

Militarinone A induces differentiation in PC12 cells via MAP and Akt kinase signal transduction pathways

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Abstract The fungal metabolite militarinone A (MILI A) promotes neurite outgrowth in PC12 cells. This study was conducted to investigate the signaling pathways involved in the cellular differentiation processes induced by the compound, with a focus on cascades implicated with nerve growth factor (NGF)-mediated neuritogenesis. MILI A possessed pronounced amphiphilic properties. The compound rapidly accumulated in the cell membrane and was slowly released into the cytoplasm. In primed PC12 cells, an early activation of protein kinase B (Akt), representing a downstream target of phosphoinositol 3 (PI₃) kinase, and a delayed phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), and of transcription factor cAMP responsive element binding protein (CREB) was found. The NGF-dependent activation of c-Jun amino terminal kinase (SAPK/JNK1) was potentiated. Morphological differentiation of cells and the phosphorylation of specific signal molecules were blocked by the MAP kinase (MEK1) inhibitor PD098059, the PI₃-kinase (PI₃K) inhibitor wortmannin and the adenylyl cyclase inhibitor 9-cyclopentyladenine.

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1. Introduction

The development of effective treatments for neurodegenerative diseases represents a major challenge for our aging society. Prevention of neuronal apoptosis by supplementation with endogenous trophic factors has been proposed as one of the several new therapeutic strategies [1]. Clinical trials with neurotrophic factors, however, have been rather disappointing [2,3]. One reason for the failure was related to the obstacles of delivering therapeutic proteins to the central nervous system [4]. The development of non-peptidic small molecules acting on signaling pathways for neurotrophins has been proposed as an alternative [5]. Such compounds could exert neurotrophin-like activities while penetrating through the blood–brain barrier (BBB) without major difficulties.

We recently identified a novel fungal alkaloid, militarinone A (MILI A) (Fig. 1), in the screening of natural products for neuritogenic properties in PC12 cells [6–8]. Given that MILI A was the first representative of a new chemical class of neurotrophic compounds, we decided to analyze in more detail the signal transduction pathways underlying its effects in PC12 cells.

The PC12 cell line has been extensively used as model for neuronal differentiation because of its ability to differentiate into sympathetic neuron-like cells when treated with nerve growth factor (NGF) [9]. The binding of NGF to its high affinity tyrosine kinase A (TrkA) receptor triggers mainly two cascades of cellular signaling responses that mediate neurotrophic effects [10,11]. After binding to phosphotyrosine-containing recognition elements of the receptor, signal molecules on the MAPK Erk1/2 and inositol triphosphate (PI₃) kinase pathways are phosphorylated and thus activated, triggering effects on gene transcription and regulation of the cytoskeletal machinery.

There is increasing evidence that besides the Ras/ERK cascade, another pathway involving stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) has substantial influence on differentiation events. An approximately tenfold overactivation of SAPK/JNK was found in a variant PC12 cell line that spontaneously differentiates and extends neurites [12]. Recently, it was observed that the neuronal growth-associated protein SCG10, which is enriched in the growth cones of neurons, is specifically phosphorylated by SAPK/JNK. SCG10 destabilizes microtubules and thus contributes to the assembly and disassembly of microtubules upon extracellular signals transmitted by phosphorylation [13]. JNKs are rapidly activated following nerve injury and this activation persists for weeks until successful regeneration or neuronal cell death. In contrast, the stress activated kinase p38 is rapidly but only transiently activated after axotomy [14].

In addition to direct receptor tyrosine kinase (RTK) mediated signal transduction, other pathways and molecules are considered to induce biological responses such as neuronal differentiation and survival. Cyclic AMP can act as inducer of such effects, either on its own or via activation of RTKs [15]. In PC12 cells, cAMP elevation induces the development of neurites and the activation of ERK similar to the treatment with NGF [16]. Forskolin, an activator of adenylyl cyclase, increases the intracellular cAMP level, which in turn is followed by neuronal differentiation [17,18]. The transcription factor cAMP-responsive element binding protein (CREB) appears to be required for NGF-mediated induction of primary response

