

SVP-B5 peptide from *Buthus martensii* Karsch scorpion venom exerts hyperproliferative effects on irradiated hematopoietic cells

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Abstract. Previous studies have demonstrated the radioprotective efficacy of scorpion venom peptide, fraction II (SVPII) from the venom of *Buthus martensii* Karsch. In the present study, the SVP-B5 polypeptide, which is one of the active components of SVPII, was purified using a two-step chromatographic process. SVP-B5 significantly promoted the proliferation of irradiated M-NFS-60 mouse-derived myelocytic leukemia cells. In addition, SVP-B5 effectively and persistently promoted hematopoietic recovery and expansion of hematopoietic cells after irradiation as demonstrated by cobblestone area forming cell and long-term bone marrow culture assays. Treatment of M-NFS-60 cells with SVP-B5 upregulated the expression of interleukin 3 receptor and activated the Janus kinase-2/signal transducer and activator of transcription 5 signaling pathway. In conclusion, the present study demonstrated that SVP-B5 has growth factor-like properties and may be used as a therapeutic modality in the recovery of severe myelosuppression, which is a common side effect of radiotherapy.

Introduction

Radiation therapy combined with chemotherapy is the standard of care for several types of malignancies, including carcinoma.

These therapies directly target the bone marrow niche, which are the predominant site of hematopoiesis and the differentiation of blood cells (1,2). Myelosuppression and hematopoietic dysfunction are the most common clinical complications for patients receiving chemo- and radiotherapy. These insults damage hematopoiesis by targeting either rapidly proliferating hematopoietic stem cells (HSCs) or the microenvironment or both (1,2). Therefore, it becomes pertinent to promote the recovery of hematopoiesis from myelosuppression. Different cytokines and growth factors have been studied and are clinically in use to combat myelosuppression. The recovery of hematopoiesis relies on the proliferation and differentiation of undamaged HSCs and recovery of the microenvironment (3). Researchers are constantly in search of therapeutic agents, which effectively relieve radiation damage and restore the hematopoietic functions of bone marrow.

A broad range of bioactive peptides have already been purified and characterized from scorpion venoms, with the total number estimated to approach 100,000, among which only 1% is fully known (4). These scorpion venom polypeptides (SVPs) have been demonstrated to have a diverse array of biological activities with high specificities to their targeted sites, including anti-tumor, anti-epileptic, analgesic and ion channel blocking properties (5-9). Our group has focused on the SVP and SVPII from the *Buthus martensii* scorpion venom (10-12). A recent study by our group identified SVP-B5, a novel peptide from the SVPII, which mitigates radiation-induced DNA damage and improves the survival rate through the reactive oxygen species-p16/p21 pathway (10). In another study by our group, hematopoietic growth factor-like effects of SVPII were reported (12). The present study demonstrated that the principal component of SVPII, namely SVP-B5, promotes the proliferation of irradiated hematopoietic cells. It therefore has the potential to be considered as a therapeutic modality in radiation therapy for the recovery of hematopoiesis; however, this warrants further study.

Materials and methods

Purification of SVP-B5. A two-step chromatography method was used to purify the SVP-B5 peptide from the crude

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scorpion venom (SVC; Zhengzhou scorpion farm; Zhengzhou, China). In the first step, SVC was passed through a Sephadex G-50 chromatography column (55x500 mm). The elutions obtained were then further analyzed by a CM Sepharose FF ion exchange chromatography column (55x1,000 mm) and phosphate buffers (buffer A, 0.05 M Na₂HPO₄-NaH₂PO₄, pH 6.4; buffer B: Buffer A supplemented with 0.3 M NaCl) using a linear gradient at a flow rate of 2 ml/min. The fractions with the peptide were concentrated and desalinated using a Vivaflow 50 membrane package and dried to a powder in a vacuum freeze-dryer. The purity of each SVP component was further analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC; Inertsil ODS-3 C18 chromatographic column, 4.6x250 mm). The chromatographic conditions were as follows: Mobile phase A, 0.1% trifluoroacetic acid in water; mobile phase B, 0.1% trifluoroacetic acid in 70% acetonitrile in water at a flow rate of 0.5 ml/min. The composite gradient of mobile phase B developed from 0 to 100% in 60 min.

