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Jaspine B Hydrochloride-induced Apoptosis in HeLa Cells Is Associated With Disrupted Sphingolipid Metabolism and Ceramide Overload

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Abstract. *Background/Aim: A series of experiments on HeLa cells were conducted to provide new information concerning the anti-cancer properties of jaspine B hydrochloride (JBH). Materials and Methods: HeLa cells treated with 0.5 μmol/l JBH for 24, 48, and 72 h underwent flow cytometric analysis of the cell cycle, and measurement of phosphatidylserine externalization, mitochondrial membrane potential (MMP), casp-3 activation, cleavage of PARP, ceramide levels, aSMase activity, and Bcl-2 release. nSMase activity was measured by a colorimetric assay. Gene expression was determined by qRT–PCR. Immunocytochemistry was performed to detect p21 and p27 expression. Results: JBH-induced apoptosis in HeLa cells associated with externalization of phosphatidylserine, reduced MMP, activation of casp-3, and cleavage of PARP as well as up-regulation of TNF-α, FasL, and casp-8. Significant increase in nSMase activity, ceramide levels, Bcl-2 release (predominantly in the inactive form), and pro-apoptotic nuclear localization of p21 and p27 were also detected. Conclusion: JBH-induced apoptosis in HeLa cells is associated with disrupted sphingolipid homeostasis resulting in increased ceramide levels.*

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Cervical cancer (CC) is the fourth most common type of malignancy and cause of cancer death in women worldwide (1). Even though the incidence and mortality rates have declined in the developed countries over past few decades, CC still remains the most common gynecological cancer (2). Furthermore, women treated for cervical intraepithelial neoplasia are also at increased risk to develop invasive CC (3). Most commonly, the treatment involves combination of surgery, radiotherapy, cisplatin-based chemotherapy and brachytherapy (4). Although, current approaches have improved patient survival, treatment is still associated with significant reduction in quality-of-life. Therefore, the search for new promising anticancer agents with lower toxicity has to continue to increase the efficiency and tolerability of treatment.

Natural compounds play an important role in cancer prevention and treatment (5). The marine environment provides a unique reservoir of biologically active substances, which represent a promising source of potential novel anticancer agents. Primitive multicellular sea sponges from the genus *Jaspis* (family *Jaspidae*) represent a rich source of structurally novel natural compounds acting as antifungal

Table I. *Flow cytometric analysis*.

Analysis	Staining solution	Manufacturer
Cell cycle	PI (working solution: 0.2% Triton X-100, 0.5 mg/ml ribonuclease A and 0.025 mg/ml PI 500 μl PBS)	Molecular Probes, Eugene, OR, USA
Phosphatidylserine (PS) externalization	Annexin V/PI	BD Biosciences Pharmingen, San Diego, CA, USA
MMP (mitochondrial membrane potential)	TMRE (working solution: 0.1 μM TMRE)	Molecular Probes, Eugene, OR, USA
Caspase 3 (casp 3)	Caspase-3-PE	BD Biosciences Pharmingen, San Diego, CA, USA
Cleavage of PARP	Cleaved-PARP (Asp214) XP® Rabbit mAb (PE Conjugate)	Cell Signaling Technology, Danvers, MA, USA
Bcl-2 Phospho Bcl-2	Bcl-2 (124) Mouse mAb (PE Conjugate) Phospho-Bcl-2 (Ser70) Rabbit Rabbit mAb Alexa Fluor® 488 Conjugate	Cell Signaling Technology, Danvers, MA, USA
Ceramide	Anti-ceramide Mouse IgM mAb (BSA conjugate)	Enzo Life Sciences, Inc., New York, NY, USA
aSMase (acid sphingomyelinase)	Anti-Acid sphingomyelinase Mouse mAb	Abcam, Cambridge, UK