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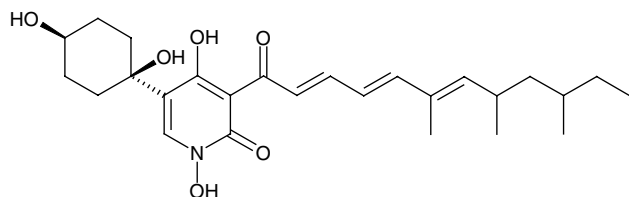


Fig. 1. Chemical structure of militarinone A (MILI A).

genes encoding transcription factors like *c-fos* and thus playing a role in the initiation and regulation of subsequent responses to NGF [19,20]. CREB binds to several separate sequences within the *c-fos* promotor, suggesting that it is a general mediator of stimulus-dependent transcription of *c-fos* [21,22]. Phosphorylation of CREB is critical for learning processes, mainly in the conversion of short-term to long-term memory [23,24].

With regard to the neuritogenic effects of MILI A in PC12 cells, we here focused on an analysis of the signaling pathways implicated in NGF-mediated differentiation processes.

2. Materials and methods

2.1. Chemicals

Antibodies that recognize CREB and the phosphorylated forms of ERK1/2 (Thr202/Tyr204), SAPK (Thr183/Tyr185), p38 (Thr180/Tyr183), Akt (Ser 473) and CREB (Ser 133) were from New England Biolabs (Beverly, USA). Anti-Akt, anti-ERK1 and anti-ERK2 were from BD Biosciences (San Diego, USA). PD098059 and wortmannin were obtained from Merck (Darmstadt, Germany) and 9-cyclopentyladenine from Sigma–Aldrich (Taufkirchen, Germany). Murine 7S NGF was purchased from Roche Diagnostics (Mannheim, Germany). MILI A was previously isolated in our laboratory [6]. The purity of the compound was >95%, as determined by HPLC and NMR. The 10 mM methanolic stock solution of MILI A was diluted in the medium to a final methanol concentration of 0.4% in the assay.

2.2. Cell culture

Rat PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Biochrom, Berlin, Germany), 10% horse serum (HS, Biochrom), 100 IE/ml penicillin and 100 µg/ml streptomycin in 5% CO₂ atmosphere.

2.3. Neurite outgrowth assay

PC12 cells were treated for 4 days with 50 ng/ml NGF in DMEM under serum starved conditions (1% HS, 0.5% FBS). This NGF-priming leads to higher TrkA receptor density and higher sensitivity to stimuli [25]. The cells were washed three times with ice cold medium to remove traces of NGF. Cells were plated in 24-well plates coated with collagen (10⁵ cells/well) and after 2 h of cultivation incubated with test substances and inhibitors, respectively, for 16–24 h. Cells were analyzed by phase contrast microscopy and photographed.

2.4. Subcellular fractionation

PC12 cells were incubated in 10 ml of DMEM in 6-well plates (2×10^7 cells/well) with MILI A (60 µM final concentration) or with 0.4% methanol as negative control. Cells were harvested at defined times, washed with phosphate-buffered saline and resuspended in ice cold lysis buffer lacking detergent Igepal CA-630 to preserve the cell membranes. After incubation for 10 min on ice, cells were homogenized by a threefold freeze/thaw cycle with liquid nitrogen. The disruption of cells was monitored by microscopy. The homogenates were centrifuged ($24\,000 \times g$, 10 min, 4 °C) to obtain a membrane pellet and a cytosolic fraction. The membrane pellet was extracted for 30 min with lysis buffer containing 1% Igepal, followed by a second centrifugation step at $24\,000 \times g$. The concentration of MILI A was determined at 380 nm from aliquots of the cytosolic fraction and membrane extract (supernatant of second centrifugation step).

2.5. Western blot analysis

Native PC12 cells were serum-starved overnight in serum-reduced DMEM (0.5% FBS, 1% HS) to reduce basal levels of phosphorylation. After treatment with test substances, cells were resuspended in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% Igepal CA-630, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mg/ml aprotinin and 1 mg/ml leupeptin and incubated for 10 min on ice. Cell lysates were separated by centrifugation at $24\,000 \times g$ for 10 min at 4 °C. The protein concentration of resulting supernatants was measured by the Bradford method (Coomassie Protein Assay, Pierce, Rockford, USA) and the same amounts of total protein were loaded in each lane.