Cell culture. The M-NFS-60 cell line (CRL-1838™) was purchased from the American Type Culture Collection (Manassas, VA, USA) and was maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS; both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin, 100 U/ml streptomycin, 5.958 g/l hydroxyethyl piperazineethanesulfonic acid, and 62 µg/l recombinant human macrophage colony-stimulating factor (rhM-CSF; cat. no. 300-25; PeproTech Inc., Rocky Hill, NJ, USA). The M-NFS-60 cell line was derived from a myelogenous leukemia induced with the Cas-Br-MuLV wild mouse ecotropic retrovirus. The cells are responsive to interleukin 3 (IL3) and macrophage (M)-CSF. FBMD-1 murine stromal cells at passage five were a kind gift from Dr Daohong Zhou (Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, Fayetteville, AR, USA) and maintained as described previously (13).

Isolation of BM-MNCs. Bone marrow mononuclear cells (BM-MNCs) were isolated aseptically from 8-10 week-old male C57BL/6 mice (18-22 g, n=24; Animal Center of Guangzhou Medical University, Guangzhou, China) as described previously (12,13). Mice were housed in specific-pathogen-free (SPF) conditions at 24-25°C, with ~70% humidity and a 14/10 h light/dark cycle, with access to food and water *ad libitum*. The mice were anesthetized with 2% isoflurane (Sigma Aldrich; Merck KGaA) in 98% oxygen prior to sacrifice. Harvested BM-MNCs were irradiated with X-rays (2 Gy at 300 MU/min). All animal experimental protocols were approved by the Research Ethics Committee of Guangzhou Medical University.

Cell proliferation assay. Cell proliferation was measured using a commercially available kit (CCK-8; Dojindo, Kumamoto, Japan). In brief, cells were seeded in a 96-well plate at a concentration of 5x10³ cells/100 µl/well in complete media without M-CSF and incubated with different concentrations of purified peptide fractions for 24 or 48 h. A total of 10 µl CCK-8 reagent was directly added to each well and the incubation was continued for an additional 3 h. The absorbance was measured at 450 nm.

Cobblestone area forming cell (CAFC) assay. The hematopoietic functions of hematopoietic stem cells (HSCs) and progenitors were analyzed by the cobblestone area-forming cell (CAFC) assay. The CAFC assay was performed as described previously (13). In brief, FBMD-1 feeder stromal cells were cultured in flat-bottomed 96-well plates at a density of 6x10⁵ cells per well for two weeks. BM-MNCs with different treatments (with/without SVP-B5, 1.0 µg/ml, with/without irradiation, 2 Gy) were then overlaid on the feeder stromal cell layer. Cultures were fed weekly by changing 50% of the medium. The frequencies of CAFCs were determined at days 14 and 35. Wells were scored positive if at least one phase-dark hematopoietic clone (containing 5 or more cells) was seen. The frequency of CAFC was then calculated by using Poisson statistics as described previously (13).

Colony-forming cell (CFC) assay. Irradiated bone marrow cells were cultured with different concentrations of SVP-B5 (0.5 or 1.0 µg/ml) in MethoCult M3534 medium (cat. no. 03534; StemCell Technologies, Vancouver, BC, Canada) in 24-well plates at a cell density of 1x10⁵/ml for 7 days. After completion of incubation, colony-forming unit granulocyte and macrophage (CFU-GM) colonies were counted under the microscope. A mass consisting of >50 cells was defined as 1 CFU (10).

