Bioorganic & Medicinal Chemistry 18 (2010) 3834–3840

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

The anticancer activity of 3- and 10-bromofascaplysins is mediated by caspase-8, -9, -3-dependent apoptosis

Alexandra S. Kuzmich [a], Sergey N. Fedorov [a,*], Valeria V. Shastina [a], Larisa K. Shubina [a], Oleg S. Radchenko [a], Nadezda N. Balaneva [a], Maxim E. Zhidkov [b], Joo-In Park [c], Jong Y. Kwak [c], Valentin A. Stonik [a]

[a] Pacific Institute of Bioorganic Chemistry, 159 Prospect 100-Let Vladivostoku, Vladivostok 690022, Russian Federation
[b] Far East National University, 8 Sukhanova St., Vladivostok 690001, Russian Federation
[c] Medical Research Center for Cancer Molecular Therapy, Dong-A University, Busan 602-714, Republic of Korea

ARTICLE INFO

Article history:
Received 19 October 2009
Revised 26 February 2010
Accepted 16 April 2010
Available online 21 April 2010

Keywords:
3- and 10-Bromofascaplysins
Cancer preventive
p53, AP-1 nuclear factors
Apoptosis

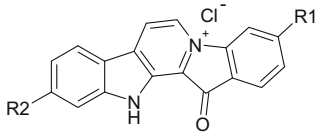
ABSTRACT

3- and 10-Bromofascaplysins was previously found to possess cytotoxic activity. In this study, we investigated their cancer preventive and proapoptotic properties. These effects were tested on mouse skin epidermal JB6 P^+ Cl41 cell line, its stable transfectants, and human tumor HL-60, THP-1, SNU-C4, SK-MEL-28, DLD-1, MDA-MB-231, and HeLa cells using a variety of assessments, including a cell viability (MTS) assay, flow cytometry, anchorage-independent soft agar assay, luciferase assay, mitochondrial permeability assay, and Western blotting. 3- and 10-Bromofascaplysins were effective at submicromolar concentrations as the anticancer agents, which exerted their action, at least in part, through the induction of caspase-8, -9, -3-dependent apoptosis.

1. Introduction

Marine organisms are receiving an increased level of interest as a rich source of compounds possessing anticancer properties. As a part of our investigation of marine natural products that inhibit malignant transformation of normal cells and induce apoptosis of tumor cells, we studied brominated derivatives of fascaplysin, isolated in 1988 from marine sponge *Fascaplysinopsis* sp.[1] Fascaplysin itself was previously identified as a pentacyclic alkaloid that specifically targets enzymatic activity of Cdk4 and inhibits the in vitro phosphorylation of the retinoblastoma protein Rb causing G_0/G_1 arrest of cancer cells.[2]

Chemical structures of 3- and 10-bromofascaplysins



1: R1 = Br; R2 = H

2: R1 = H; R2 = Br

This paper considers biological activity of 3- and 10-bromofascaplysins (**1** and **2**) isolated in 2003–2004 from an ascidian *Didemnum* sp.[3,4] and later synthesized.[5] Unlike the fascaplysin, whose biological activities and mechanism of action were studied in detail,[2–4,6–8] only cytotoxic effects against various mouse and human tumor cells were known for 3- and 10-bromofascaplysins.[3,4] Herein, we report anticarcinogenic and proapoptotic activities of **1** and **2** and provide some details regarding the action of **1** on the p53 and AP-1 nuclear factors and main mitogen-activated protein kinases (MAPKs) in mouse JB6 Cl41 cells.

2. Results and discussion

Low molecular weight natural products that prevent normal cell transformation and suppress tumor cell proliferation are attracting more and more attention as good candidates in chemopreventive or chemotherapeutic anticancer strategies.[9–15] To evaluate the inhibition by 3-bromofascaplysin of tumor promoter-induced neoplastic transformation of mouse epidermal JB6 P^+ Cl41 cells or colony formation of human cancer cell lines, we used the well-accepted anchorage-independent assay in soft agar and EGF (10 ng/ml) as a promoter of JB6 P^+ Cl41 cells transformation.[16–19] The obtained results indicated that 3-bromofascaplysin inhibited JB6 P^+ Cl41 cell transformation induced by EGF in dose-dependent manner (Fig. 1). Specifically, a 50% inhibition of JB6 P^+ Cl41 cells colony formation ($INCC_{50}$) by 3-bromofascaplysin was achieved at as low concentration as 106.4 nM.

Similar effects were observed for suppression by 3-bromofascaplysin of colony formation of several human tumor cell lines in soft agar (Fig. 2). For example, the $INCC_{50}$ for inhibition values of colony

* Corresponding author. Tel.: +7 4232 311168; fax: +7 4232 314050.
E-mail address: fedorov@piboc.dvo.ru (S.N. Fedorov).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.04.043

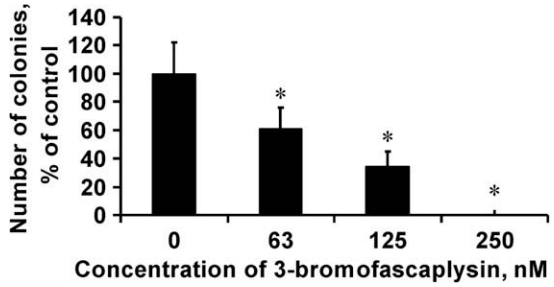


Figure 1. The inhibition of epidermal growth factor (EGF)-induced JB6 P$^+$ Cl41 cells transformation by 3-bromofascaplysin (**1**) in soft agar (anchorage-independent assay). Data represent the percentage of EGF-activated, 3-bromofascaplysin-treated cell colonies compared to percentage of EGF-activated, untreated cells. Each bar represents the mean ± SD from six samples of two independent experiments. * indicates a significant inhibition ($p < 0.05$).

