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Melia toosendan Regulates PC12 Cell Differentiation via the Activation of Protein Kinase A and Extracellular Signal-Regulated Kinases

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Key Words

Melia toosendan · Extracellular signal-regulated kinase · Protein kinase A · Neuronal differentiation · Mitogen-activated protein kinase · PC12 cells

Abstract

With a history of several thousand years, traditional Chinese medicine has been well documented to be effective in the treatment of various disorders. We have investigated the activities of potential neuroactive compounds in traditional Chinese medicine such as *Melia toosendan* using an in vitro model system, rat pheochromocytoma PC12 cells. We report here that treatment of PC12 cells with a crude extract of the fruits of *M. toosendan* reduces cell growth in a dose-dependent manner without detectable cytotoxicity. Upon treatment with *M. toosendan*, PC12 cells exhibit robust neurite outgrowth, to a greater extent than that observed with nerve growth factor. Results obtained with specific kinase inhibitors and protein kinase A-deficient PC12 cells indicate that the actions of *M. toosendan* are mediated by the activation of protein kinase A and extracellular signal-regulated kinases.

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Introduction

Neurodegenerative diseases such as Alzheimer's and Parkinson's are prevalent among the elderly. They are progressive and ultimately fatal neurological disorders for which there is no effective treatment at present. Most of the therapy currently available is targeted at the replacement of the neurotransmitter(s) or inhibition of the degrading enzyme [1]. Promising results have been reported for the neuroprotective effects of neurotrophic factors in the animal models of neurodegenerative diseases, with nerve growth factor (NGF) as the prime candidate [1, 2]. Recent studies also showed that glial cell line-derived neurotrophic factor (GDNF) supports the survival of dopaminergic neurons in vitro and in animal models of Parkinson's disease [3]. However, difficulty encountered in the delivery of NGF or GDNF protein to the brain [3, 4] and poor bioavailability at the desired target sites [5] severely hampered the progress of clinical trials involving neurotrophic factors.

Natural compounds in traditional Chinese medicine (TCM) provide a unique element of molecular diversity and biological activity that has proven to be invaluable in the search for drug leads. As such, natural products offer major opportunities in the discovery of novel drug candidates as potential therapeutic agents. For example, recent studies indicate that huperzine A, a lycopodium alkaloid

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from the leaves of the Chinese herb, *Huperzia serrate*, protects PC12 cells from hydrogen peroxide-induced injury [6] and holds promise in improving memory and learning ability in Alzheimer's patients [7]. These studies highlight the potential of TCM as a valuable source of drug leads and suggest that further research focused on delineating the molecular mechanisms underlying the actions of TCM may yield valuable information for potential drug development.

We have recently investigated the presence of neuroactivities in TCM such as *Melia toosendan* Sieb. et Zucc. (hereon *M. toosendan*) [8]. A previous report indicates that *M. toosendan* is effective for the treatment of malaria and stomach aches caused by roundworm [9]. The chemical structures of several limonoids isolated from the methanolic fraction of *M. toosendan* have been identified [10]. The anti-tumor effect of several of these limonoid compounds has been suggested to result from the growth-inhibitory effects observed in human cancer cell lines [10]. Moreover, toosendanin, a triterpenoid derivative extracted from the bark of *M. toosendan*, has been reported to act as a presynaptic blocker by decreasing the number of vesicles at the neuromuscular junction and, more recently, to induce apoptosis of PC12 cells [9, 11, 12]. However, the molecular mechanisms mediating the neuroactive effects of *M. toosendan* have not been elucidated.

In the present study, we have examined the ability of *M. toosendan* to affect neuronal differentiation using rat pheochromocytoma PC12 cells as an in vitro model system. We report here that treatment of PC12 cells with *M. toosendan* results in reduced cell growth and enhanced neurite outgrowth. These effects of *M. toosendan* are mediated by the activation of protein kinase A (PKA) and extracellular signal-regulated kinases (ERKs). Taken together, our findings demonstrate the presence of neuroactive compounds in *M. toosendan* that promote neuronal differentiation and add this herb to the class of agents that can activate the PKA/ERK-signaling pathway.