Long-term bone marrow culture (LTBMC). LTBMC was performed as described previously (13). A total of 2x10⁶/ml irradiated BM-MNCs in Iscove's modified Dulbecco's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 20% FBS were seeded in a 24-well plate and maintained for 14 days in the presence of purified scorpion venom peptide. Half the volume of the media was replaced with fresh media every week. The number of hemopoietic colonies (at least 1x10⁶ cells per colony) was counted at 7 and 14 days.

Expression of IL-3 receptor (IL-3R) and phosphorylation of Janus kinase 2 (JAK2) and STAT5. The effect of SVP-B5 on the expression of IL-3R was determined in M-NFS-60 cells by immunofluorescence using a laser scanning confocal microscope (Leica DM1 4000B; Leica Microsystems, Wetzlar, Germany) as described previously (12). The M-NFS-60 cells were treated with different concentrations of SVP-B5 for 24 and 48 h. Cells treated with 10 ng/ml Recombinant Human IL-3 (cat. no. AF-200-03; PeproTech Inc., Rocky Hill, NJ, USA) were used as a positive control. Furthermore, for western blot analysis, and membrane proteins were extracted with a ReadyPrep™ Protein Extraction kit (Membrane I) (cat. no. 1632088; Bio-Rad Laboratories, Inc., Hercules, CA, USA) to determine the expression of IL-3R. Protein concentrations were measured using Lowry's method. Total proteins (30 µg) extracted from cultured cells were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were subsequently blotted with antibodies against IL-3R (1:1,000; cat. no. sc-30007), GAPDH (1:1,000; cat. no. sc-365062; both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), JAK2 (1:1,000; cat. no. 3230), p-JAK2 (1:1,000; cat. no. 3771), STAT5 (1:1,000; cat. no. 9363; Cell Signaling Technology, Inc.), and p-STAT5 (1:1,000; cat. no. 9359; all from Cell Signaling Technology, Inc., Danvers,

MA, USA). Primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000; cat. nos. sc-2004 and sc-2005; Santa Cruz Biotechnology, Inc.) were each incubated for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Inc.) and exposed to X-ray films (Kodak, Tokyo, Japan).

Statistical analysis. Values are expressed as the mean $\pm$ standard deviation. One-way analysis of variance was used for statistical analysis, performed with SPSS software (version 15.0; SPSS, Inc., Chicago, IL, USA).

Results

Characterization of scorpion venom. Three different peaks, SVP I-III, were obtained in the first step (Fig. 1A). Furthermore, seven peaks (B1, B2, B3, B4, B5, B6, and B7) were obtained from SVP II using CM-Sephadex FF ion exchange column chromatography (Fig. 1B). These peaks were characterized using RP-HPLC (data not shown). Based on the area normalization method, B5 showed the highest purity (95%; retention time, 48.609 min) followed by B4 (retention time, 37.858 min). By contrast, components B3, B6 and B7 had numerous peaks and were relatively less pure (data not shown).

Effects of SVP-B4 and SVP-B5 on M-NFS-60 cell proliferation. Next, the effect of SVP-B5 and SVP-B4 on the proliferation of M-NFS-60 cells was determined. A significant increase in the cell proliferation was observed when cells were treated with SVPB5 for 24 and 48 h when compared with that in the control group (Fig. 2A and B). However, no significant difference in cell proliferation was observed when the cells were treated with SVP-B4. As SVPB4 had no significant effect on the proliferation of M-NFS-60 cells, this peptide was not used in the further experiments, while SVP-B5 was used at doses of 0.5 and 1 μ g/ml.

Effect of SVPB5 on CAFC and CFU-GM. CAFC is a colony-forming assay widely applied to assess the hematopoietic functions of HSCs. Irrespective of irradiation, a significant increase in the CAFC in mouse bone marrow cells was found when treated with 1.0 μ g/ml SVP-B5 for 14 days compared with untreated cells ($P < 0.05$; Fig. 3A). However, no statistically significant difference was found in the number of CAFCs at day 35 (Fig. 3B). Furthermore, the proportions of CFU-GMs in mouse bone marrow cells were determined. Irradiated BM-MNCs were used in this experiment. Similar to the CAFC results, a significant increase in CFU-GMs was observed in the SVP-B5-treated group compared with that in the untreated group (61.7 ± 1.4 vs. 50 ± 4.5 per 2×10^4 bone marrow monocytes; $P < 0.05$; Fig. 3C and D). These results indicated that SVP-B5 supports hematopoietic cell expansion.