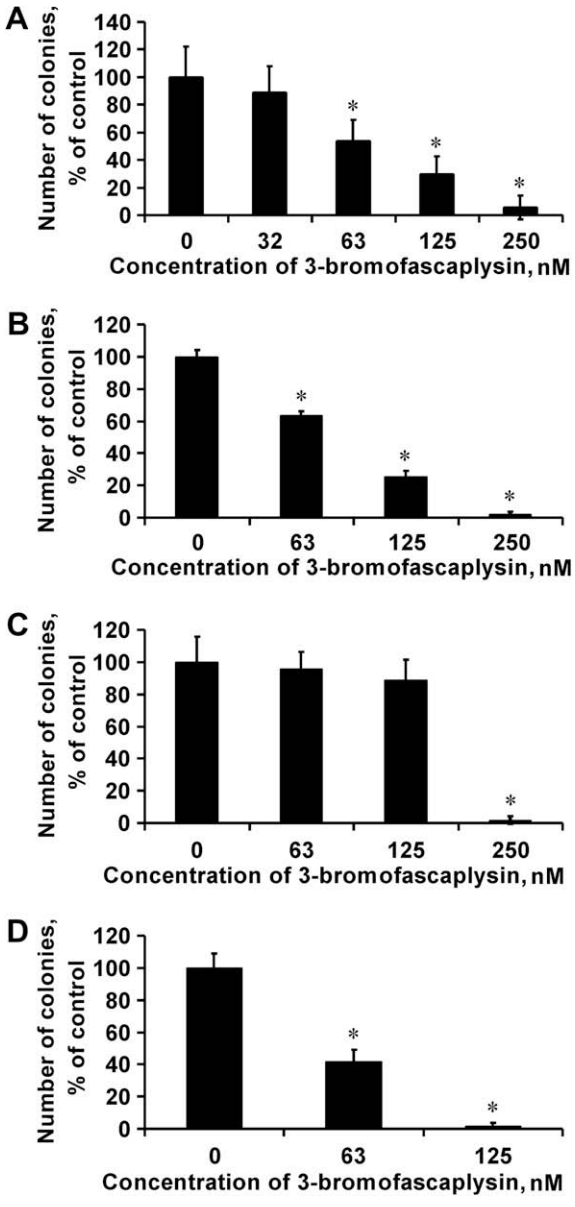


Figure 2. The inhibition by 3-bromofascaplysin (**1**) of colony formation of HeLa (A), HL-60 (B), SK-MEL-28 (C), and THP-1 (D) cells in soft agar (anchorage-independent assay). Data represent the percentage of 3-bromofascaplysin-treated cell colonies compared to percentage of untreated cells. Each bar represents the mean ± SD from six samples of two independent experiments. * indicates a significant inhibition ($p < 0.05$).

formation of HeLa (cancer of cervix; Fig. 2A), HL-60 (promyelocytic leukemia; Fig. 2B), SK-MEL-28 (melanoma, Fig. 2C), or THP-1 (monocytic leukemia; Fig. 2D) cells were 108.1, 103.5, 162.9, or 59.7 nM, respectively.

Cytotoxicity of 3- and 10-bromofascaplysins against JB6 Cl41 or human cancer cell lines was determined by the [5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium inner salt] (MTS) cell viability assay.[20] Results are represented in Table 1 as the corresponding IC_{50} values. They indicate that **1** inhibited JB6 P$^+$ Cl41 cell transformation induced by EGF or colony formation of several human cancer cell lines at non-cytotoxic concentrations. Really, the concentrations ($INCC_{50}$) at which **1** prevents JB6 Cl41 cells transformation, or HeLa, HL-60, SK-MEL-28, or THP-1 cells colony formation was found to be 2.7, 3.1, 5.3, 4.8, or 8.7 times lower than the concentrations (IC_{50}, Table 1) at which this compound inhibit proliferation of the corresponding cells. Results presented in Table 1 showed that 10-bromofascaplysin possesses higher cytotoxic activity than 3-bromofascaplysin towards most of the cancer cells studied. Interestingly, both 3- and 10-bromofascaplysins were the least active against human melanoma SK-MEL-28 cells. This finding may be explained by the fact that malignant melanoma is a highly metastatic and drug resistant cutaneous cancer.[21] Various studies demonstrate that melanoma chemoresistance is due to deregulated apoptosis through the intrinsic and extrinsic pathways.[22]

Apoptosis plays an essential role as a protective mechanism against tumorigenesis in the organism. Many chemopreventive substances may act through the induction of programmed cell death or apoptosis, which eliminates genetically damaged cells and cells induced improperly to divide.[23,24] To elucidate a possible molecular mechanisms of the anticancer action of 3- and 10-bromofascaplysins, we first studied their proapoptotic properties using flow cytometry. THP-1 cells were treated with increasing concentrations of 3-bromofascaplysin and harvested after 24 h. Apoptosis was clearly induced by **1** in a dose-dependent manner (Fig. 3) at a wide range of concentrations.

To show the apoptotic effects of both 10- and 3-bromofascaplysins we also used HL-60 cells. Cells were treated with different concentrations of **1** or **2** for 24 h and apoptotic changes were measured using annexin V-FITC/PI staining method. HL-60 cells, treated by the substances in a range of concentrations from 50 nM to 100 nM induced a relatively low level of total apoptosis (the number of Annexin V-positive/PI-negative and Annexin V-positive/PI positive cells), not more than ~20–38% (Fig. 4A). By increasing the concentration of **1** or **2** the total apoptosis of HL-60 cells gradually increased in dose-dependent manner and achieved a maximum at 1000 nM concentration (~90% of total cells number). The quantity of early apoptotic cells first increased in a range of 50–250 nM (from ~5–10% to 40–60%) and then decreased (to ~20%, Fig. 4B). According to obtained data 10-bromofascaplysin is a more potent inducer of apoptosis than 3-bromofascaplysin. The effects of **1** or **2** on procaspases-3, -8, -9, proapoptotic protein

Table 1
Cytotoxicity of 3- and 10-bromofascaplysins against mouse JB6 Cl41 P$^+$ and human cancer cell lines determined by the MTS method

Cell line	1	2
JB6 Cl41 P$^+$	284	144
HL-60	549	142
THP-1	521	161
HeLa	337	86
MDA-MB-231	575	173
DLD-1	243	118
SNU-C4	238	125
SK-MEL-28	785	>1000

Data are represented as IC_{50} in nM.