After 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Invitrogen, Karlsruhe, Germany), the separated proteins were transferred to PVDF-membranes (BioRad, München, Germany). Immune complexes with antibodies were visualized by enhanced chemiluminescence detection system (Pico Substrate, Pierce, Rockford, USA) and X-ray films.

3. Results and discussion

3.1. Militarinone A accumulates in the cell membrane

Differentiation, survival and neuronal development are regulated by many different external signals. Membrane bound receptors like TrkA (NGF-receptor), or signal molecules such as adenylyl cyclase transmit signals to the cytoplasmatic pathways and the nucleus. The structure (Fig. 1) and chromatographic behavior of MILI A [6] suggested pronounced amphiphilic properties and, hence, the possibility for interaction with membrane associated proteins. We therefore determined the concentration of MILI A in the soluble cytoplasmic fraction and in the nuclear and cytoplasmic membrane extracts after exposure of PC12 cells for different times of incubation. The concentration of MILI A was determined by UV–Vis spectroscopic measurement at the absorption maximum of the compound. The curves obtained for the membrane and cytosolic fractions are shown in Fig. 2. A maximal concentration in the cytosolic fraction was reached after 6 h with a value of OD₃₈₀ 6. The concentration in the membrane extract was fourfold higher (OD₃₈₀ 24). A further increase of absorption (OD_{max} of 36 after 24 h) was detected in the membrane extracts.

A direct interaction with the extracellular and/or trans-membrane domains of signal proteins like TrkA, but also of membrane bound molecules such as adenylyl cyclase was, in principle, possible. On the other hand, the enrichment of MILI A in the membrane could change membrane fluidity and thus modulate the function and activity of signal molecules in a non-specific manner [26,27]. Given that a portion of the administered MILI A reached the cytosol, a direct effect on intracellular was not excluded at this point.

3.2. Militarinone A activates MAP kinases

To examine whether the MILI A-mediated neurotrophic effect involved the activation of MAP kinases ERK1 and ERK2, we checked the phosphorylation state of these kinases in PC12 cells after different times of treatment. A noticeable increase of phosphorylated ERK1/2 was found after 2 h of treatment and a maximal effect was obtained after 24 h (Fig. 3A). The degree of phosphorylation was comparable to that obtained by treatment with NGF (10 ng/ml). There was, however, a significantly different time course of the activation. NGF caused rapid activation of ERK1/2 and a return to basal levels after 20 h (data not shown). The delayed onset

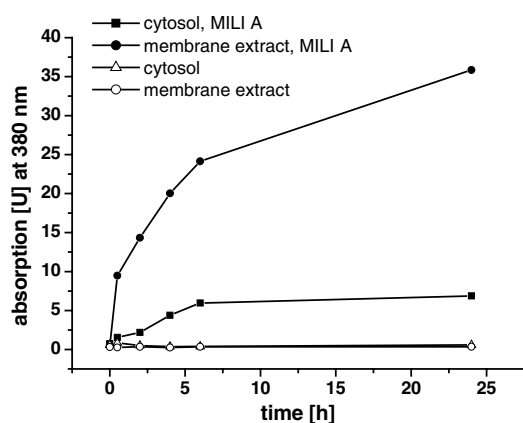


Fig. 2. Time-dependent distribution of MILI A in PC12 cell fractions. The graph shows the concentration of MILI A in the soluble (cytosol) fraction and in the pellet (cell membrane and nucleus) when cells were exposed to the compound over an incubation time of up to 24 h. The relative concentrations were determined via measurement at the absorption maximum of MILI A at 380 nm.

and prolonged activation of these MAPKs by MILI A seemed in accord with its slow appearance in the cytosolic fraction.

Magnitude and duration of ERK activation determine the nature of the cellular response [28]. In PC12 cells, a prolonged activation of the MAPKs caused by NGF via the TrkA receptor induces differentiation, whereas the phosphorylation of ERK1/2 as a consequence of EGF receptor activation is transient and stimulates proliferation. It therefore seems plausible that the late and prolonged activation of ERK1/2 by MILI A could be responsible for the neuritic differentiation.