Effect of SVPB5 on LTBM. LTBMCs were established with bone marrow cells. A significant increase in the number of hematopoietic colony-forming cells was observed at day 14 when cells were cultured in the presence of 0.5 μ g/ml SVPB5 compared with that in the control group. However, an

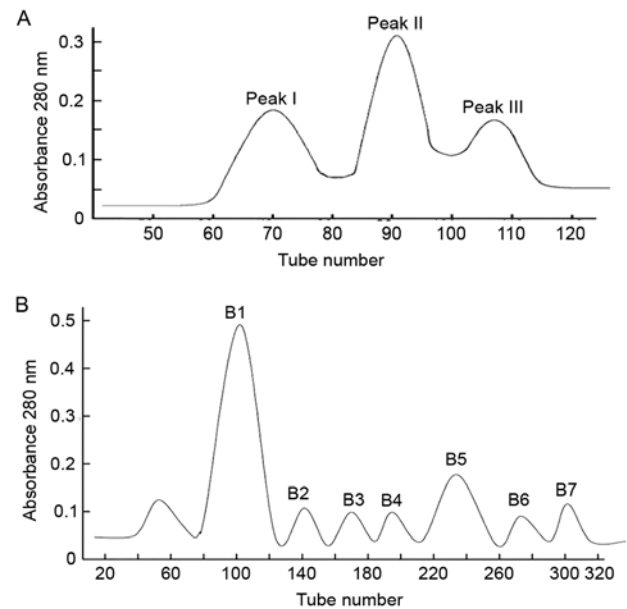


Figure 1. Isolation, purification and identification of scorpion venom. (A) Three protein peaks were identified in the scorpion venom crude extract (peaks I, II, and III) obtained Sephadex G-50 gel chromatography. (B) Seven peaks (B1-B7) were identified within peak II separated by CM-Sephadex FF ion chromatography. Products were further characterized by reverse-phase high-performance liquid chromatography.

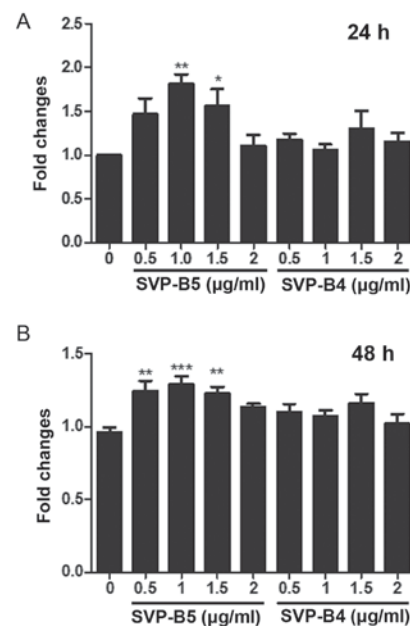


Figure 2. Effects SVP-B4 and SVP-B5 on cell proliferation. Irradiated M-NFS-60 cells treated with SVP-B4 and SVP-B5 for (A) 24 and (B) 48 h. The cell proliferation rate is expressed as the fold increase compared with the control group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ vs. untreated.

increased dose of SVPB5 (1 μ g/ml) did not cause any significant change in the number of hematopoietic colony-forming cells. Furthermore, 7 days of incubation was not sufficient to cause any change in the number of colonies at any dose of SVPB5 (Table I). These results demonstrated that SVP-B5 treatment at a low dose had a high stimulatory effect on the proliferative ability of these bone marrow cells at 14 days. In this experiment, BM-MNCs were irradiated.