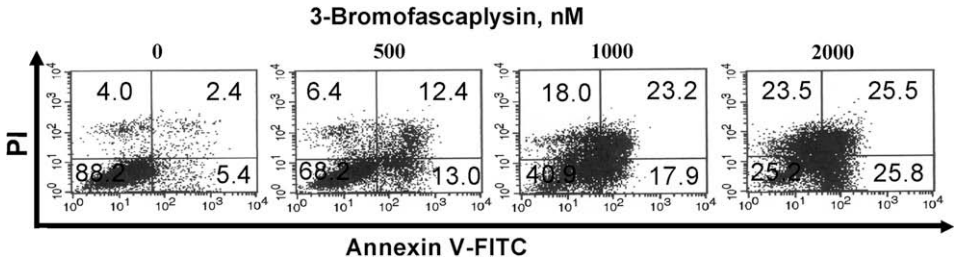


Figure 3. The induction of apoptosis by 3-bromofascaplysin in human leukemia THP-1 cells. A representative experiment is shown.

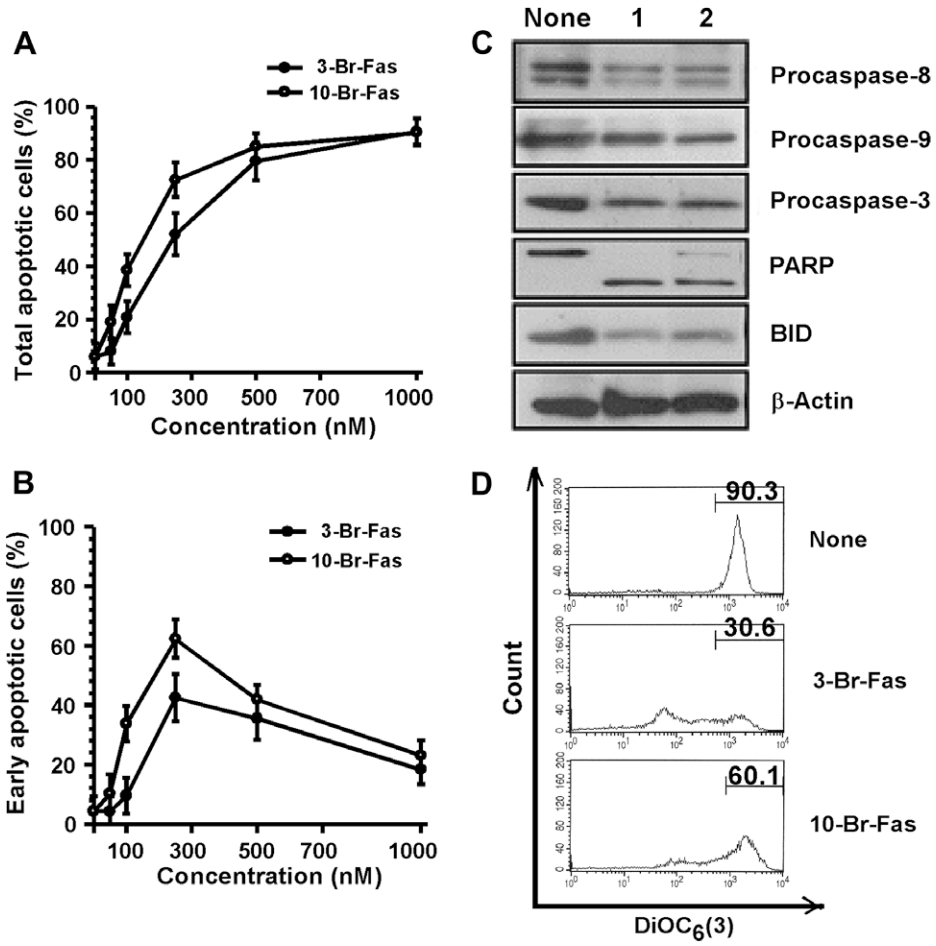


Figure 4. The apoptotic effects induced by 3- and 10-bromofascaplysins in HL-60 cells. The induction of total (A) and early (B) apoptosis by 3- and 10-bromofascaplysins in HL-60 cells. The cells were treated with indicated concentrations of 3-Br-Fas and 10-Br-Fas for 24 h. The apoptotic cells were determined by annexin V-FITC and PI staining. Each bar represents the mean ± SD from three independent experiments. Effect of **1** and **2** on the intracellular levels of the apoptotic proteins (C). The HL-60 cells were treated with 500 nM or 250 nM of **1** or **2**, respectively, for 24 h and the expression of PARP and BID proteins was detected by Western blot analysis. The expression of β-actin protein was examined and served as a loading control. Effect of **1** or **2** on the mitochondrial permeability (D) in HL-60 cells. The cells were treated with 500 nM or 250 nM of 3-Br-Fas and 10-Br-Fas respectively for 24 h. Cells were incubated with 40 nM $DiOC_6(3)$ for 30 min and subjected to flow cytometry. The representative experiments are shown.

Bid, and PARP—a specific substrate of activated caspase-3 were then investigated. The activation of caspases cascade is the most common and important characteristic of apoptosis, which can be triggered by several groups of so called initiators (e.g., caspases-8, -9) and effectors (e.g., caspases-3, -7, -6).[25,26] The initiatory procaspases in turn can be activated by different ways. Thus, procaspase-8 is initiated through membrane-associated complexes of proteins, in contrast with intrinsic mitochondrial-dependent initiation of procaspase-9.[26,27] Bid, a proapoptotic Bcl-2 family member, is a specific proximal substrate of caspase-8.[28] The cleaved truncated form of Bid (tBid) can activate downstream caspases including caspase-3, committing the cells to apoptosis.[29]