Materials and Methods

Preparation of M. toosendan *Extracts*

Fruits of *M. toosendan* (20 kg) were cut into small pieces and incubated with 95% ethanol in a bath. After reflux for 2 h, the solution was filtered with cotton gauze. The filtrate was then collected and the incubation procedure was repeated twice with the residue mixed with 95% ethanol. All the filtrates were pooled together and concentrated with a rota-evaporator under reduced pressure at 70–80°C. After the concentration procedure, 500 g of *M. toosendan* extract was obtained. The extract was dissolved in DMSO (final culture concentration, <0.5%). Our studies indicated that the solvent, at the concentration used, had no effect on cell viability.

Cell Culture

PC12 cells were routinely cultured in growth medium, i.e. Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% heat-inactivated fetal bovine serum (HIFBS, v/v), 6% heat-inactivated horse serum (HIHS, v/v), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). PC12 cell differentiation was induced by switching the growth medium to low serum medium, i.e. supplemented with 1% HIFBS (v/v) and 1% HIHS (v/v) and adding 50 ng/ml NGF (Boehringer Mannheim) for various periods of time as indicated. The amount of DMSO added to the control cultures was equivalent to that used in the *M. toosendan*-treated group. PKA-deficient PC12 cell line, A126–1B2, was a gift from Dr. Kevin A.W. Lee (Department of Biology, Hong Kong University of Science and Technology). These cells were normally cultured on collagen-coated dishes and maintained under the same condition as wild-type PC12 cells. Mouse neuroblastoma cells (Neuro2A; N2A), mouse muscle cells (C2C12) and monkey kidney cells (COS-7) were routinely cultured in DMEM supplemented with 5, 20 or 10% fetal bovine serum, respectively, according to protocols from American Type Culture Collection (ATCC).

Cell Growth of PC12 Cells

Total viable cells were assessed by a modified MTT assay (Roche). PC12 cells were plated on 96-well plates (2,000 cells/well) in growth medium. After overnight culture, cells were treated with increasing concentrations of *M. toosendan*. MTT assays were performed as previously described [13]. Viability is expressed as percent of solvent-treated control ± SEM (n = 6 dishes).

Cytotoxicity Assays

The Cytotoxicity Detection Kit (Roche) used was based on the detection of lactate dehydrogenase (LDH) released from dead cells as a result of cytotoxicity. Cell-free culture supernatants from *M. toosendan*-treated PC12 cells were collected and then transferred to multi-titer plates. A substrate mixture containing tetrazolium salts was added, and then incubated for 0.5 h. The formazan dye formed was quantitated by measuring the absorbance at 490 nm.

The activity of caspase enzyme was measured by the cleavage of a peptide substrate for caspase-3 (AcDEVD-AMC). After incubation for at least 12 h, PC12 cells were lysed with Tris-lysis buffer as suggested by manufacturer (Promega). Forty m*M* HEPES containing 10% glycerol and 2 m*M* DTT was then added to the lysate with 1 µl of 7-amino-4-methoxy coumarin. The mixture was incubated for 75 min at 37°C. The resulting optical density at 360 and 460 nm wavelengths was determined.

Protein Preparation, Western Blot Analysis and Antibodies

PC12 cells were lysed on ice in RIPA buffer [13]. Aliquots of each sample containing equal amounts of proteins were resolved on SDS-PAGE gels (7.5 or 10%) and subjected to Western blot analysis as previously described [13]. Monoclonal mouse antibodies specific for the phosphorylated and unphosphorylated forms of neurofilament-160 (Zymed) were used as neuronal markers in the study. Rabbit polyclonal anti-sera detecting the phosphorylated and unphosphorylated forms of p44/42 mitogen-activated protein (MAP) kinases (ERKs; ERK1/2), c-Jun NH_2-terminal kinase (JNK1/2), p38 and TrkA were obtained from New England Biolabs.