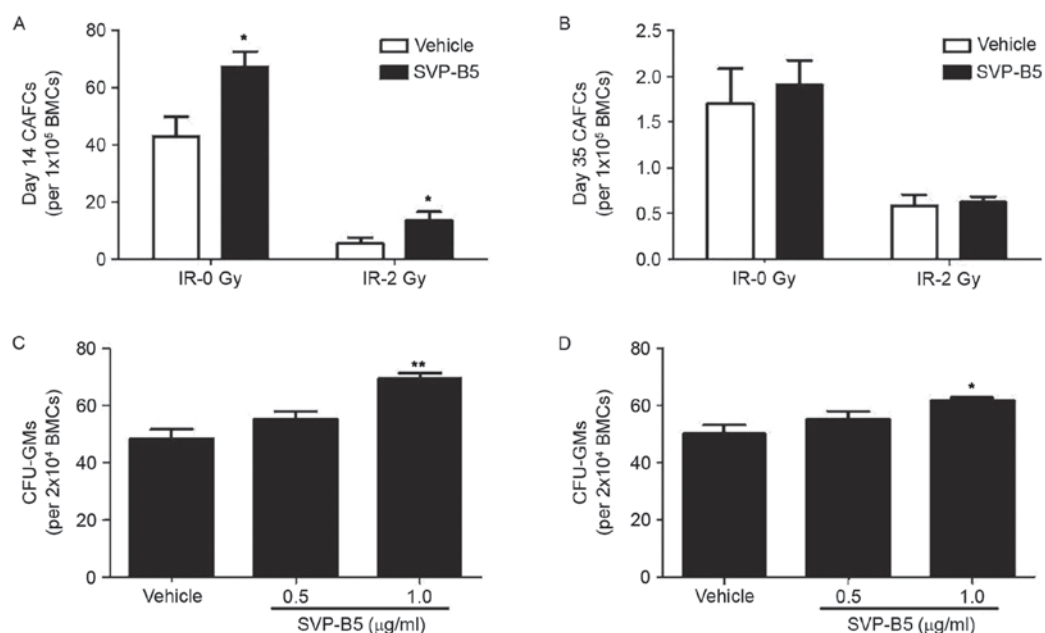


Figure 3. Effect of SVP-B5 on CAFCs and CFU-GMs. CAFC results at (A) 14 days and (B) at 35 days. CFU-GM results at (C) day 7 and (D) day 14. PBS was used as a vehicle in the control cells. * $P<0.05$ and ** $P<0.01$ vs. vehicle. CAFCs, cobblestone area forming cells; CFU-GMs, granulocyte-monocyte colony forming units; IR-0 Gy, not irradiated; IR-2 Gy, irradiated with X-rays at 2 Gy; BMCs, bone marrow cells.