In our study, Western blot analysis showed that after treatment of HL-60 cells with **1** or **2** (500 or 250 nM, respectively) the levels of procaspase-8, -9, -3 and also protein Bid were significantly decreased indicating their cleavage to the active truncated forms (Fig. 4C). An additional confirmation of caspase-3 activation after the treatment of HL-60 cells with **1** or **2** was the decrease of the level of PARP—a specific substrate of activated caspase-3 (Fig. 4C). Another well known hallmark of apoptosis is the mitochondrial dysfunction including changes in membrane permeability and release of mitochondria inner proteins from intermediate space into the cytosol.[30] The decreased levels of Bid and procaspase-9 in HL-60 cells after **1** or **2** treatment presumably indicate

that bromofascaplysins could also provoke apoptotic death through the mitochondrial pathway. In order to clarify the role of mitochondria in **1**- or **2**-induced apoptosis we examined changes of mitochondrial permeability using the $DiOC_6(3)$ staining assay. As shown in Figure 4D, after treatment with 500 nM of 3-bromofascaplysin or 250 nM of 10-bromofascaplysin, the permeability of mitochondria notably changed (90.3% vs 30.6% and 60.1%, respectively). These data indicate that **1** and **2** could induce apoptosis partially through the intrinsic mitochondrial pathway. On the basis of obtained data, we can speculate that bromofascaplysins **1** and **2** may activate caspase-8, which in turn causes Bid truncation and then activates mitochondria-mediated downstream events with sequential activation of caspases-9 and -3. There is ample evidence that cross talk between these two apoptotic pathways, extrinsic through activation of caspase-8 and intrinsic mitochondrial, is very effective for the execution of apoptosis.[23,24]

The effect of **1** on p53- or AP-1-dependent transcriptional activity was then investigated in JB6 Cl41 p53 or AP-1 cells stably expressing a luciferase reporter gene controlled by a p53 or AP-1 DNA binding sequence. A number of factors and signaling pathways in a cell can lead to apoptosis and one of the most important pathways extends through activation of the p53 nuclear factor. The tumor suppressor protein p53 is a part of the cell's emergency response team that functions to negatively regulate cell growth following damage by inducing cell cycle arrest and apoptosis.[31–33] p53 is also involved in the chemopreventive effects of many natural compounds.[34–38] On the other hand, some cancer-preventive compounds, like gingerol, curcumin, and polyprenylated benzoquinones and hydroquinones, act through the inhibition of the p53 activity.[39–43] In our study, 3-bromophascaplysin at active concentrations 250–500 nM time- and dose-dependently also inhibited basal p53-dependent transcriptional activity (Fig. 5A).

Beside p53-dependent transcriptional activity, the time- and dose-dependent effect of **1** on the viability of JB6 Cl41 p53 cells (Fig. 5B) was also evaluated. As shown, about 90–50% of cells were still alive after 6 h of the treatment with the active concentrations 375–500 nM of **1**. At these conditions, the p53-dependent transcriptional activity was inhibited to the level of 60–30%, when compared with untreated control cells. After 24 h of the incubation with **1**, even cells treated with the low concentrations of 3-bromofascaplysin (125–250 nM) showed 40–60% decrease in the p53-dependent transcriptional activity compared to untreated cells (Fig. 5A). Per contra, it is known that p53 can induce apoptosis in a transcription-independent manner.[44,45] For example, p53 is able to interact with anti-apoptotic proteins Bcl-2, Bcl-X_L, and Mcl-1 at the mitochondrial membrane compromising their ability to stabilize the membrane and thereby induces the release of cytochrome *c* from the intermembrane space of mitochondria.[46,47] Therefore, further investigations are necessary to answer the question if 3-bromofascaplysin-induced apoptosis is indeed p53-indepenent or not.

The AP-1 transcription factor regulates a variety of cellular processes, including proliferation, differentiation, and apoptosis and, in distinction from p53, has been considered primarily to be an oncogene.[17,18,48,49] Recently, some of the AP-1 proteins, such as Jun-B and c-Fos, were shown to have tumor-suppressor activity both in vitro and in vivo.[50,51] Activation of another AP-1 protein, c-Jun, is required for the induction of apoptosis in PC12 and HL-60 cells.[52,53] Activation of both AP-1 and NF-κB nuclear factors is necessary for DNA damaging agents- and ceramide-induced apoptosis in T lymphocytes and Jurkat T cells.[54,55] Anticancer drugs, such as vinblastine and vincristine, which inhibit microtubules, activate AP-1 in cancer cells. This activation is required for efficient apoptosis induced by these drugs.[56–58] Our results showed that 3-bromofascaplysin also induced basal AP-1-dependent transcriptional activity in JB6 Cl41 AP-1 cells in a time- and dose-dependent manner (Fig. 6).

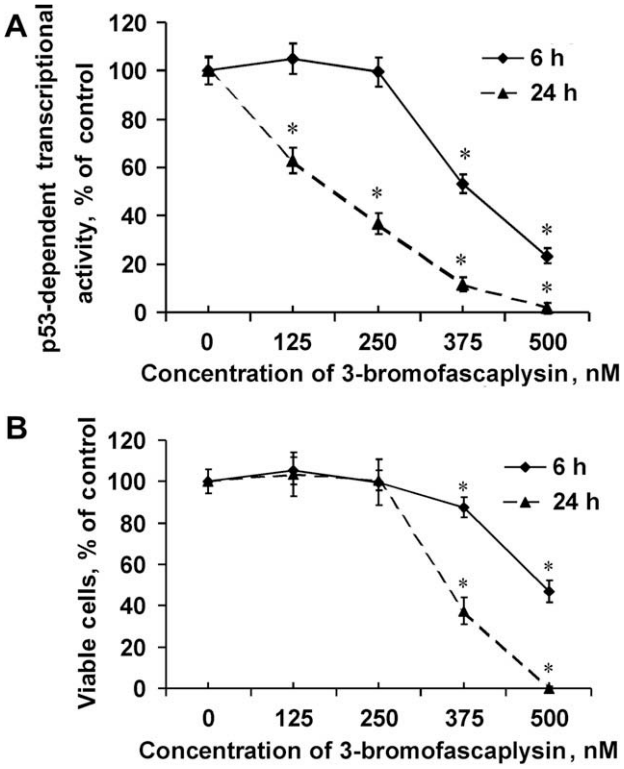


Figure 5. The dose- and time-dependent inhibition by 3-bromofascaplysin (**1**) of basal p53-dependent transcriptional activity (A) in JB6 Cl41 p53 cells. The effect of **1** on the cell viability of JB6 Cl41 p53 cells (B) evaluated after 6 or 24 h of incubation with the compound. Each datapoint represents the mean ± SD from six samples of two independent experiments. $^*p < 0.05$.

Specifically, AP-1 activity increased two times as much compared to untreated control cells after 6 h of the treatment with 125–250 nM concentrations of **1** (Fig. 6). After 24 h of the incubation, cells treated with 125–250 nM concentrations of **1** also showed 1.5–2-fold increase in the AP-1-dependent transcriptional activity compared to untreated cells. These results suggest that the cancer preventive effect of 3-bromofascaplysin might be mediated by the AP-1 nuclear factor.