(6), antitumoral (7), antibiotic, and/or antihelmintic agents (8). Jaspamide is the most studied sponge-derived compound with a cyclodepsipeptide structure (7). The study of jaspamides led to the unexpected discovery of the novel biologically active substance jaspine B, first isolated in 2002 by Kuroda *et al*. (9). Independently, one year later Ledroit *et al*. (10) isolated the same compound and named it pachastrissamine (*Pachastrissa sp*.). Since isolation of naturally occurring sponge-derived compounds provides limited quantities (11), jaspine B has become the target of chemical synthesis. Cytotoxic and antiproliferative effects of jaspine B hydrochloride (JBH) were evaluated in several cancer cell lines with remarkable higher sensitivity of HeLa cells with IC_{50} value of 0.61±0.27 μmol/l compared to IC_{50} of 4.6±0.9 mmol/l in NIH 3T3 mouse embryonic fibroblasts (12). JBH possesses a sphingosine moiety and has a structure similar to sphingosine, thus may interfere with sphingolipid metabolism. The balance between sphingolipid metabolites, ceramide and S1P (*sphingosine-1-phosphate),* the "ceramide/S1P rheostat", is playing a critical role in cell survival and cancer progression. Accordingly, accumulation of ceramide is associated with proapoptotic effects while accumulation of S1P with antiapoptotic effects (13). Therefore, the principal objective of the present study was to investigate the possible mechanism of antiproliferative action of an anhydrophytosphingosine derivative JBH in cervical carcinoma HeLa cells with a more detailed analysis of ceramide signaling pathway.

Materials and Methods

Tested compound. JBH was synthetized at the Faculty of Science, P.J. Šafarik University in Košice, Slovakia (12). The structure was confirmed using Mass spectrometry and 1H, 13C NMR, IR spectroscopy. Studied compound was dissolved in dimethyl sufloxid (DMSO). The final concentration of DMSO in the culture medium was lower than 0.2% and exhibited no cytotoxicity.

Cell culture. The HeLa cell line (human cervical carcinoma) was maintained in RPMI 1640 medium (Biosera, Kansas City, MO, USA) supplemented with 10% fetal bovine serum and 1× HyClone™ antibiotic/antimycotic solution (PNC/STR/AMB) (GE Healthcare, Little Chalfont, UK) in humidified air atmosphere at 37˚C containing 5% CO_2. Cell viability was estimated using the trypan blue dye exclusion test and was greater than 95% before each experiment. HeLa cells (1×10^6) were treated with 0.5 μmol/l JBH concentration for 24, 48, and 72 h.

Flow cytometry. HeLa cells treated with JBH for 24, 48, and 72 h at the concentration of 0.5 μmol/l were harvested, washed in PBS, and divided for analysis according to the manufacturer's instructions (Table I). After 15 min of incubation at room temperature in the dark, fluorescence was measured by BD FACSCalibur flow cytometer using filter 585/42 FL-2 (Becton-Dickinson, San Jose, CA, USA). A minimum of 1×10^4 events was analyzed per analysis.

Table II. *Primer sequences used in real-time PCR analysis.*

Gene name	Forward primer	Reverse primer
GADPH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'
Bax	5'-CCCGAGAGGTCTTTTTCCGAG-3'	5'-CCAGCCCATGATGGTTCTGAT-3'
Bcl-2	5'-TCCATGTCTTTGGACAACCA-3'	5'-CCAGCCCATGATGGTTCTGAT-3'
Bcl-xL	5'-TTACCTGAATGACCACCTA-3'	5'-ATTTCCGACTGAAGAGTGA-3'
Casp 3	5'-TGGTTCATCCAGTCGCTTTG-3'	5'-CATTCTGTTGCCACCTTTCG-3'
Casp 7	5'-AGTGACAGGTATGGGCGTTCG-3'	5'-GCATCTATCCCCCCTAAAGTGG-3'
Casp 8	5'-AGAGTCTGTGCCCAAATCAAC-3'	5'-GCTGCTTCTCTCTTTGCTGAA-3'
TNFα	5'-GGCGTGGAGCTGAGAGATAAC-3'	5'-GGTGTGGGTGAGGAGCACAT-3'
FasL	5'-CACTTTGGGATTCTTTCCAT-3'	5'-GTGAGTTGAGGAGCTACAGA-3'
p53	5'-TTGCAATAGGTGTGCGTCAGA -3'	5'-AGTGCAGGCCAACTTGTTCAG -3'
p21	5'-GCAGACCAGCATGACAGATTT-3'	5'-GGATTAGGGCTTCCTCTTGGA-3'
p27	5'-AACGTGCGAGTGTCTAACGG-3'	5'-CCAGGAGTTACTTCTATGCCTGA-3'
PI3K	5'-CCTGATCTTCCTCGTGCTGCTC-3'	5'-ATGCCAATGGACAGTGTTCCTCTT-3'
Akt	5'-GCAGCACGTGTACGAGAAGA-3'	5'-GGTGTCAGTCTCCGACGTG-3'
NFκB	5'-CACTGCTCAGGTCCACTGTC-3'	5'- CTGTCACTATCCCGGAGTTCA-3'
IκB	5'-GCAATTTCTGGCTGGTTGG-3'	5'-GATCCGCCAGGTGAAGGG-3'
aSMase	5'-CAGTTCTTTGGCCACACTCA-3'	5'-TCCGGGGTAGTTTCCATCTA-3'
nSMase	5'-ACGTGCTTTACAAGGCAGTC-3'	5'-AGGACACACAGCAACACCAG-3'
S1pp1	5'-GGGCTGATTCTCATTCCCTG-3'	5'-AAATCAAGTGAAGCCCGATG-3'
Sgpl1	5'-GATGTCTGCTAAGGGGTGGA-3'	5'-ATAGATGGCACCCATTCCTG-3'
CerS2	5'-TGCCAAGATGTTTAACTACGC-3'	5'-TAGCCAAAGAAGGCAGGGTA-3'
CERK	5'-GTCCTTCCTCCCAGCACAG-3'	5'-GCACTTCCGGATAAGGATGA-3'
AC	5'-GCCCAGTGGGTAGGGTTTAT-3'	5'-CACATACCATCTGCCATGCT-3'
Spk1	5'-GATGCATGAGGTGGTGAATG-3'	5'-AACAGCAGTGTGCAGTTGAT-3'
Spk2	5'-GAAGGCATTGTCACTGTGTC -3'	5'-GCAGAGAAGAAGCGAGCAGT-3'
S1P1	5'-GGATCGCGCGGTGTAGAC-3'	5'-GCTAGAGGGCGAGGTTGAG-3'
S1P2	5'-CACTATGTGCTCTGCGTGGT-3'	5'-GGCGATGTAGGCATATGCA-3'
S1P3	5'-CCTCATCACCACCATCCTCT-3'	5'-TGGAGTAGAGGGGCAAGATG-3'