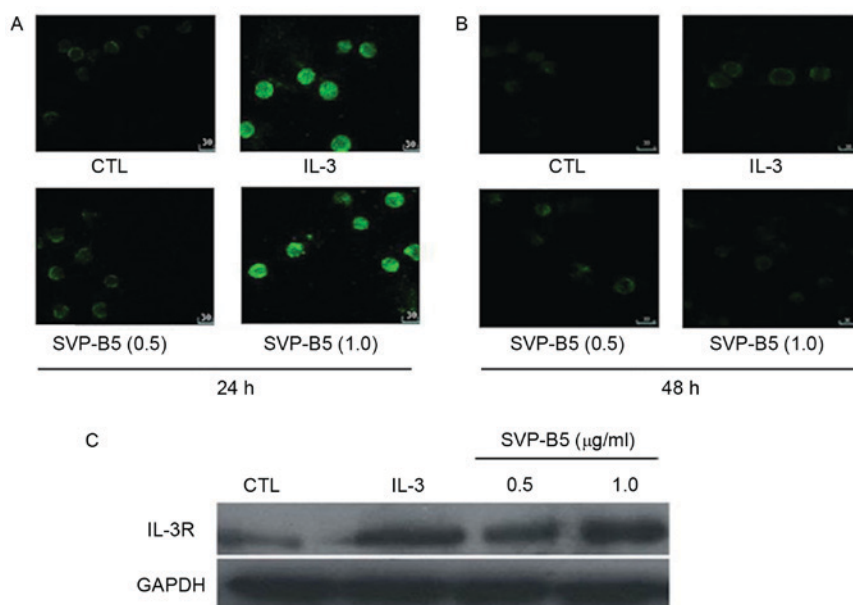


Figure 4. Effect of SVP-B5 on the expression of IL-3R. (A and B) M-NFS-60 cells were treated with SVP-B5 for (A) 24 and (B) 48 h and expression of IL-3R was observed by immunohistochemistry (magnification, x400). (C) Representative western blot showing the increased expression of IL-3R in M-NFS-60 cells treated with SVP-B5 for 24 h. GAPDH served as a loading control. Cells treated with IL-3 served as a positive control, and an untreated CTL group was also used. CTL, control; IL-3R, interleukin 3 receptor.

Effect of SVPB5 on IL-3R expression. SVP-B5 was found to dose-dependently increase in the expression of IL-3R in M-NFS-60 cells compared with that in the controls at 24 and 48 h, as determined by immunohistochemistry (Fig. 4A and B). Similar results were observed when the expression of IL-3R was determined by western blot analysis at 24 h (Fig. 4C).

Effect of SVPB5 on the protein expression and phosphorylation of JAK2 and STAT5. Next, the effect of SVP-B5 treatment on the activation of the JAK2/STAT5 pathway was determined.

It was observed that SVP-B5 increased the expression of p-JAK2 and p-STAT5 in M-NFS-60 cells in a time-dependent manner. However, the expression of total JAK2 and STAT5 remained unchanged with SVP-B5 treatment (Fig. 5).

Discussion

In search of novel therapeutic modalities, the use of natural products as well as purification and characterization of their active components have been pursued as a successful strategy

Table I. Effect of SVP-B5 on irradiated long-term bone marrow cell cultures.

Group	Number of colonies	
	7 days	14 days
IR-	16.00±1.73	9.00±1.00
IR ⁺	1.83±1.04	5.50±2.00
IR ⁺ +SVPB5 (0.5 µg/ml)	1.17±0.29	11.67±3.25 ^a
IR ⁺ +SVPB5 (1.0 µg/ml)	1.50±0.87	6.00±3.46

^aP<0.05 vs. IR⁺ group. Values are expressed as the mean ± standard deviation (n=3). IR⁺, irradiation.

employed by modern medicinal researchers for numerous years. Our laboratory is continuously working on characterizing components from SVP and defining their effects on the post-radiation recovery of hematopoietic cells. The novel principal component SVP-B5 was isolated from the previously defined SVPII fraction (10). It was demonstrated that SVP-5B has hyperproliferative effects on irradiated hematopoietic cells. Treatment with SVP-B5 increased IL-3R expression and led to the activation of the JAK/STAT5 pathway in M-NFS-60 cells.

Proliferation and differentiation of HSCs are the major concern during radiation therapy in the recovery phase. SVPII augments the proliferation of these cells in a concentration-dependent manner (12). The results of the present study demonstrated that the principal component in the SVPII, namely SVP-B5, promotes the proliferation of the M-NFS-60 mouse-derived myelocytic leukemia cell line. These results corroborate with the findings of previous studies by our group and emphasize that purified SVP-B5 is the peptide to which the properties of the crude SVP II fraction of scorpion venom may be ascribed. Furthermore, hematopoietic functions of HSCs were evaluated using different methods, including CAFc, CFU and LTBMc assays. SVP-B5 promoted colony formation of BM-MNCs and enhanced HSC recovery. These observations demonstrated that SVP-B5 is the active principal component within SVP II, which has the ability to promote the proliferation of these cells, exerts growth factor-like properties, and therefore warrants future study, whereas the other purified peptide, SVPB4, failed to do so. These results clearly emphasized the potential of SVPB5 as a hematopoietic growth factor.