We used dominant negative mutant (DNM) JB6 Cl41 cells and the MTS method to elucidate the role of the main MAPKs in the 3-bromofascaplysin-induced cytotoxic effects. Three main MAPKs have been identified in mammalian cells: extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38. In general, JNKs and p38 are primarily activated by environmental stresses, whereas ERKs respond mainly to mitogenic and proliferative stimuli.[59] The ERKs pathway has long been associated with cell growth, proliferation, and survival.[60,61] ERKs-mediated survival signaling has been proposed to be mediated mainly through activation of RSK kinase, which in turn activates anti-apoptotic Bcl-2, Bcl-X_L, and Bcl-1 proteins.[60,62] JNKs signaling pathway can mediate survival, proliferation, or apoptosis, depending on the stimuli and cellular conditions.[63,64] In our experiments, 3-bromofascaplysin demonstrated different cytotoxic activity in respect to various JB6 Cl41 DNM cells. Really, **1** was significantly more active against DN-ERK2 or DN-JNK1 cells than against DN-p38 or non-transfected JB6 Cl41 cells (Fig. 7).

Specifically, at 125 nM concentration, **1** did not induce cytotoxicity in non-transfected JB6 Cl41 or DN-p38 cells, whereas only 60% and 30% of DN-JNK1 or DN-ERK2 cells, respectively, survived at this

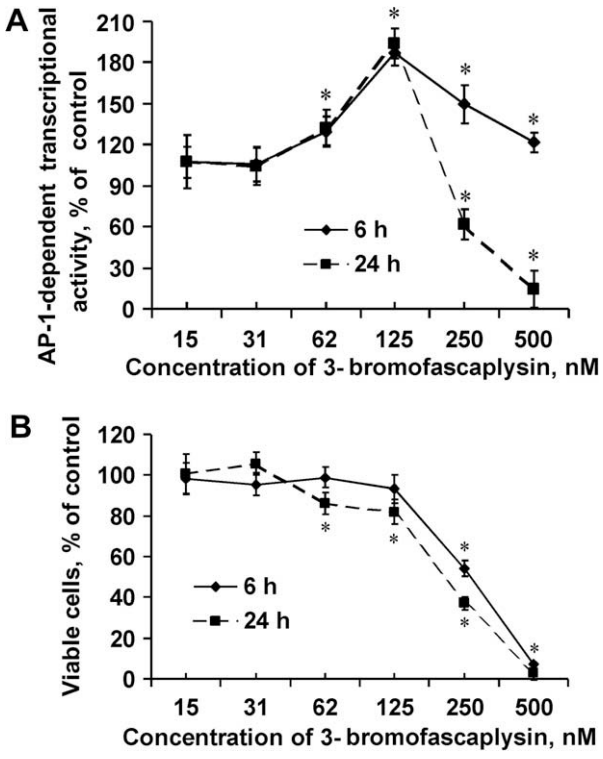


Figure 6. The dose- and time-dependent inhibition by 3-bromofascaplysin (**1**) of basal AP-1-dependent transcriptional activity (**A**) in JB6 Cl41 AP-1 cells. The effect of **1** on the cell viability of JB6 Cl41 AP-1 cells (**B**) evaluated after 6 or 24 h of incubation with the compound. Each datapoint represents the mean ± SD from six samples of two independent experiments. $^{*}p < 0.05$.

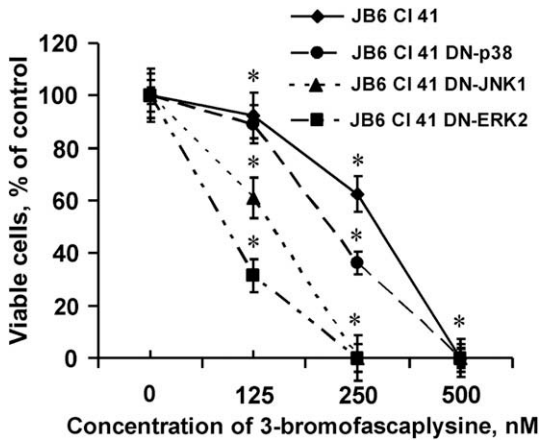


Figure 7. The dose-dependent inhibition by 3-bromofascaplysin (**1**) of the cell viability of various JB6 Cl41 DNM cells. Each datapoint represents the mean ± SD from six samples of two independent experiments. $^{*}p < 0.05$.

condition. At 250 nM concentration of **1**, 100% of DN-JNK1 or DN-ERK2 cells were MTS-positive, but about 60% or 40% of JB6 Cl41 or DN-p38 cells were still viable. These results suggest that JNKs and/or ERKs signaling pathways might be involved in cell response to 3-bromofascaplysin.

In conclusion, our findings indicate that 3- and 10-bromofascaplysins show anticancer cytotoxic effects against seven different human cancer cell lines, HL-60, THP-1, HeLa, MDA-MB-231, DLD-1, SNU-C4, and SK-MEL-28. 3-Bromofascaplysin also shows cancer-preventive effect at non-cytotoxic concentrations in JB6 Cl41 cells. These activities of **1** or **2** are mediated at least in part through the induction of caspase-3, -8, -9-dependent apoptosis. Furthermore, our study indicates that 10-bromofascaplysin is a more potent inducer of apoptosis than 3-bromofascaplysin. The induction of the AP-1- and inhibition of the p53-dependent transcriptional activity in JB6 Cl41 cells were also shown for 3-bromofascaplysin indicating that the effects induced by **1** in JB6 cells at least in part might be mediated by the AP-1 nuclear factor. Our results also show that at least two of three main MAPK signaling pathways, JNKs and ERKs, might be involved in cell response to the treatment with 3-bromofascaplysin. Therefore, further study of fascaplysin derivatives as anticancer compounds is important and may lead to the development of promising cancer-preventive agents.