Neutral sphingomyelinase activity assay. Proteins for colorimetric nSMase activity assay (Sphingomyelinase Assay Colorimetric Kit, Abcam, Cambridge, UK) were extracted from HeLa cells by Laemmli lysis solution [1 mol/l Tris/HCl (pH=7.4), glycerol, 20% SDS (sodium dodecyl sulfate), deionized H_20] containing protease inhibitors followed by subsequent sonication. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). To assay for nSMase activity the manufacturer's instructions were followed and Cytation™ 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT, USA) was used.

RNA extraction and reverse transcription. Total RNA was isolated from HeLa cells using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and reverse transcription of total RNA (0.5 μg) into cDNA was performed using the commercial RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer's instructions and further used for quantitative PCR.

Quantitative PCR. Real-time PCR was performed on Applied Biosystems® 7500 RT-PCR device with a fast system using Luna® Universal qPCR Master Mix (BioLabs, New England Biolabs, Ipswich, MA, USA). The list of used primer sequences is given in Table II. Changes in the expressions of i) proapoptotic *casp-3, -6, -7, -8, p53, Bax, TNFα, FasL*, ii) antiapoptotic *Bcl-2, Bcl-xL, PI3K, Akt, NFκβ, Iκβ;* CDK inhibitors *p21, p27*, iii) genes involved in ceramide pathway with proapoptotic properties: sphingosine-1-phosphate phosphatase-1 (*S1pp1*), sphingosine-1-phosphate lyase (*Sgpl1*), ceramide synthase (*CerS2*), *aSMase, nSMase*, and iv) genes involved in ceramide pathway with antiapoptotic properties: acid ceramidase (*AC*), ceramide kinase (*CerK*), sphingosine-1-phosphate receptors 1, 2, 3 (*S1P1, 2, 3*), sphingosine kinase 1, 2 (*SpK1, 2*) were analyzed. The PCR program started by denaturation at 95˚C for 5 min followed by 40 cycles: denaturation at 95˚C for 30 s, hybridization at 55˚C for 45 s, and extension at 72˚C for 30 s. The data were analyzed by the comparative Ct method and normalized in each sample to the expression level of the reference *GADPH* gene. A melting curve was generated for each PCR reaction to verify the purity of the amplification product.