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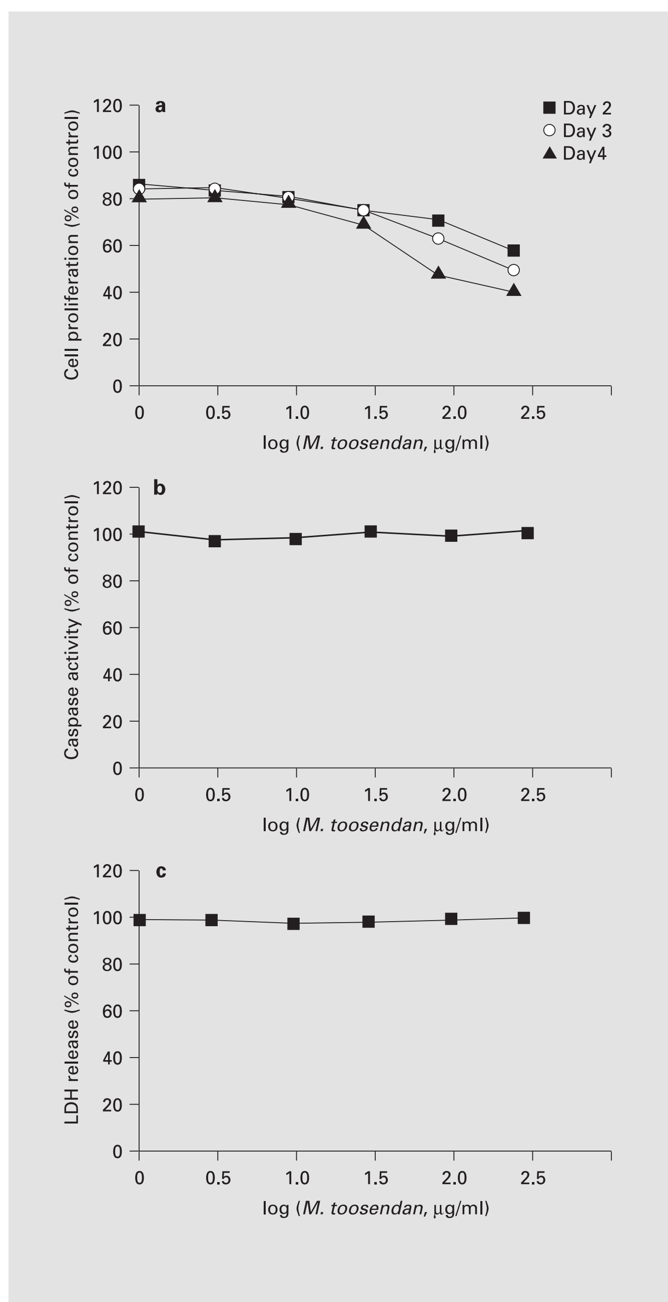

Fig. 1. *Melia toosendan* treatment of PC12 cells resulted inreduction of cell growth without detectable cytotoxicity. **a** Effect of *M. toosendan* on PC12 cell growth. Cell survival was quantified by MTT assay after exposure to various concentrations (1–300 µg/ml) of *M. toosendan* for 2–4 days. **b** Caspase-3 activity in PC12 cells treated with *M. toosendan* (1–300 µg/ml) for 4 days. The mean fluorescence units per microgram of protein was shown as percent of solvent control. Treatment of PC12 cells with staurosporine (1 µ*M*) resulted in >700% increase in caspase-3 activity (data not shown). **c** LDH release to the culture medium following treatment of PC12 cells with *M. toosendan* (1–300 µg/ml) for 18 h. Results from three independent experiments were expressed as percent of solvent control ± SEM (n = 4 dishes). Error bars were too small to be depicted.

Protein Kinase A Assay

To measure the PKA activity, total cell extracts of PC12 cells were prepared as described. The PKA activity was measured by using the PepTag assay for non-radioactive detection of cAMP-dependent PKA (Promega) as recommended by the manufacturer. The final reaction products were analyzed on 1% agarose gel. Only the phosphorylated peptide was quantitated.

Statistical Analysis

Data are presented as mean value ± SEM. For simple statistical comparisons, such as treatment compared with control, one-way analysis of variance (ANOVA) was carried out to determine the significance, followed by Dunnett's t test. For dose- or time-dependent effects, one-way ANOVA followed by Fisher's protected least-significant difference (PLSD) test were used. A p value of <0.01 is considered significant.

Results

M. toosendan *Inhibited the Cell Growth of PC12 Cells with No Detectable Cytotoxicity*

As a first step to identify the presence of neuroactive compounds in *M. toosendan*, we examined the ability of this herb to induce neuronal differentiation in PC12 cells. The effect of *M. toosendan* on the cell growth of PC12 cells was examined by monitoring the metabolic activities of PC12 cells after treatment with increasing concentrations (1–300 µg/ml) of *M. toosendan* for 2–4 days (fig. 1a). A significant reduction in cell growth was observed following treatment with *M. toosendan* (100 µg/ml) for 2–4 days when compared with the solvent control. The effect of this herb extract on cell growth observed in PC12 cells was reminiscent of the growth inhibition reported in cancer cell lines [10].