The MEK inhibitor PD098059 (20 μ M) completely blocked both the MILI A induced phosphorylation of ERK1/2 (Fig. 3C) and the outgrowth of neurites (Fig. 5B). Interestingly, also the PI₃K inhibitor wortmannin (100 nM) caused a complete block of neuritogenesis (Fig. 5C). These data suggest that other pathways such as the PI₃ kinase and Ras/ERK cascades could be involved in the signal transduction modulated by MILI A.

The exposure of PC12 cells to MILI A potentiated the NGF-mediated SAPK/JNK activation (Figs. 3A and 4). The weak phosphorylation signal after NGF addition was transient and characterized by a rapid onset (within 30 min) [31]. In our experiments, MILI A did not influence the duration of NGF-mediated SAPK/JNK activation.

SAPK/JNK was initially considered as an exclusively stress activated kinase, but there is increasing evidence for specific physiological functions in cell differentiation [13]. Activation of SAPK/JNK is involved in the spontaneous neuritogenesis in PC12 cells and may be an alternative pathway in the regulation of neuronal differentiation [12]. An active cross-talk between SAPK/JNK and MAPK/ERK pathways results in the regulation of c-Jun expression and its activation state and finally in differentiation response [31]. This cooperation appears necessary for regulation and maximal activation of the neurofilament light chain promoter (NFLC), which is essential for the expression of major proteins of the neuronal cytoskeleton [32].

In contrast to the effects on MAP kinases ERK1/2 and SAPK/JNK, treatment of PC12 cells with MILI A did not activate the stress activated MAP kinase p38 at defined time of

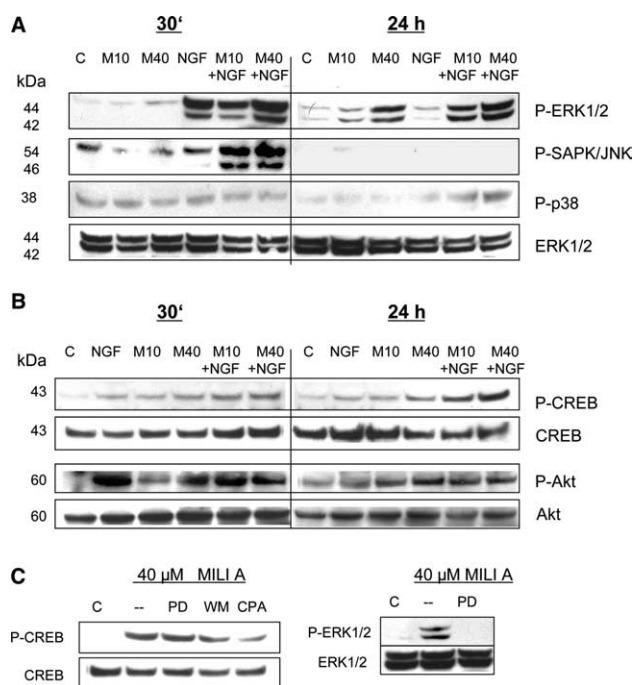


Fig. 3. The activation state of different signaling cascades was investigated by immunoblotting of total PC12 cell lysates. Cells were exposed to MILI A (10 and 40 μ M final concentrations) and/or NGF (10 ng/ml) for 30 min or 24 h, respectively, and subsequently transferred in lysis buffer. Equal amounts of cellular proteins were subjected to SDS-PAGE, electrotransferred to PVDF-membrane and treated with phosphorylation state specific antibodies. Uniform protein loading was checked using anti-ERK1/2 antibodies. The loading control also represents the other two MAP kinases because of parallel loadings of same extracts. (A) MILI A stimulated ERK1/2 activation and potentiated the NGF-mediated SAPK/JNK phosphorylation. Immunoblots with phospho-MAP kinase antibodies (p-ERK1/2, p-SAPK/JNK, p-p38). (B) Activation of CREB and Akt (after 30 min). (C) Inhibition of ERK1/2 activation by PD098059 and of CREB activation by 9-cyclopentyladenine. Prior to MILI A exposition, PC12 cells were pretreated for 30 min with selective inhibitors of MEK (PD098059, 20 μ M), PI₃K (wortmannin, 100 nM) and adenylyl cyclase (9-cyclopentyladenine, 200 μ M). C, control; M10, MILI A 10 μ M; M40, MILI A 40 μ M; PD, PD098059; WM, wortmannin; CPA, 9-cyclopentyladenine.

