously [17].

2.6. Annexin V/Propidium Iodide dual staining

NSP-B treated K562 cells were washed with PBS and subsequently stained with fluorescein-conjugated annexin-V antibody and propidium as described previously [16].

2.7. Live/dead assay

K562 and U937 were treated with increasing doses of NSP-B for 48 h. Live/dead stain was prepared as per manufacturer's protocol, and cells were then stained for 20 min. EVOS FLoid Cell Imaging System from Invitrogen (Thermo Fisher Scientific) was used to view the cells and images were captured at 20X magnification [18].

2.8. Cell lysis and immunoblotting

K562 and U937 cells were treated with NSP-B for 48 h, and after incubation, the lysate was prepared as described earlier [16]. An aliquot of 50 μg of proteins was loaded and run the Western blotting and transferred the gel into polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Billerica, MA, United States). Immunoblotting was performed using different antibodies, and the status of protein was visualized by ChemiDoc System (Amersham, Bio-Rad, United States).

2.9. DNA laddering

DNA was isolated from NSP-B treated and untreated cells using all prep DNA/RNA mini kit according to the manufacturer's protocol [19]. Isolated DNAs were loaded on agarose gel electrophoresis. DNA laddering images were obtained using a ChemiDOC imaging system.

2.10. Measurement of mitochondrial membrane potential

K562 and U937 cell lines were treated with increasing doses of NSP-B and incubated at 37^0C for 48 h. MMPs were determined by staining with 7-aminoactinomycin D (7-AAD) according to the manufacturer's protocol. Muse Cell Analyzer (Merck Millipore, USA) was used to measure the mitochondrial membrane potential [20].

2.11. Statistical analysis

GraphPad Prism software (version 5.0 for Windows, GraphPad Software Inc., San Diego, CA, http://www.graphpad.com) was used for the statistical analysis of the data. Data are shown as the mean ± S.D. Values of *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 are statistically significant.

3. Results

3.1. NSP-B inhibits cell proliferation of leukemic cells

We investigated whether NSP-B treatment can suppress the cell viability of K562 and U937 cells. Leukemic cells were treated with 0, 0.5, 1, 2.5, 5, 10, 20, and 40 μM of NSP-B for 48 h. Following the completion of treatment, Cell Counting Kit-8 (CCK-8) was used to determine cell viability. We observed a dose-dependent decrease in cell growth in both cell lines (Fig. 1 B). However, NSP-B treatment did not affect the cell viability of control normal PBMCs (**Supplementary Fig. 1A**). The growth inhibition by NSP-B was statistically significant at most of the doses used. Next, we determined whether inhibition of cell viability was due to cell death, and to measure this parameter, live/dead assays were performed. K562 and U937 cells were treated with NSP-B and stained with live and dead assay kit as described in methods. As shown in Fig. 1C, there was an increase in dead cells in a dose-dependent manner in response to NSP-B treatment.

3.2. NSP-B induces apoptosis in leukemic cells

As shown in Fig. 1D, the sub-G1 population, a marker of apoptosis, increased from 4.52% in control to 42.9.87%, 44.3, and 49.1 at 1, 5, and 10 μM of NSP-B, respectively in K562 cells. A similar result was observed in U937 cells (data not shown). The increase in the sub-G1 population indicating apoptotic cell death was further confirmed by annexin V-FITC/PI staining.

As shown in Fig. 1E, the untreated cells exhibited a lower percentage of cells in early and late apoptosis; however, with NSP-B treatment, a dose-dependent increase in the percentage of early and late apoptosis was seen in K562 cells.

A dose-dependent increase in the expression of p-H2AX (a marker of double-stranded breaks) was also seen in NSP-B treated leukemic cells (Fig. 1 F). Finally, we analyzed DNA fragmentation, which is another hallmark of apoptosis. K562 and U937 cells were treated with increasing doses of NSP-B, and DNA was isolated using AllPrep DNA/RNA Mini Kit (Qiagen). As shown in Fig. 1G, dose-dependent DNA fragmentation was seen in K562 and U937 cells.