To examine whether the reduction in cell growth was due to cytotoxicity, caspase-3 activity and release of LDH were monitored in PC12 cells after treatment with *M. toosendan* (1–300 µg/ml). It was observed that *M. toosendan* did not induce any detectable change in caspase-3 activity or LDH release when compared with DMSO-treated control cells at 12 h (data not shown) or after 4 days (fig. 1b, c).

M. toosendan *Induced the Neuronal Differentiation of PC12 Cells Maintained in Growth Medium*

To examine the morphological effect of *M. toosendan* on PC12 cells, we exposed the cultures maintained in growth medium to NGF (50 ng/ml) or *M. toosendan* (30 µg/ml) for 5 days. While PC12 cells did not extend neurites in the absence of NGF on day 3, morphological changes occurred in PC12 cells treated with NGF or *M. toosendan* (fig. 2a). PC12 cells treated with *M. toosendan* exhibited neuronal phenotype, including compaction

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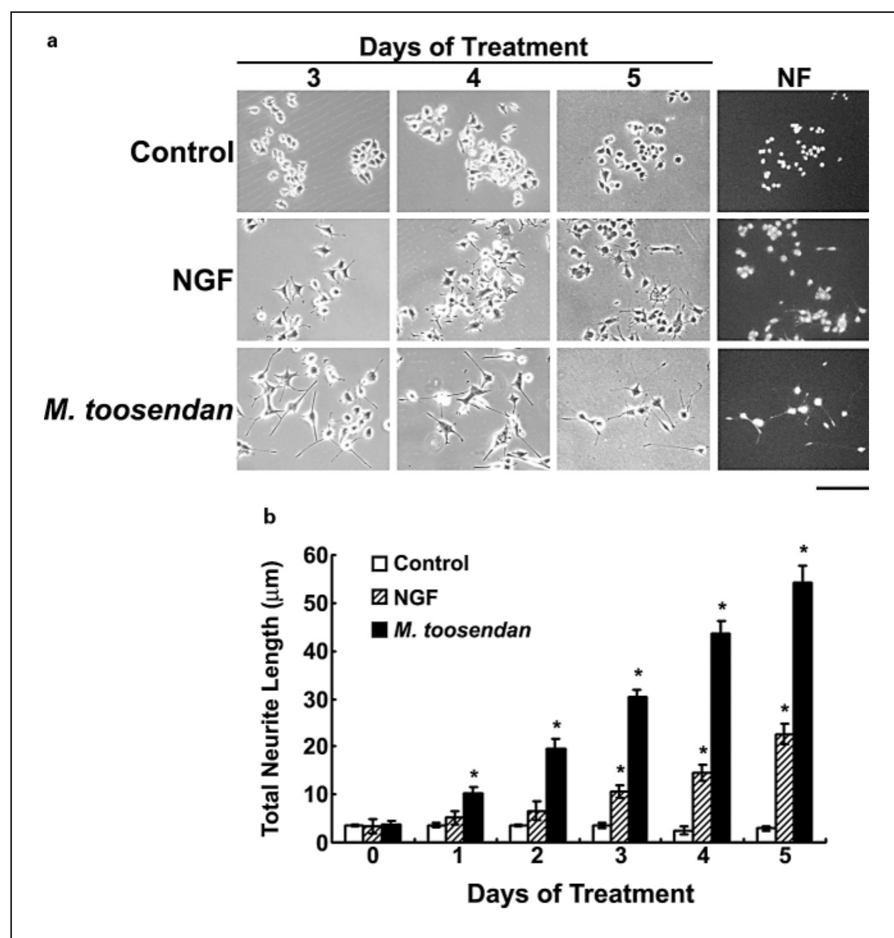

Fig. 2. *Melia toosendan* induced neurite growth of PC12 cells. **a** Phase-contrast photomicrographs showing time-dependent induction of neurite outgrowth by *M. toosendan* (30 μg/ml). The right panels show the immunofluorescent staining of phospho-neurofilament H (NF) on day 5. Scale bar: 50 μm. **b** Neurite outgrowth was quantified as described in Materials and Methods. Results represent the mean value expressed in neurite length per cell ± SEM (n = 4 dishes). * $p < 0.01$ compared with DMSO controls on the same day.

of cell bodies and extension of neurites. After 5 days of *M. toosendan* treatment, more than 90% of the PC12 cells bore numerous and elongated neurite extensions (~54.2 μm/cell; fig. 2). When maintained under mitogenic conditions, PC12 cells treated with NGF only displayed moderate neurite extension (~22.6 μm/cell; fig. 2) and did not establish a neurite network until later time points (data not shown). Interestingly, treatment with *M. toosendan* (30 μg/ml) resulted in more robust extension of neurites when compared to NGF treatment (fig. 2a). Expression of a neuronal marker, phosphorylated neurofilament (pNF160), in these neurites was confirmed by immunocytochemical analysis (fig. 2a).