Immunocytochemistry (ICC). HeLa cells (1×10^6) were incubated with JBH (0.5 μmol/l) for 24, 48, and 72 h, respectively. After treatment, cells were washed with PBS and loaded into an automated cytospin machine StatSpin Cytofuge 2 (Beckman Coulter, Atlanta, GA, USA) following the manufacturer's instructions, and centrifuged at 500 rpm for 5 min. Prepared cytospine slides were air-dried at room temperature. Immunocytochemical staining was performed with the EnVision FLEX System (Agilent, Santa Clara, CA, USA). Primary antibodies p21 (Monoclonal Mouse Anti-Human, Clone SX118; dilution 1:50; DakoCytomation, Glostrup, Denmark) and p27 (Monoclonal Mouse Anti-Human p27Kip1, Clone SX53G8; dilution 1:50; DakoCytomation) were added to the slides and incubated at

Table III. *Effect of jaspine B hydrochloride on the cell cycle progression of HeLa cells after 24, 48 and 72 h incubation at concentration 0.5 μmol/l.*

	sub-G_0/G_1	G_1	S	G_2/M
CTRL	2.7 (±0.8)	64.7 (±5.25)	15.4 (±0.99)	17.3 (±2.8)
24 h	9.3 (±1.83)[a]	64.4 (±0.5)	16.5 (±3.37)	9.8 (±0.38)[b]
48 h	33.3 (±6.2)[c]	48.3 (±5.0)[b]	12.2 (±0.35)	6.2 (±0.86)[b]
72 h	38.7 (±3.55)[c]	46.0 (±2.55)[b]	11.3 (±0.75)	4.1 (±0.22)[b]

The results are presented from three independent experiments as mean±SD; (Significance *vs*. untreated control: [a]$p<0.05$, [b]$p<0.01$, and [c]$p<0.001$).

Table IV. *Induction of apoptosis in HeLa cells after treatment with jaspine B hydrochloride using Annexin V-FITC/PI staining.*

	An^-/PI^-	An^+/PI^-	An^+/PI^+	An^-/PI^+
CTRL	84.6 (±5.1)	2.1 (±0.31)	8.3 (± 2.12)	5.1 (±1.12)
24 h	50.8 (±0.42)[b]	19.2 (±1.84)[b]	15.8 (±0.39)[a]	14.3 (±2.21)[a]
48 h	39.8 (±2.35)[b]	28.7 (±2.33)[b]	15.6 (±1.27)[a]	16.0 (±2.13)[a]
72 h	28.2 (±1.61)[c]	41.0 (±1.39)[c]	19.6 (±2.61)[a]	11.3 (±2.31)

The results are presented from three independent experiments as mean±SD. Living cells are presented as An^-/PI^- events, cells in the early stage apoptosis are presented as An^+/PI^-, late apoptotic and necrotic cells as An^+/PI^+ and death cells as An^-/PI^+ (significance to untreated control: [a]$p<0.05$, $p<0.01$, [c]$p<0.001$).

room temperature in a humid chamber for 20 min. The slides were washed with PBS, counterstained with hematoxylin and dehydrated with a mixture of benzyl alcohol and xylene. ICC localization of p21 and p27 was observed by light microscope (Olympus BX40 F4, Tokyo, Japan) equipped with digital camera (Olympus E 420) and PC-based image analyzing/recording software (QuickPHOTO Industrial, Promicra, Prague, Czech Republic).

Statistical analysis. Results are expressed as mean±standard deviation (SD). Statistical analysis of the data was performed with one-way ANOVA followed by Bonferroni multiple comparisons test. Values of $p<0.05$ were considered to be statistically significant.

Results

Flow cytometry. Flow cytometric cell cycle analysis of HeLa cells exposed to 0.5 μmol/l JHB for 24, 48, and 72 h revealed a time-dependent effect with a significant increase in the cell population in the sub-G_0/G_1 phase, considered as apoptotic, with the highest significance after 48 (33.3%) and 72 (38.7%) h of incubation. The increase in apoptosis was associated with decrease in G_0/G_1 and G_2/M. Quantification of the percentage of cells in the different cell cycle phases is shown in Table III. To confirm the proapoptotic effect of JHB on HeLa cells we performed Annexin V staining to detect phosphatidylserine (PS) externalization and PI staining do detect increase in nuclear membrane permeability. Analysis of HeLa cells treated with 0.5 μmol/l of JHB for 24, 48 and 72 h showed a significant increase in the An^+/PI^+ cells considered late apoptotic compared to An^+/PI^- cells considered early apoptotic. After 24 h of incubation, 19% of the cells were observed in the early phase of apoptosis (An^+/PI^-) and 15% in the late stage of apoptosis (An^+/PI^+). With prolonged time of incubation, the percentage of cells increased and after 72 h, 41% of HeLa cells was found in the early phase and 19.6% in the late phase of apoptosis (Table IV). Disruption of mitochondrial membrane potential (MMP, $\Delta\Psi_{mt}$) is involved in the process of cell death. Therefore, we performed TMRE staining to detect the percentage of HeLa cells with declined MMP after JBH (0.5 μmol/l) treatment. As demonstrated in