3.3. NSP-B mediated cell death involves mitochondrial apoptotic pathways in leukemic cells

We sought to determine whether NSP-B mediated apoptosis involves the activation of caspase-8 signaling, and we observed that NSP-B treatment of K562 and U937 cells activated caspase-8, leading to the truncation of Bid as indicated by a decrease in full-length Bid band (Fig. 2A). In addition, NSP-B treatment caused upregulation of proapoptotic Bax and downregulation of anti-apoptotic protein Bcl2 (Fig. 2A). The increased Bax/Bcl2 ratio enhances the pore formation in the mitochondrial membrane, causing cytochrome *c* release into the cytosol. We then sought to determine the effects of NSP-B on mitochondrial membrane potential and to perform this experiment, K562 and U937 cells were treated with NSP-B for 48 h, labeled with Mitopotential dye, and measured using Muse cell Analyzer. Loss of mitochondrial membrane potential was seen after NSP-B treatment in both cell lines (Fig. 2B). To test whether NSP-B -mediated apoptosis caused a release of cytochrome *c* from mitochondria, K562 and U937 cells were treated with NSP-B. Cell lysates were used for cytochrome *c* expression. NSP-B treatment increased cytochrome *c* expression in a dose-dependent manner (Fig. 2C), suggesting NSP-B-induces the release of cytochrome *c* by increasing the permeabilization of the outer mitochondrial membrane. We then sought to investigate whether NSP-B induced release of cytochrome *c* could activate caspase-cascade signaling. Interestingly, activated caspase-9 and -3 and the cleaved PARP were observed in leukemic cell lines when treated with NSP-B (Fig. 2D). These results show that the release of cytochrome *c* and activation of caspases involve NSP-B mediated apoptosis. This was confirmed by using z-VAD-fmk, a universal inhibitor of caspases, which abrogated activation of cleaved caspase-3 induced by NSP-B (Fig. 2E).