MAP Kinases Were Activated by Treatment of PC12 Cells with M. toosendan

It is well documented that MAP kinase pathways are crucial for NGF-induced neuronal differentiation [14]. In particular, the initiation of neurite outgrowth of PC12 cells requires activation of the Ras/ERK pathway [14]. We have therefore examined the ability of *M. toosendan* to activate ERK1/2 in PC12 cells and another neuronal cell line, Neuro 2A cells (fig. 3a). We found that treatment of these neuronal cells with *M. toosendan* resulted in ERK1/2 activation, while phosphorylation of ERK1/2 was not observed at comparable concentrations in other non-neuronal cell types such as C2C12 and COS-7 cells (fig. 3a). Consistent with these observations, *M. toosendan* also did not reduce the proliferation of non-neuronal cells (fig. 3b), suggesting that the effects of *M. toosendan* was neuro-specific.

It has been reported that sustained activation of ERK1/2 for an extended duration of more than 2 h is critical for neurite elongation and initiation [14]. We found that treatment of PC12 cells with *M. toosendan* treatment resulted in the activation of ERK1/2 in a dose- and time-dependent manner (fig. 3c). Significant ERK1/2 phosphorylation was observed upon treatment with *M. toosen-*

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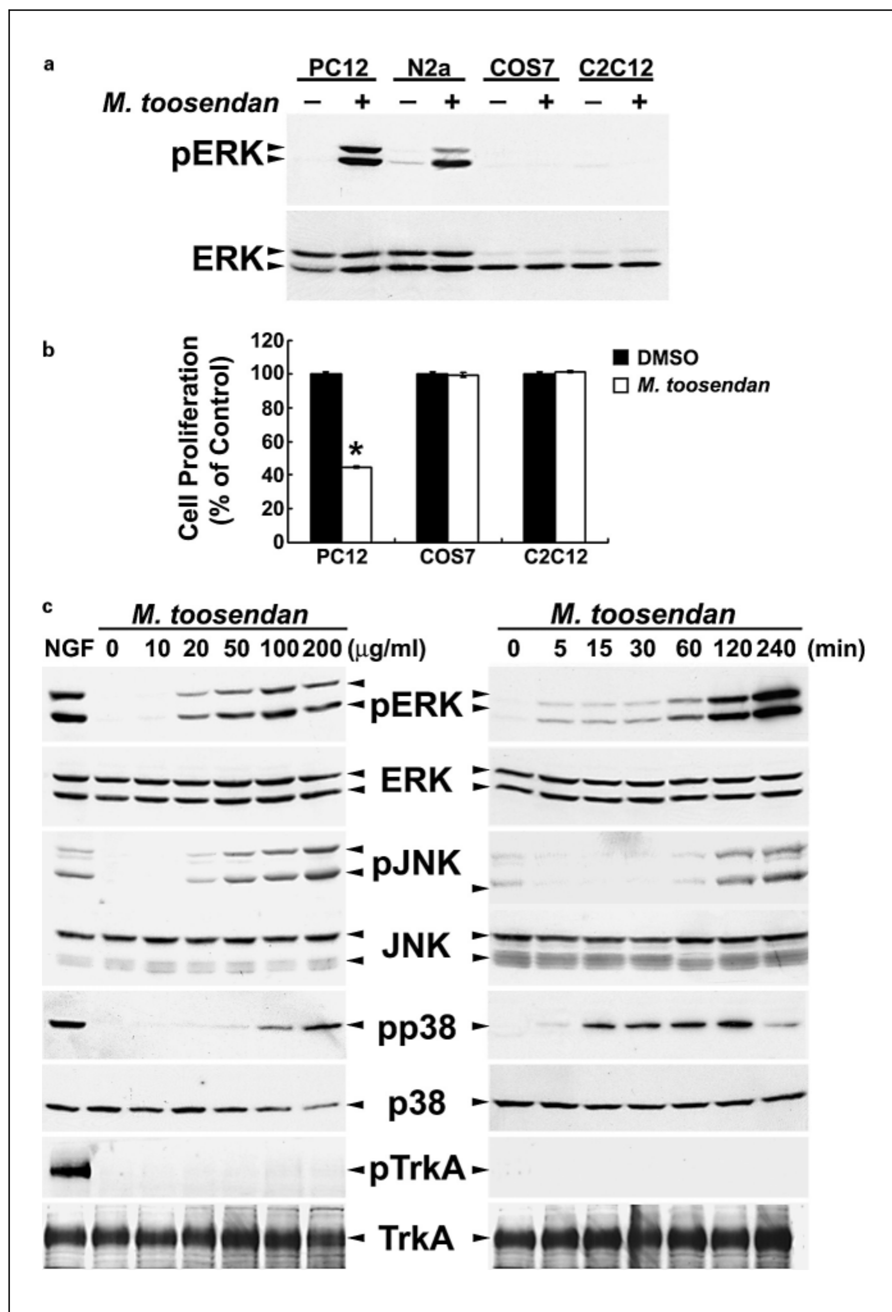