Table V. *Activation of the enzyme neutral sphingomyelinase (nSMase) after 24, 48, and 72 h treatment of HeLa cells with jaspine B hydrochloride.*

	CTRL	24 h	48 h	72 h
nSMase (mU/ml)	1 (±0. 03)	1.44 (±0.12)[a]	2.06(±0.15)[b]	1.80(±0.11)[b]

The results are presented from three independent experiments as mean±SD. Significance *vs*. untreated control: [a]$p<0.05$ and [b]$p<0.01$.

Figure 1A, 24 h treatment of HeLa cells with JBH led to a significant increase in the number of HeLa cells with decreased MMP and this effect persisted even after prolonged incubation. Changes in the permeability of the outer mitochondrial membrane are under the control of regulators of apoptosis' intracellular mechanism - Bcl family proteins. The release of antiapoptotic protein Bcl-2 from mitochondria to cytosol plays an important role in the regulation of mitochondrial apoptotic pathway. Treatment of HeLa cells with JBH (0.5 μmol/l) for 48 and 72 h led to a significant increase in both its total and phosphorylated forms (Figure 1D). However, phosphorylation of Bcl-2 is generally associated with a loss of its antiapoptotic activity. Caspases are a family of enzymes activated in the process of apoptosis and cleavage of PARP-1 is a useful hallmark of apoptosis. Flow cytometric analysis showed that JBH resulted in a significant increase in the levels of the activated form of casp-3 at all evaluated time intervals, followed by an increase in cleaved PARP levels at all time points (Figure 1B and C). Ceramides regulate various cell signaling processes, including apoptosis, where they act as second messengers in activating the mitochondrial pathway. Their production in cells in response to stress and apoptotic impulses is regulated by sphingomyelinases (SMases), among which the most important are aSMase and magnesium-dependent nSMase. In our experiments, HeLa cells showed a significant increase in ceramide levels (Figure 1E) and a slight but nonsignificant time-dependent increase in aSMase activity (Figure 1F) after