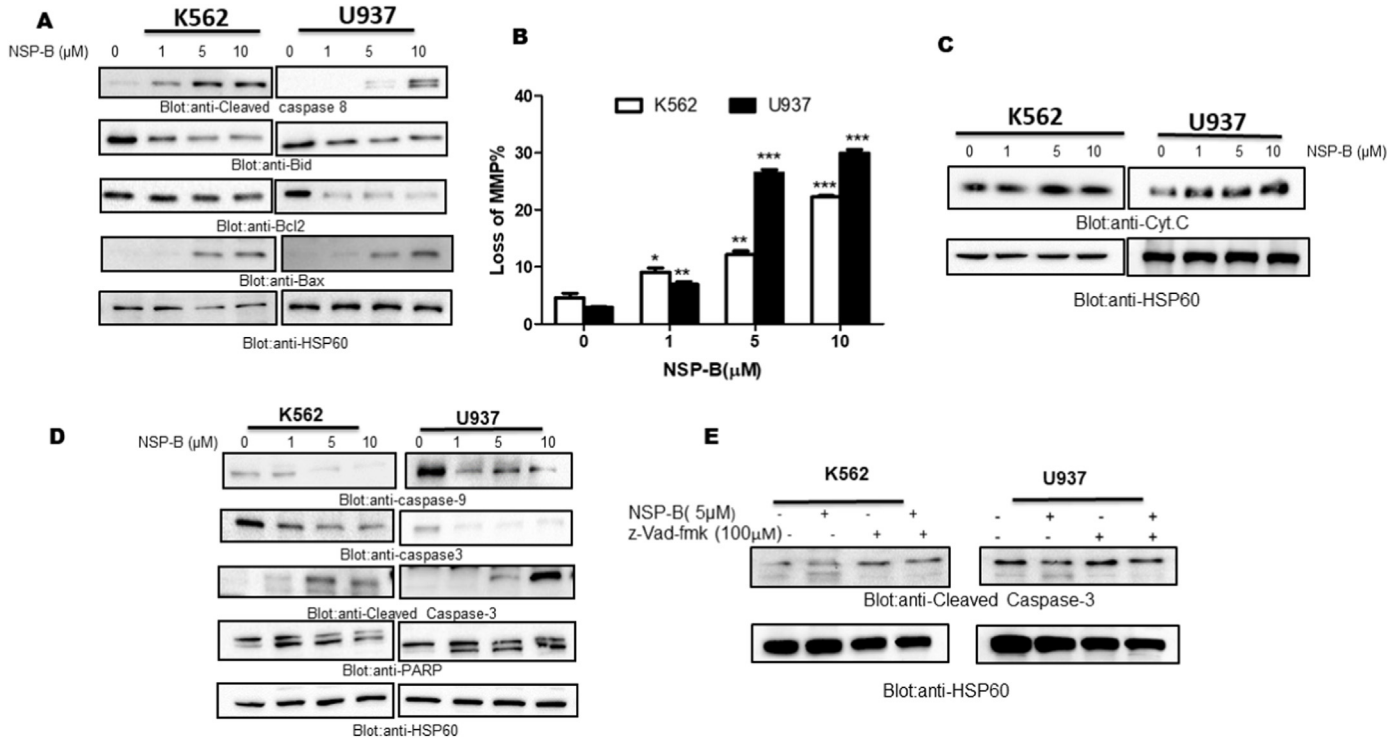

Fig. 2. NSP-B-induced mitochondrial signaling pathways in leukemic cell lines. (A) Effect of NSP-B on caspase-8/BID/Bcl2 and Bax expression in leukemic cell lines. K562 and U937 cells were treated with NSP-B as indicated, and 50 μg of protein was separated by SDS-PAGE and immuno-blotted with antibodies against cleaved caspase-8, BID, BCl2, and HSP60 as indicated. (**B**) NSP-B mediated loss of mitochondrial membrane potential in leukemic cells. K562 and U937 cells were treated with indicated doses of NSP-B for 48 h. Mitochondrial membrane potential was determined by staining with mitochondrial membrane potential reagent as described in the materials and methods section and analyzed. The graph displays the mean ± SD of three independents of experiments. *p < 0.05, **p < 0.01,***p < 0.001. (**C**) The NSP-B-induced release of cytochrome *c*. K562 and U937 cells were treated with NSP-B, and proteins were separated on SDS-PAGE and immunoblotted with antibodies against cytochrome *c* and HSP60. (**D**) NSP-B *mediates caspase activation in leukemic cells.* K562 and U937 cells were treated with NSP-B, and proteins were separated on SDS-PAGE and immunoblotted with antibodies against caspase-9, caspase-3, cleaved caspase-3, PARP, and HSP60. **(E)** Effect of z-VAD-FMK on NSP-B induced apoptosis. K562 and U937 cells were pretreated with z-VAD-FMK for 1 h, followed by NSP-B treatment, and analyzed by western blot for antibodies against cleaved caspase-3 and HSP60.

3.4. NSP-B inhibited AKT/PKB and suppressed SKP2 and MTH1 expression in leukemic cell lines

To determine whether NSP-B inhibits the constitutive phosphorylation/activation of the AKT/PKB pathway, K562 and U937 cells were treated with NSP-B at different doses (0,1,5, and 10uM). We observed that NSP-B caused de-phosphorylation of AKT at Ser^{473} in both cell lines without affecting the AKT expression (Fig. 3A). Similar effects of NSP-B on p-AKT were seen in serum-starved leukemic cells (**Supplementary Fig. 1B**). In addition, NSP-