Fig. 3. Activation of MAP kinases was induced by treatment with *Melia toosendan.* ERK1/2 phosphorylation (**a**) and cell proliferation (**b**) were determined in PC12, Neuro 2A (N2A), COS7 and C2C12 cells following treatment with (+) or without (–) *M. toosendan* (50 μg/ml). * $p < 0.01$ compared with DMSO controls. **c** MAP kinases were activated following treatment with *M. toosendan.* PC12 cells were serum-starved for 4 h, and then treated with *M. toosendan* (0–200 μg/ml) for 1 h (left panels) or *M. toosendan* (50 μg/ml) for various time periods (right panels) as indicated. NGF (50 ng/ml) was used as the positive control. The antibodies used for this study are specific for the phosphorylated (p) form or total TrkA, ERK1/2 (ERK), JNK and p38 proteins as indicated. Experiments were repeated at least three times and similar results were obtained.

dan (20–200 μg/ml) within 5 min and was sustained for at least 4 h. Other members of MAP kinase family, such as JNK1/2 and p38 MAPK, have previously been reported to be phosphorylated during NGF-induced differentiation of PC12 cells [15, 16]. We found that both JNK1/2 and p38 were phosphorylated by *M. toosendan* in a dose- and time-dependent manner (fig. 3a).

ERK1/2 Activated by M. toosendan *Was TrkA-Independent and PKA-Dependent*

Since the Ras/ERK cascade can be initiated by the NGF-induced tyrosine phosphorylation of TrkA [17, 18], we examined the ability of *M. toosendan* to activate TrkA. It was observed that *M. toosendan* did not induce detectable phosphorylation nor protein expression of TrkA at concentrations up to 200 μg/ml (fig. 3c). To further inves-