IL-3 promotes pluripotent hematopoiesis by stimulating the self-renewal of early pluripotent stem cells as well as the proliferation and differentiation of marrow-derived progenitor cells, resulting in the continued production and survival of mature blood cells. IL-3 exerts its biological activities by binding to its specific high-affinity receptor, IL-3R, on hematopoietic and other cell types (14,15). In the present study, SVP-B5 significantly increased the expression of IL-3R in the M-NFS-60 mouse-derived myelocytic leukemia cell line. It was speculated that the increment in cell proliferation and the increased expression of IL-3R by SVP-B5 may be functionally linked; however, this remains to be elucidated.

The JAK2/STAT5 pathway is an important signaling pathway for a majority of cytokines in the regulation of HSC

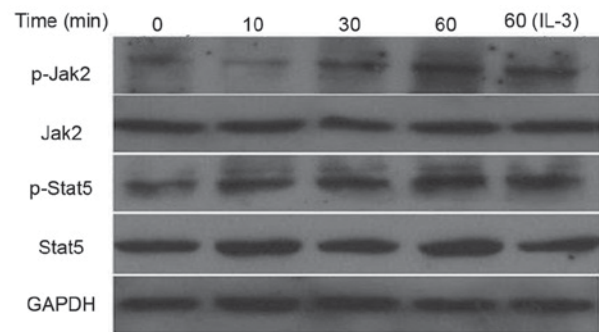


Figure 5. Effect of SVP-B5 on the phosphorylation of JAK2 and STAT5. Representative western blots displaying increased levels of P-JAK2 and P-STAT5 after SVPB5 (1 µg/ml) treatment with SVP-B5 for different periods. Cell treated with IL-3 served as a positive control. P-JAK2, phosphorylated Janus kinase 2; STAT5, signal transducer and activator of transcription 5; IL, interleukin.

and progenitor cell proliferation, self-renewal and differentiation. In addition, the role of IL-3 and its cognate receptor IL-3R in different hematopoietic expansions by the activation of JAK2/STAT5 is well documented in the literature (16). Considering the growth factor-like properties and the enhanced expression of IL-3R post-treatment with SVP-B5, the present study investigated whether SVP-B5 activates the JAK2/STAT5 pathway. The results clearly demonstrated that SVP-B5 activated the JAK2/STAT5 pathway in the M-NFS-60 cells.

The sequence of the SVP-B5 peptide has been previously discussed (10). Using the Basic Local Alignment Search Tool protein database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), it was found that SVP-B5 shares >80% homology with the sequence of the α -toxins family (data not shown). Importantly, SVP-B5 contains the eight intrinsic cysteines and four pairs of disulfide bonds, which is the characteristic feature of the α -toxins (17-19). The α -toxins are known to induce a prolongation of the action potential of nerves and muscles by fast inactivation of sodium channel receptor affinity dependent upon membrane potential (20). This property of SVP-B5 and further characterization should be explored in the near future.

The ability of natural toxins to bind specifically to various cellular domains upholds new hope for anti-cancer drug development. Latest developments in nanotechnology illustrate how researchers are tuning drug candidates to target specific sites. Recently, usage of scorpion venom encapsulated in nanoparticles (NanoVenin) has been demonstrated to have the potential to treat breast cancer (21). The assets of purified SVP-B5 discussed in the present study put forward its potential use as a novel drug candidate; however, further investigation is required prior to commencing their clinical application. As sufficient gaps exist in the literature regarding the appropriate form of drug administration, the exact molecular mechanism of its action, toxicological studies, and drug-drug interactions, additional research work in this direction using a rigorous system biology approach is required to address the important issues highlighted above.

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