3. Experimental

3.1. General experimental procedures

Cell colonies in the anchorage-independent transformation assay were scored using the Olympus CKX31 inverted microscope (Olympus, Japan). The MTS reduction assay to determine cell viability was carried out using the μQuant microplate reader (Bio-Tek instruments, Inc., USA). The onset of apoptosis was analyzed by flow cytometry using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA). Data analysis was performed using CellQuest software (Becton-Dickinson, CA, USA). The luminescence assay for p53 or AP-1 nuclear factor-dependent transcriptional activity was measured using the Luminoscan Ascent Type 392 microplate reader (Labsystems, Helsinki, Finland). The statistical computer program STATISTICA 6.0 for Windows (StatSoft, Inc., Tulsa, OK, USA, 2001) was used for analysis of the obtained data. Non-parametric Mann–Whitney *U* test was used to compare two independent groups of data. Method of regressions was used to compute IC_{50} or $INCC_{50}$ in corresponding experiments.

3.2. Reagents

3- and 10-Bromofascaplysins were synthesized as described previously[5] and were pure in accordance with NMR, MS, and C, H, N—analysis data. Minimum essential medium (MEM) and RPMI medium were purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA, USA); penicillin/streptomycin and gentamycin were from Bio-Whittaker (Walkersville, MD, USA); and L-glutamine was from Mediatech, Inc. (Herndon, VA, USA). Epidermal growth factor (EGF) was obtained from Collaborative Research (Bedford, MA, USA). The luciferase assay substrate and Cell Titer 96 Aqueous One Solution Reagent (MTS) kit for the cell viability assay were from Promega (Madison, WI, USA). The Annexin V-FITC Apoptosis Detection kit was from Medical & Biological Laboratories (Watertown, MA, USA).

3.3. Cell culture

The JB6 P^{+} Cl41 mouse epidermal cell line and its stable transfectants JB6 Cl41 DN-JNK1 mass1, JB6 Cl41 DN-p38 G7, JB6 Cl41 DN-ERK2 B3 mass1, JB6 Cl41-Luc-p53 (PG-13), and JB6 Cl41-Luc-AP-1 cells were cultured in monolayers at 37 °C and 5% CO_2 in MEM containing 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.[65] The human cancer cell lines, HL-60, THP-1, HeLa, SNU-C4, DLD-1, MDA-MB-231, and SK-MEL-28, were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured at 37 °C and 5% CO_2 in RPMI, DMEM (for MDA-MB-231) and MEM (for SK-MEL-28) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Information regarding the genetic background of these cell lines is available online.

3.4. Anchorage-independent transformation or colony formation assay

The cancer-preventive effect of 3-bromofascaplysin was evaluated using an anchorage-independent neoplastic transformation or colony formation assay. For evaluation of the anticancer effects of 3-bromofascaplysin in human cancer cell lines using a colony formation assay, no additional stimulus was required. EGF (10 ng/ml) was used for stimulating neoplastic transformation of JB6 P^+ Cl41 cells. The assay was carried out in six-well tissue culture plates. Human HeLa, HL-60, THP-1, SK-MEL-28, or mouse JB6 P^+ Cl41 cells (8×10^3/ml) were treated with various concentrations of 3-bromofascaplysin in 1 ml of 0.33% basal medium Eagle (BME) agar containing 10% FBS over 3 ml of 0.5% BME agar containing 10% FBS and various concentration of 3-bromofascaplysin. The cultures were maintained in a 37 °C, 5% CO_2 incubator for 1 week. Then, cell colonies were scored.

3.5. Cell viability assay

The effect of **1** or **2** on cell viability was evaluated using MTS reduction into its formazan product.[20] The cells were cultured for 12 h in 96-well plates (6000 cells/well) in the corresponding media (100 μl/well) containing 10% FBS. The media was then replaced with 10% FBS-media containing the indicated concentrations of **1** or **2**, and the cells were incubated for 22 h. Then, 20 μl of MTS reagent was added into each well, and MTS reduction was measured 2 h later spectrophotometrically at 492 and 690 nm as background.

3.6. Apoptosis assay

The onset of early and late apoptosis was analyzed by flow cytometry using Annexin V–FITC and propidium iodide (PI) double staining. Cells, 1×10^6/10 cm dish, in 10% FBS-RPMI were treated with various concentrations of **1** or **2** for 24 h. After incubation, cells were washed with PBS by centrifugation at 1000 rpm for 5 min, and processed for detection of apoptosis using Annexin V-FITC and PI staining according to manufacturer's protocol. In brief, 1×10^5–5×10^5 cells were resuspended in 500 μl of 1× binding buffer (Annexin V-FITC Apoptosis Detection Kit). Then, 5 μl of Annexin V-FITC and 5 μl of PI were added, and the cells were incubated at room temperature for 15 min in the dark and were analyzed by flow cytometry.

3.7. Assay for AP-1 or p53-dependent transcriptional activity

The ability of 3-bromofascaplysin to influence p53- or AP-1-dependent transcriptional activity in the mouse JB6 Cl41 cell line was evaluated using the luciferase method. JB6 Cl41-Luc-p53 (PG-13) or JB6 Cl41-Luc-AP-1 cells (6×10^3) suspended in 100 μl 5% FBS-MEM were added into each well of a 96-well plate. Plates were incubated for 12 h and then treated with various concentrations of the substances in 100 μl of 5% FBS-MEM. After incubation with the substances for 24 h, the cells were extracted for 1 h at room temperature with 100 μl/well of lysis buffer [0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM dithiothreitol (DTT), 2 mM EDTA]. Then, 30 μl of lysate from each well was transferred in a plate used for luminescent analysis, and luciferase activity was measured using 100 μl/well of the luciferase assay buffer [0.47 mM D-luciferin, 20 mM Tricin, 1.07 mM magnesium carbonate hydroxide pentahydrate $(MgCO_3)_4 \times Mg(OH)_2 \times 5H_2O$, 2.67 mM $MgSO_4 \times 7H_2O$, 33.3 mM DTT, 0.53 mM ATP, 0.27 mM CoA, and 0.1 mM EDTA (pH 7.8)].