exposure (30 min, 24 h), neither in the presence of NGF nor on its own (Fig. 3A). This result suggests that MILI A does not induce a non-specific stress reaction in the cells represented by activation of MAP kinases.

As previously shown [6], neuritic processes developed after 16–24 h stimulation of NGF-primed PC12 cells with MILI A, whereas the control cells without further treatment retained a spherical shape. The pre-treatment of cells (priming) with NGF under serum-reduced conditions has a significant influence on intensity and occurrence of the MILI A effect [7]. It is known that a continuous presence of NGF is required for complete differentiation of PC12 cells. The reaction of PC12 cells to this pre-treatment is not clear in detail, but an increase of TrkA receptors density following the stimulation of a transcription-dependent synthesis and accumulation of material, which enables the cells to produce neuritic processes, have been discussed [25,29]. This is in agreement with our findings concerning the noticeable increase in responsiveness of TrkA overexpressing PC12 cells [30] to MILI A (data not shown).

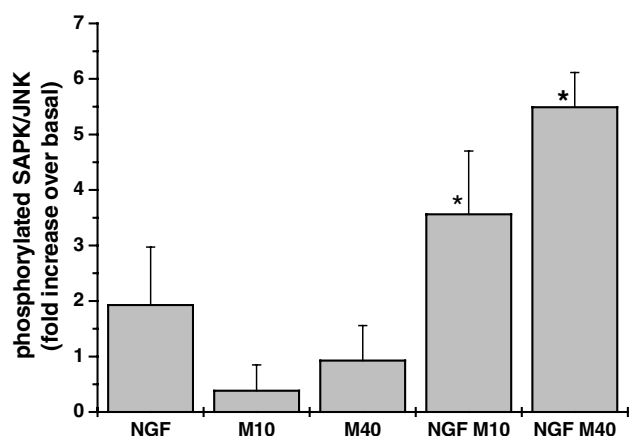


Fig. 4. Potentiation of NGF-mediated SAPK/JNK activation by MILI A. Quantitative analysis of band density (54 kDa) of three independent Western blot analyses. PC12 cells were treated for 30 min with 40 μ M MILI A, followed by separation on SDS-PAGE of total cell lysates adjusted to same protein amount, electrotransfer on PVDF-membrane and immunoblot with phospho-specific antibody against SAPK/JNK. The data are presented as means (\pm S.D.) of three independent experiments. Significant ($P < 0.05$) increases in phosphorylation of SAPK/JNK compared to NGF control values are indicated with an asterisk.

3.3. Militarionone A induces the phosphorylation of Akt

NGF activates different intracellular signaling pathways leading to neurite outgrowth and cell survival. The inhibition of MILI A induced neurite formation by wortmannin suggested involvement of the PI_3K pathway. Indeed, microinjection of a constitutively active mutant of phosphatidylinositol-3 kinase induced neurite formation in PC12 cells through the Rac-JNK transduction pathway [33].

The effects of MILI A on the PI_3K pathway were investigated via the measurement of the phosphorylation of protein kinase B/Akt, a downstream target of PI_3K . We found a rapid and distinct increase of Akt phosphorylated at Ser473 (Fig. 3B) after 30 min of exposure to MILI A. The signal was remarkably persistent, as pAkt was detected even after 24 h of exposure. This was in contrast to the more transient response induced by treatment with NGF. The suppression of MILI A-mediated neuritogenesis by the PI_3K inhibitor wortmannin (Fig. 5) corroborates the involvement of the PI_3K pathway in the neuritogenic effect of this alkaloid.