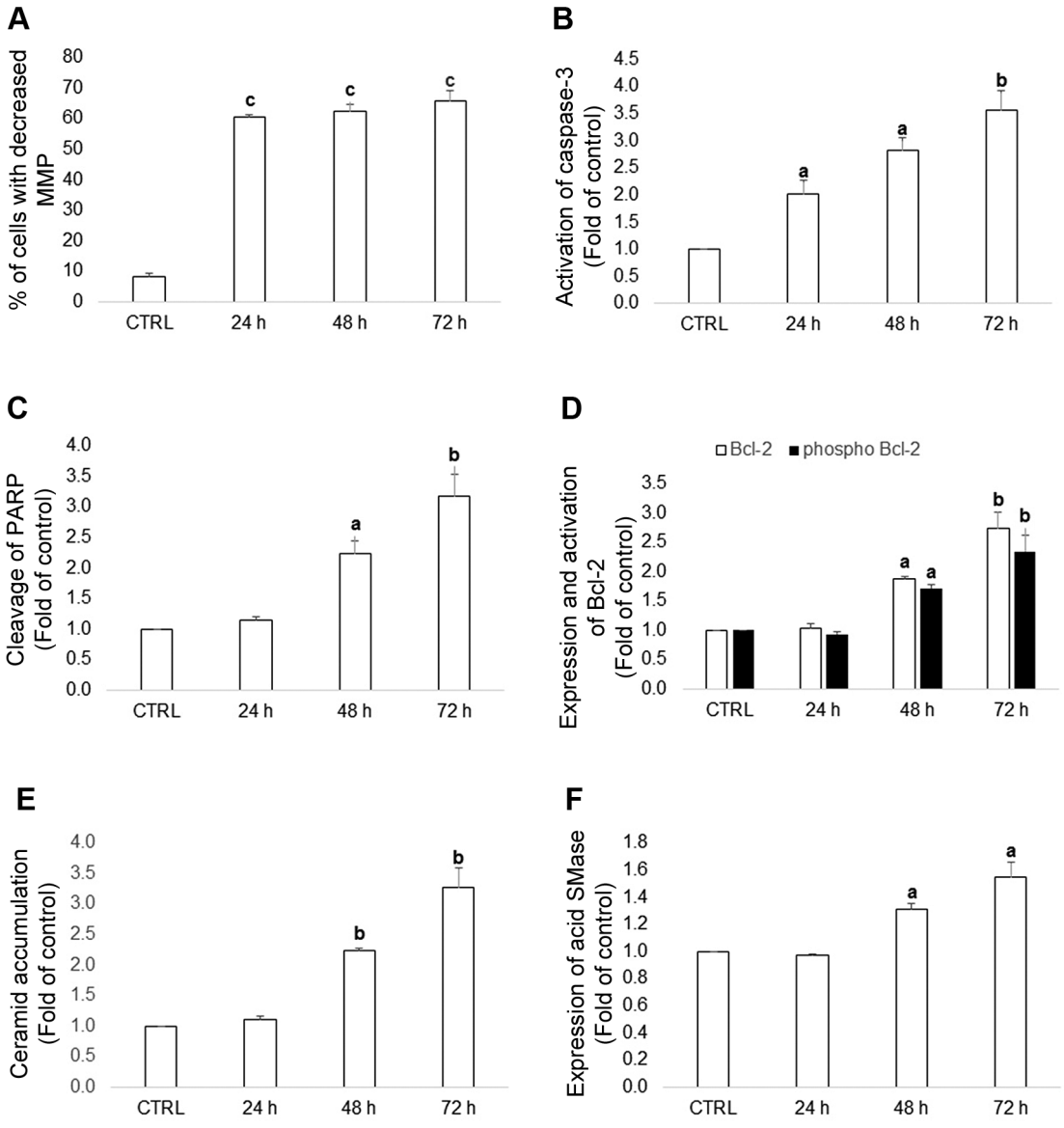

Figure 1. *Flow cytometric analysis of HeLa cells after 24, 48, and 72 h of treatment with jaspine B hydrochloride. A) Mitochondrial membrane potential changes, B) caspase 3 activation, C) cleavage of PARP, D) relative levels of Bcl-2 and phospho-Bcl-2 protein, E) intracellular ceramide levels, F) aSMase activity. Data were obtained from three independent measurements (significance vs. untreated control: $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$).*

48 and 72 h treatment with JBH (0.5 μmol/l). The colorimetric assay used to detect nSMase activity revealed increased nSMase activity in HeLa cells treated with JBH (0.5 μmol/l) at all time points with a peak at 48 h (Table V).

Gene expression analysis. qRT-PCR analysis was used to study the alterations at the gene transcription level after treatment of HeLa cells with JBH (0.5 μmol/l). A significant increase in proapoptotic *TNFα, FasL,* and *p53* after 24 h was detected. After 48 h of incubation, the significant increase in proapoptotic *TNFα* and *FasL* persisted, and a significant increase in *bax*, *p53,* and *casp-8* was noticed. The studied antiapoptotic genes *Bcl-2, Bcl-xL, PI3K, Akt, NFκβ, Iκβ* showed decreased expression after 24 and 48 h of treatment. CDK inhibitors *p21* and *p27* were significantly down-regulated after 24 h of treatment. The analysis of gene expression in the ceramide pathway showed a significant upregulation in the proapoptotic *aSMase* and antiapoptotic *CerK* after 24 h of incubation, which persisted even after prolonged incubation. After 48 h of incubation, significant over-expression of antiapoptotic *S1P1*

Table VI. *The effect of jaspine B hydrochloride (0.5 μM) on gene expression in HeLa cells.*