3.8. Western blot analysis

HL-60 cells treated with 3- or 10-bromofascaplysins for 24 h were harvested, washed with ice-cold PBS, and treated with lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1 mM NaF, 1% Triton X-100 with 0.1 mM sodium orthovanadate, and protease inhibitors (1 mM PMSF, 20 U/ml aprotinin). The concentration of proteins in each lysate was determined using Bio-Rad Protein Assay Reagent (Bio-Rad Lab., Richmond, CA, USA) following manufacturer's protocol. Then, 25 μg of proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Amersham Life Science, Inc., Piscataway, NJ, USA). The membranes were blocked with a blocking buffer (10 mM Tris–HCl, 0.15 M NaCl, 0.1% NaN_3, and 5% skim milk) for 1 h at 25 °C and incubated with primary polyclonal antibodies directed against procaspase-3, -8, -9 and β-actin (Cell Signaling Technology, Beverly, MA, USA), PARP, and BID (Santa-Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000 dilution) in a blocking buffer overnight at 4 °C. The membranes were incubated with the secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) for 1 h at 25 °C. The signals were detected using ECL chemiluminescence (Amersham, Buckinghamshire, UK) following manufacturer's instructions.

3.9. Mitochondrial permeability assay

3- or 10-Bromofascaplysin-treated HL-60 cells (1×10^6/ml) were incubated with 40 nM 3,30-dihexyloxacarbocyanine iodide [DiOC6(3), Molecular probes] for 30 min at 37 °C and then washed with PBS. Stained cells were analyzed by flow cytometry.

Acknowledgments

This work was supported by the Grant 2813.2008.4 for Support of the Leading Russian Science Schools, Program of Presidium of RAS 'Molecular and Cell Biology' and FEB RAS Grants 09-III-A-05-146 and 09-III-B-05-155. The Korean co-authors are grateful for financial support from the NRF of Korea Grant funded the Korean government through the Medical Research Center for Cancer Molecular Therapy at Dong-A University (R13-2002-044-04001-0). Authors are grateful to Professor Zigang Dong (Hormel Institute of Minnesota University, USA) who kindly presented JB6 cell lines, which were used in the present study.

References and notes

1. Roll, D. M.; Ireland, C. M.; Lu, H. S. M.; Clardy, J. *J. Org. Chem.* **1988**, *53*, 3276.
2. Soni, R.; Muller, L.; Furet, P.; Schoepfer, J.; Stephan, C.; Zumstein-Mecker, S.; Fretz, H.; Chaudhuri, B. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 877.
3. Segraves, N. L.; Lopez, S.; Johnson, T. A.; Said, S. A.; Fu, X.; Schmitz, F. J.; Pietraszkiewicz, H.; Valeriote, F. A.; Crews, P. *Tetrahedron Lett.* **2003**, *44*, 3471.
4. Segraves, N. L.; Robinson, S. J.; Garcia, D.; Said, S. A.; Fu, X.; Schmitz, F. J.; Pietraszkiewicz, H.; Valeriote, F. A.; Crews, P. *J. Nat. Prod.* **2004**, *67*, 783.
5. Zhidkov, M. E.; Baranova, O. V.; Balaneva, N. N.; Fedorov, S. N.; Radchenko, O. S.; Dubovitskii, S. V. *Tetrahedron Lett.* **2007**, *48*, 7998.
6. Charan, R. D.; McKee, T. C.; Gustafson, K. R.; Pannell, L. K.; Boyd, M. R. *Tetrahedron Lett.* **2002**, *43*, 5201.
7. Aubry, C.; Jenkins, P. R.; Mahale, S.; Chaudhuri, B.; Sutcliffe, M. J. *Chem. Commun.* **2004**, 1696.
8. Popov, A. M.; Makarieva, T. N.; Fedoreev, S. A.; Stonik, V. A. *Chemother. Tumors USSR (Rus.)* **1991**, *56*, 61.
9. Delmas, D.; Lancon, A.; Colin, D.; Jannin, B.; Latruffe, N. *Curr. Drug Targets* **2006**, *7*, 423.
10. Lu, Y. P.; Low, Y. R.; Xie, J. G.; Peng, Q. Y.; Liao, I.; Yang, C. S.; Huang, M. T. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12455.
11. Rogozin, E. A.; Lee, K. W.; Kang, N. J.; Yu, H.; Nomura, M.; Miyamoto, K. I.; Conney, A. H.; Bode, A. M.; Dong, Z. *Carcinogenesis* **2008**, *29*, 1228.
12. Fedorov, S. N.; Bode, A. M.; Stonik, V. A.; Gorshkova, I. A.; Schmid, P. C.; Radchenko, O. S.; Berdyshev, E. V.; Dong, Z. *Pharm. Res.* **2004**, *21*, 2307.