3.4. Activation of transcription factor CREB

Activation of the ERK/RSK pathway in PC12 cells by a treatment with NGF leads to the phosphorylation of CREB at Ser-133 [34]. MILI A had a similar effect. Incubation for 30 min resulted in the phosphorylation level of CREB comparable to that induced by NGF (Fig. 3B). The combination of NGF and MILI A led to a remarkable enhancement and persistence of phosphorylated CREB and was still pronounced after an exposure of 24 h.

To elucidate if the activation of CREB resulted from adenylyl cyclase-mediated elevation of cAMP levels, we applied the inhibitor 9-cyclopentyladenine (CPA; 200 μ M). A moderate decrease of activated CREB was observed (Fig. 3C). Interestingly, CPA completely blocked the neuritogenesis in PC12 cells (Fig. 5D). The possibility of a direct interaction of MILI A with adenylyl cyclase was checked in assays with

isolated PC12 membranes (data not shown). However, there was no indication of an activating effect similar to that of the well-known activator of adenylyl cyclase activity forskolin [16]. In contrast to 9-cyclopentyladenine, both the MEK inhibitor PD098059 (10 μ M) and the PI_3 kinase inhibitor wortmannin (100 nM) had no detectable effect on MILI A mediated CREB phosphorylation (Fig. 3C). This suggests that the activation of CREB occurs not via the ERK and PI_3 kinase pathways but rather by processes like adenosine receptor coupled modulation of adenylyl cyclase activity or other cAMP elevating mechanisms [35]. Given the interaction of MILI A with membrane bilayers, the effect on adenylyl cyclase could be indirectly caused by a change in membrane properties. The membrane fluidity reportedly affects intracellular cAMP levels [26,27].

In summary, the fungal metabolite MILI A exerts its neurotogenic activity in PC12 cells via activation of pathways that are involved in NGF-mediated neuritic differentiation. However, MILI A activates the MAP kinase and Akt/ PI_3 kinase pathways with a time course that clearly differs from a NGF-mediated response. It is thus unlikely that MILI A could act as a direct ligand of TrkA and thereby trigger the MAP kinase cascade. Our data suggest rather an indirect effect on the TrkA receptor via the elevation of the intracellular cAMP levels, which is followed by transphosphorylation of the receptor. The potentiation of NGF-mediated phosphorylation of SAPK/JNK corroborates this notion. As a next step, the time course of TrkA receptor phosphorylation will be studied. Also, the possibility of an activation of kinases downstream of TrkA needs to be addressed.

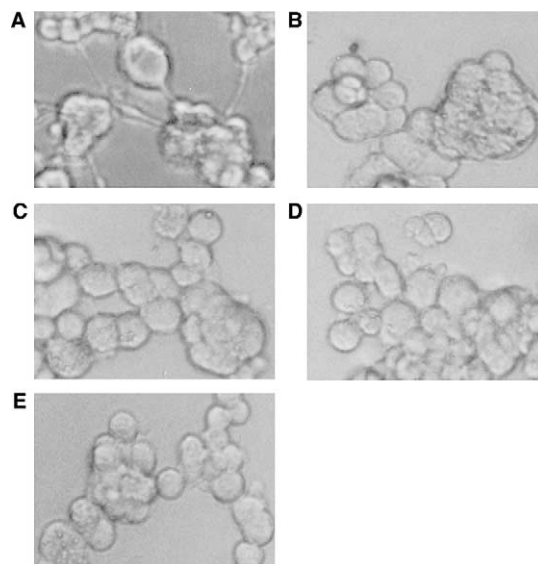


Fig. 5. Selective inhibition of MILI A-induced neuritic differentiation of NGF-primed PC12 cells. (A) Neuritogenesis following MILI A treatment (40 μ M final concentration) was blocked by pretreatment for 30 min with (B) the MEK inhibitor PD098059 (20 μ M), (C) the PI_3K inhibitor wortmannin (100 nM), and (D) the adenylyl cyclase inhibitor 9-cyclopentyladenine (200 μ M). (E) Primed PC12 cells treated with solvent (methanol 0.25% final concentration) alone were used as negative control. These cells did not show any spike formation.

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