Proapoptotic				Antiapoptotic			
Gene	24 h	48 h	72 h	Gene	24 h	48 h	72 h
Bax	0.441[a]	2.143[b]	12.0[c]	*Bcl-2*	0.099[b]	0.126[b]	0.361[a]
Casp. 3	0.559	0.566	2.684[a]	*Bcl-xL*	0.28[b]	0.769	3.079[c]
Casp. 7	0.048[c]	0.314[a]	3.492[b]	*PI3K*	0.114[b]	0.911	1.228
Casp. 8	1.04	7.919[c]	12.813[c]	*Akt*	0.252[b]	0.993	3.474[c]
TNFα	4.999[c]	9.417[c]	7.394[c]	*NFκB*	0.176[b]	0.96	13.055[c]
FasL	7.742[c]	8.193[c]	10.423[c]	*IκB*	0.617	1.329	14.239[c]
p53	1.521[a]	4.229[c]	5.178[c]				
Ceramide pathway							
aSMase	1.899[a]	4.071[c]	4.446[c]	*CERK*	1.739a	1.457	8.797[c]
nSMase	0.217[b]	0.593	9.57[c]	*AC*	0.115	0.46	13.28
S1pp1	0.44[a]	0.382[a]	8[c]	*Spk1*	0.408[a]	0.922	2.07[a]
Sgpl1	0.39	0.68	11.79[c]	*Spk2*	0.235[b]	0.836	14.56[c]
				S1P1	1.16	4.36[c]	5.46[c]
				S1P2	0.54	0.252[b]	5.92[c]
				S1P3	0.67	0.636	3.818[c]
CDK inhibitors							
p21	0.128[b]	0.622	2.82[b]				
p27	0.02[c]	0.242[c]	1.892[a]				

Data were analyzed using the ΔΔCt comparison method and normalized to the expression level of the reference *GADPH* gene for each sample (significance *vs.* GADPH control: [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$).

was observed. After 72 h of treatment, significant upregulation of the monitored proapoptotic: *casp-3, -6, -7, -8, p53, Bax, TNFα, FasL, S1pp1, Sgpl1, CerS2*, *aSMase, nSMase*, and antiapoptotic: *Bcl-xL, PI3K, Akt, NFκβ, Iκβ, AC, CerK, S1P1, 2, 3, SpK1, 2* genes was observed (Table VI).

ICC. CDK inhibitors p21 and p27 play dual role in the process of apoptosis. The cytoplasmic localization of p21 and p27 is associated with antiapoptotic effects whereas the nuclear localization is related to apoptosis (14, 15). ICC was used to detect changes in the localization of p21 and p27. A significant nuclear localization of p21 and p27 was observed in HeLa cells after 24 h of incubation with JBH (0.5 μmol/l), which also persisted during longer periods of incubation (Figure 2).

Discussion

The antiproliferative effects of JBH against selected human cancer cell lines were described already in 2006 and later by our group in 2014 (12, 16). Our current study is so far the first to describe the potential underlying mechanism of the proapoptotic effect in cervical carcinoma cells. In our experiments, cell cycle analysis of HeLa cells treated with JBH revealed a significant increase in the apoptotic subG_0/G_1 population. Cell cycle progression is regulated by CDKs and CDKIs such as p21, which expression can be directly regulated by the transcription factor p53, and p27 (17, 18). Quantitative gene expression profiling showed significant over-expression of *p53*, whereas *p21* and *p27* expressions were rather decreased. The ability of JBH to induce apoptosis was also confirmed by phosphatidylserine externalization, a hallmark of an early apoptotic stage (19). Similar effect has also been observed in JBH-treated melanoma cells (13).

The extrinsic apoptotic pathway, mediated *via* surface death receptors such as Fas-R, TNF-R, TRAIL-R, involves the activation of initiator caspases-8 and -10 followed by activation of executioner caspases (20, 21). Our gene expression analysis revealed significant over-expression of *TNF-α, FasL*, and *Casp. 8* indicating that JBH activated the extrinsic apoptotic pathway in HeLa cells. Furthermore, treatment of HeLa cells with JBH led to down-regulation of *NF*κB and *IκB* as well as to increase in the levels of activated casp-3 resulting in PARP cleavage.

Apoptosis can also be triggered by different death stimuli and by intracellular molecules such as ceramide (22). Flow cytometric analysis revealed a significant increase in intracellular ceramide levels in HeLa cells treated with JBH.