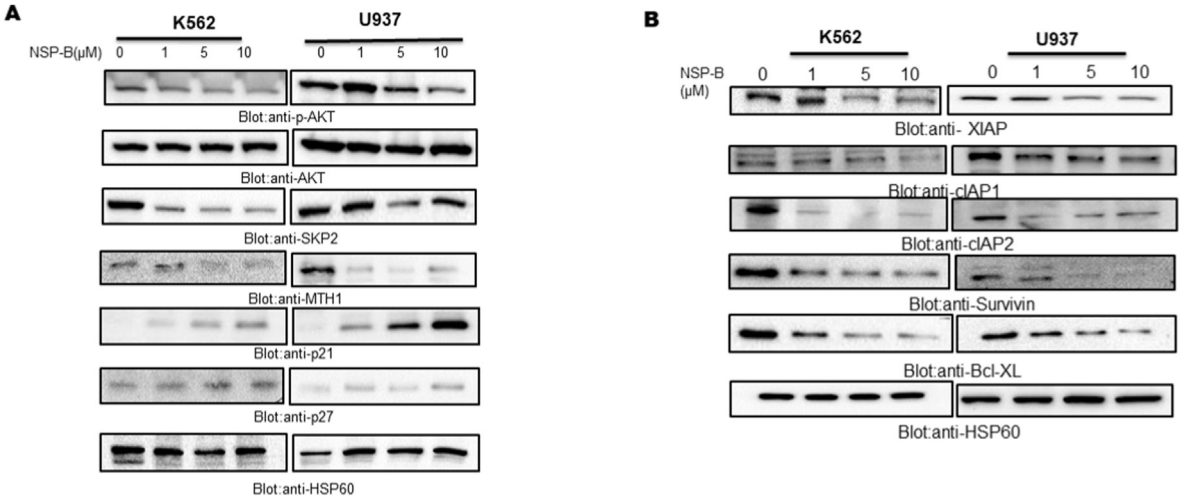

Fig. 3. NSP-B inhibits activated AKT/PKB, induces downregulation of SKP2, MTH1, and antiapoptotic proteins in leukemic cell lines. (**A**) NSP-B treatment caused the inactivation of AKT and down-regulated the expression of SKP2 MTH1 and enhanced the expression levels of P27 and P21. K562 and U937 cells were treated with various doses of NSP-B, and equal amounts of proteins were immuno-blotted with antibodies against p-AKT, AKT, SKP2, MTH1,p27, and p21 as indicated(**B)** NSP-B-mediated downregulation of antiapoptotic proteins. . K562 and U937 cells were treated various doses of NSP-B. After cell lysis, equal amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against XIAP, c-IAP1, c-IAP2, Survivin, Bcl-XL, and HSP60 as indicated.

B suppressed the expression of SKP2 and MTH1 but increased the expression of p21Cip1 and p27/kip1 (Fig. 3A). Finally, we tested the expression of inhibitors of apoptosis protein (IAP)in K562 and U937 cells after NSP-B treatment. As shown in Fig. 3B, NSP-B treatment caused downregulation of XIAP, cIAP1,cIAP2, Bcl-XL, and survivin protein. These results indicate that inhibiting IAP proteins could be one of the mechanisms in NSP-B -induced apoptosis.

4. Discussion

Fungi represent an invaluable source of natural products for drug discovery and development [21–23]. Several natural products derived from fungi have demonstrated anticancer properties against cancer cell lines of different types [17,24]. NSP-B, a fungal meroterpenoid secondary metabolite isolated from *Neosetophoma* sp., was reported to possess anticancer activity against a panel of cancer cell lines [4]. Our findings showed that NSP-B causes efficient apoptotic cell death in leukemic cells. Programmed cell death or apoptosis is an important physiological function that helps remove damaged cells [25]. This can happen in two ways: the extrinsic or receptor-mediated and the Intrinsic or mitochondrial-mediated [26]. NSP-B mediated its apoptotic effects in leukemic cells via the Bax/Bcl2 ratio enhancement that leads to the loss of mitochondrial membrane potential and the release of cytochrome C in the cytosol. In the cytosol, cytochrome *c* activates caspase-cascade by forming an apoptosome complex which then converts inactive procaspase 9 to active caspase 9 that relays the death signal for cleavage of PARP via caspase-3 activation. Activated PARP is the hallmark of apoptosis that eventually results in DNA fragmentation and cell death [27]. Our results showed that NSP-B activated the intrinsic caspase-mediated apoptosis pathway by triggering caspase-3 and PARP cleavage in a dose-dependent manner. Furthermore, NSP-B treatment caused the activation of H2AX, a marker of DNA double-stranded break, which can also result in the degradation of DNA, thus inititating apoptosis [28].

The proteasome signaling pathway is involved cell cycle progression and apoptosis [29]. SKP2 is a well-studied F-box protein that promotes cancer cell growth, thus acting as an oncogene [30]. SKP2 overexpression and elevated AKT activity were observed in many types of cancer [31]. The activated AKT induces cytosolic localization of SKP2, and its inhibition prevents the movement of SKP2 into the cytosol [32]. Our data showed that the NSP-B treatment of leukemic cells dephosphorylated AKT, accompanied by downregulation of SKP2 and MTH1 with concomitant accumulation of p27 and p21. These data demonstrate that NSP-B targets AKT/SKP2/MTH1 axis for its anticancer activity in leukemic cells.

In summary, our results showed that NSP-B inhibits the growth of leukemic cells via inhibition of AKT and SKP2 signaling. NSP-B induces cell death by triggering an intrinsic apoptosis signaling pathway. These findings may aid in developing new, less toxic cancer chemotherapies based on natural compounds and highlight the potential benefits of generating anticancer medicines that target the AKT and SKP2 proteins in leukemia.

Declaration of competing interest

Authors do not have any conflicts of interest.

Acknowledgment

This work was supported by grant funded by the Medical Research Center (MRC), Hamad Medical Corporation, Doha, Qatar (MRC-01-21-301). The authors thank Qatar National Library for open access support of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.02.071.

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