13. Fedorov, S. N.; Shubina, L. K.; Bode, A. M.; Stonik, V. A.; Dong, Z. *Cancer Res.* **2007**, *67*, 5914.
14. Fedorov, S. N.; Shubina, L. K.; Kicha, A. A.; Ivanchina, N. V.; Kwak, J. Y.; Jin, J. O.; Bode, A. M.; Dong, Z.; Stonik, V. A. *Nat. Prod. Commun.* **2008**, *3*, 1575.
15. Lee, K. W.; Kang, N. J.; Heo, Y. S.; Rogozin, E. A.; Pugliese, A.; Hwang, M. K.; Bowden, G. T.; Bode, A. M.; Lee, H. J.; Dong, Z. *Cancer Res.* **2008**, *68*, 946.
16. Colburn, N. H.; Former, B. F.; Nelson, K. A.; Yuspa, S. H. *Nature* **1979**, *281*, 589.
17. Dong, Z.; Birrer, M. J.; Watts, R. G.; Matrisian, L. M.; Colburn, N. H. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 609.
18. Dong, Z.; Watts, R. G.; Sun, Y.; Colburn, N. H. *Int. J. Oncol.* **1995**, *7*, 359.
19. Strickland, J.; Sun, Y.; Dong, Z.; Colburn, N. H. *Carcinogenesis* **1997**, *18*, 1135.
20. Baltrop, J. A.; Owen, T. C.; Cory, A. H.; Cory, J. G. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 611.
21. Hocker, T. L.; Singh, M. K.; Tsao, H. *J. Invest. Dermatol.* **2008**, *128*, 2575.
22. Soengas, M. S.; Lowe, S. W. *Oncogene* **2003**, *22*, 3138.
23. Hengartner, M. O. *Nature* **2000**, *407*, 770.
24. Sun, S.-Y.; Hail, N., Jr.; Lotan, R. *J. Natl. Cancer Inst.* **2004**, *96*, 662.
25. Chang, H. Y.; Yang, X. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 821.
26. Stennicke, H. R.; Salvesen, G. S. *Biochim. Biophys. Acta* **1998**, *1387*, 17.
27. Dias, N.; Bailly, C. *Biochem. Pharmacol.* **2005**, *70*, 1.
28. Li, H.; Zhu, H.; Xu, C. J.; Yuan, J. *Cell* **1998**, *94*, 491.
29. Luo, X.; Budihardjo, I.; Zou, H.; Slaughter, C.; Wang, X. *Cell* **1998**, *94*, 481.
30. Budihardjo, I.; Oliver, H.; Lutter, M.; Luo, X.; Wang, X. *Annu. Rev. Cell Dev. Biol.* **1999**, *15*, 269.
31. Levine, A. J. *Cell* **1997**, *88*, 323.
32. Prives, C.; Hall, P. A. *J. Pathol.* **1999**, *187*, 112.
33. Vousden, K. H. *Cell* **2000**, *103*, 691.
34. Huang, C.; Ma, W. Y.; Goranson, A.; Dong, Z. *Carcinogenesis* **1999**, *20*, 237.
35. Qin, J.; Chen, H.-G.; Yan, Q.; Deng, M.; Liu, J.; Doerge, S.; Ma, W.; Dong, Z.; Li, D. W. *Cancer Res.* **2008**, *68*, 4150.
36. He, Z.; Ma, W.-Y.; Hashimoto, T.; Bode, A. M.; Yang, C. S.; Dong, Z. *Cancer Res.* **2003**, *63*, 4396.
37. Schmidt, F.; Knobbe, C. B.; Frank, B.; Wolburg, H.; Weller, M. *Oncol. Rep.* **2008**, *19*, 1061.
38. Choudhury, S. R.; Karmakar, S.; Banik, N. L.; Ray, S. K. *Invest. New Drugs* **2009**. doi:10.1007/s10637-009-9324-7.
39. Park, Y. J.; Wen, J.; Bang, S.; Park, S. W.; Song, S. Y. *Yonsei Med. J.* **2006**, *47*, 688.
40. Tsvetkov, P.; Asher, G.; Reiss, V.; Shaul, Y.; Sachs, L.; Lotem, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5535.
41. Fedorov, S. N.; Radchenko, O. S.; Shubina, L. K.; Balaneva, N. N.; Bode, A. M.; Stonik, V. A.; Dong, Z. *Pharm. Res.* **2006**, *23*, 70.
42. Wang, L.; Shen, Y.; Song, R.; Sun, Y.; Xu, J.; Xu, Q. *Mol. Pharm.* **2009**, *76*, 1238.
43. Watson, J. L.; Greenshields, A.; Hill, R.; Hilchie, A.; Lee, P. W.; Giacomantonio, C. A.; Hoskin, D. W. *Mol. Carcinog.* **2009**. doi:10.1002/mc.20571.
44. Regula, K. M.; Kirshenbaum, L. A. *J. Mol. Cell. Cardiol.* **2001**, *33*, 1435.
45. Marchenko, N. D.; Moll, U. M. *Cell Cycle* **2007**, *6*, 1718.
46. Wolff, S.; Erster, S.; Palacios, J.; Moll, U. M. *Cell Res.* **2008**, *18*, 733.
47. Marchenko, N. D.; Zaika, A.; Moll, U. M. *J. Biol. Chem.* **2000**, *275*, 16202.
48. Dong, Z.; Lavrovsky, V.; Colburn, N. H. *Carcinogenesis* **1995**, *16*, 749.
49. Young, M. R.; Li, J. J.; Rincon, M.; Flavell, R. A.; Sathyanarayana, B. K.; Hunziker, R.; Colburn, N. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9827.
50. Chiu, R.; Angel, P.; Karin, M. *Cell* **1989**, *59*, 979.
51. Passegue, E.; Jochum, W.; Schorpp-Kistner, M.; Mohle-Steinlein, U.; Wagner, E. F. *Cell* **2001**, *104*, 21.
52. Le-Niculescu, H.; Bonfoco, E.; Kasuya, Y.; Claret, F. X.; Green, D. R.; Karin, M. *Mol. Cell. Biol.* **1999**, *19*, 751.
53. Kondo, T.; Matsuda, T.; Kitano, T.; Takahashi, A.; Tashima, M.; Ishikura, H.; Umehara, H.; Domae, N.; Uchiyama, T.; Okazaki, T. *J. Biol. Chem.* **2000**, *275*, 7668.
54. Kasibhatla, S.; Brunner, T.; Genestier, L.; Echeverri, F.; Mahboubi, A.; Green, D. R. *Mol. Cell* **1998**, *1*, 543.
55. Manna, S. K.; Sah, N. K.; Aggarwal, B. B. *J. Biol. Chem.* **2000**, *275*, 1329.
56. Fan, M.; Goodwin, M. E.; Birrer, M. J.; Chambers, T. C. *Cancer Res.* **2001**, *61*, 4450.
57. Zhu, B. K.; Wang, P.; Zhang, X. D.; Jiang, C. C.; Chen, L. H.; Avery-Kiejda, K. A.; Watts, R.; Hersey, P. *Anticancer Drugs* **2008**, *19*, 189.
58. Rogozin, E. A.; Urazova, L. N.; Serebrov, V. Y. *Bulletin SO RAMS (Rus.)* **2003**, *108*, 121.
59. Kyriakis, J. M.; Avruch, J. *Physiol. Rev.* **2001**, *81*, 807.
60. Ballif, B. A.; Blenis, J. *Cell Growth Differ.* **2001**, *12*, 397.
61. Rubinfeld, H.; Seger, R. *Mol. Biotechnol.* **2005**, *31*, 151.
62. Bonni, A.; Brunet, A.; West, A. E.; Datta, S. R.; Takasu, M. A.; Greenberg, M. E. *Science* **1999**, *286*, 1358.
63. Davis, R. *Cell* **2000**, *103*, 239.
64. Weston, C. R.; Davis, R. J. *Curr. Opin. Cell Biol.* **2007**, *19*, 142.
65. Huang, C.; Ma, W.-Y.; Young, M. R.; Colburn, N.; Dong, Z. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 156.