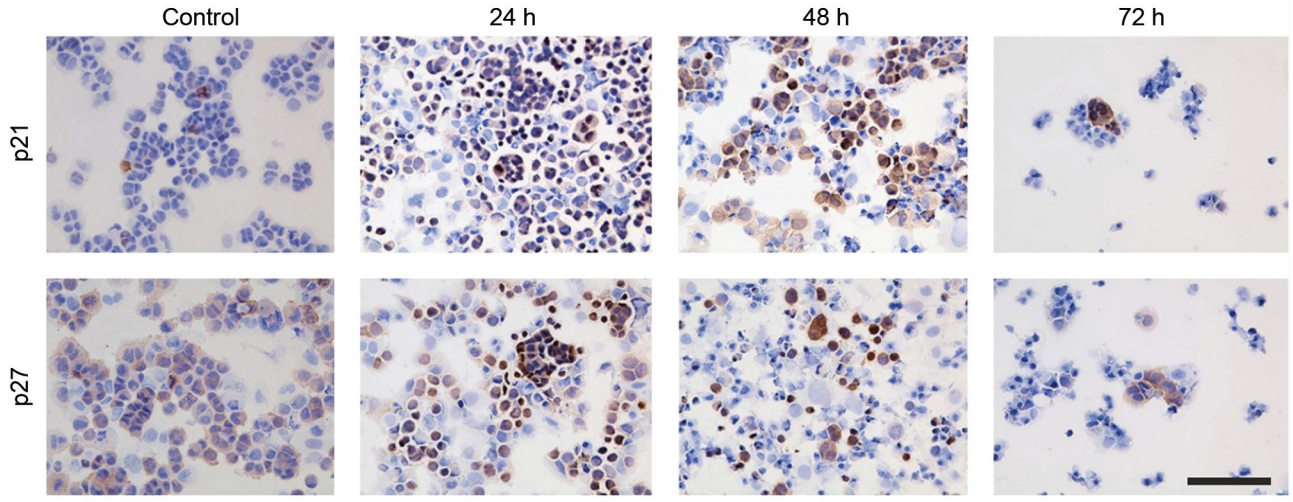

Figure 2. *Immunocytochemical detection of p21 and p27 in HeLa cells after 24, 48, and 72 h incubation with jaspine B hydrochloride. Magnification 400× (scale bar 100 μm).*

Moreover, JBH treatment down-regulated *PI3K* and *Akt*. Since PI3K/Akt signaling is known to suppress apoptosis and ceramide seems to act as a negative regulator of this pathway our data supports the anti-cancer potential of JBH (22).

It has been well documented that ceramide overload in early apoptosis is associated with permeabilization of mitochondrial membrane (23). We observed a significantly increased number of cells with reduced MMP and significant *Bax* over-expression and concomitant down-regulation of antiapoptotic *Bcl-2* and *Bcl-xL*. Although flow cytometry showed elevated levels of total Bcl-2, further detailed analysis revealed the dominance of its inactive phosphorylated (Ser70) form.

Ceramide can be generated from sphingomyelin by aSMase or nSMase in response to various stimuli such as TNF-α and/or FasL (23-25). The ceramide metabolic pathway involves numerous enzymes controlling ceramide levels (26, 27). We detected significantly increased expression of *aSMase*. Nevertheless, flow cytometric measurement of its activity showed only a slight increase in αSMase activity. However, the activity of nSMase was significantly increased. Among the tested genes encoding enzymes promoting ceramide formation, *aSMase* was the only significantly over-expressed gene, whereas all other studied genes were down-regulated. We also found significant over-expression of *CerK,* which converts the proapoptotic ceramide into prosurvival C1P. In addition, significant down-regulation of acid ceramidase (*AC*), which degrades ceramide to sphingosine, was also observed. Of note, over-expression of AC protects from TNFα-induced cell death (24, 28). Accordingly, our analysis showed significantly decreased expression of *S1P2* and *S1P3,* and upregulation of *S1P1*. S1P1 and S1P3 receptors mediate cell migration and invasion, whereas S1P2 exerts inhibitory effects. In addition, it has been shown that activation of S1P1 promoted vascular development and impaired vascular maturation *in vivo* (29). SpK, which significantly decreased following JBH treatment, converts sphingosine into prosurvival S1P (29-31) and contributes to the anti-cancer properties of JBH.

Conclusion

Based on our *in vitro* data, we may conclude that the proapoptotic effect of JHB results from its ability to interfere with the sphingolipid metabolism and subsequently increased proapoptotic ceramide levels. Moreover, the presented *in vitro* data encourage further more comprehensive *in vivo* examination of the pharmacokinetics and pharmacodynamics of JBH as a potential antitumor agent.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

M.B.P. designed and supervised the experiments. M. M. and E. M. synthesized JHB. A.B. and M.K. performed flow cytometry. A.B. performed nSMase activity assay. A.B., M.B.P., A.M., N.N. and P.T. performed qRT-PCR. A.B. and P.P. performed immunocytochemistry. M.B.P., L.M. and P.G. analyzed data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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