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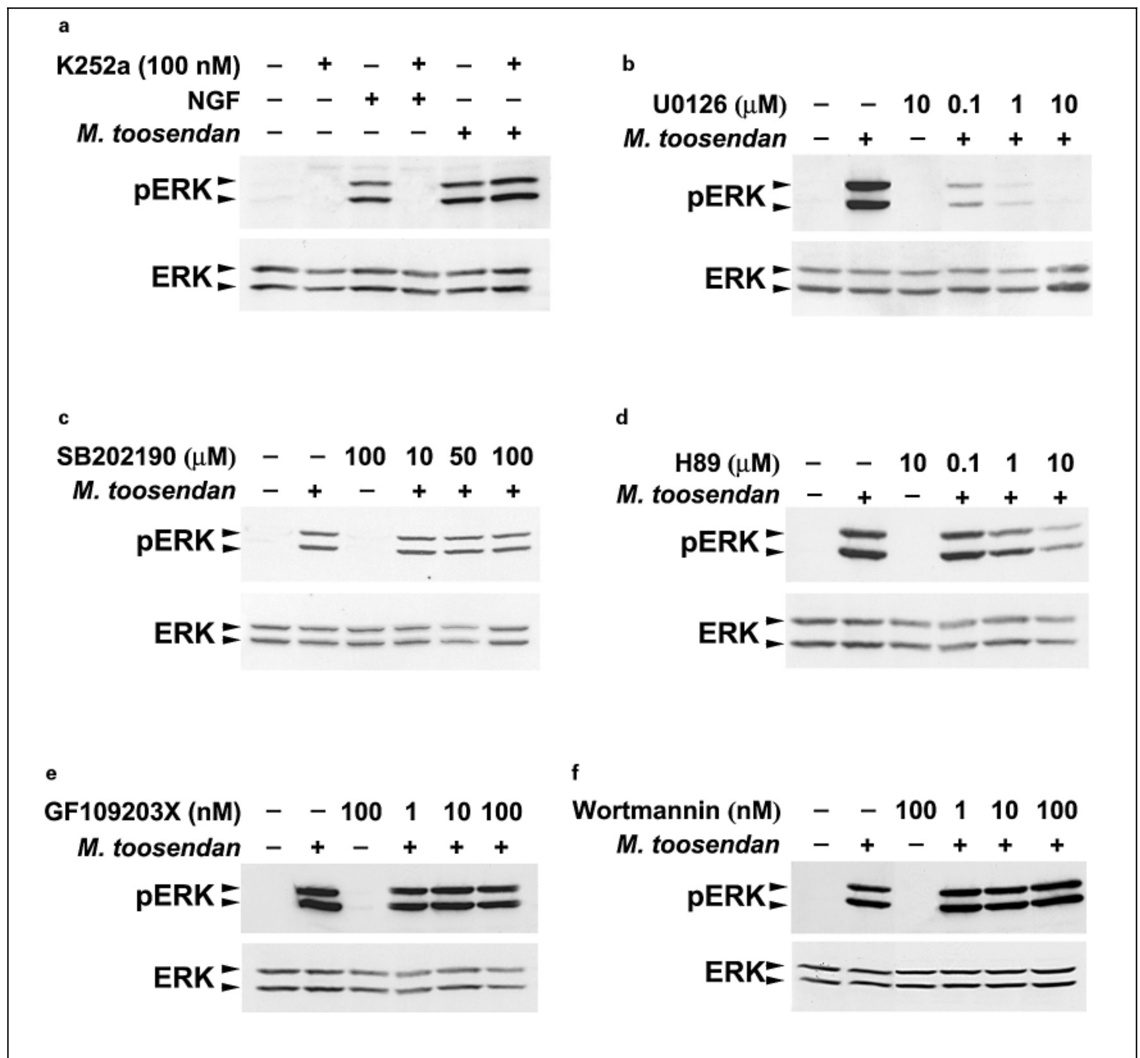

Fig. 4. Activation of ERK1/2 by *Melia toosendan* was dependent on MEK1/2 and PKA. After serum starvation, PC12 cells were treated with K252a (100 n*M*, **a**), U0126 (0.1–10 μ*M*, **b**), SB202190 (10–100 μ*M*, **c**), H89 (0.1–10 μ*M*, **d**), GF109203X (1–100 n*M*, **e**) or Wortmannin (0.1–10 μ*M*, **f**) for 1 h prior to the treatment of *M. toosendan* (30 μg/ml). The antibodies used for this study are specific for the phosphorylated (p) form or total ERK1/2 (ERK) proteins as indicated. Experiments were repeated at least three times and similar results were obtained.

tigate the potential involvement of the TrkA pathway in the activation of ERK1/2 induced by *M. toosendan*, the effect of a specific inhibitor of TrkA tyrosine kinase, K252a [19], on ERK1/2 phosphorylation was examined. While K252a (100 n*M*) completely inhibited TrkA phosphorylation induced by NGF, it did not attenuate the ERK1/2 activation induced by *M. toosendan* (fig. 4a).

As a first step to dissect the pathway that led to ERK1/2 phosphorylation, we examined whether *M. toosendan*-induced ERK1/2 activation occurred via the phosphorylation of MEK1/2 [20]. Preincubation of PC12 cells with U0126 (1 μ*M*), a selective inhibitor of MEK1/2, abolished the *M. toosendan*-induced ERK1/2 activation (fig. 4b). Pretreatment with a p38 inhibitor, SB202190, did not attenuate the phosphorylation of ERK1/2 observed (fig. 4c). To investigate if PKA, PKC or PI3-kinase was required for *M. toosendan*-induced ERK1/2 activity, we studied the effect of various inhibitors, H89 (PKA inhibitor); GF109203X (PKC inhibitor) and Wortmannin (PI-3 kinase inhibitor) on ERK1/2 activation in

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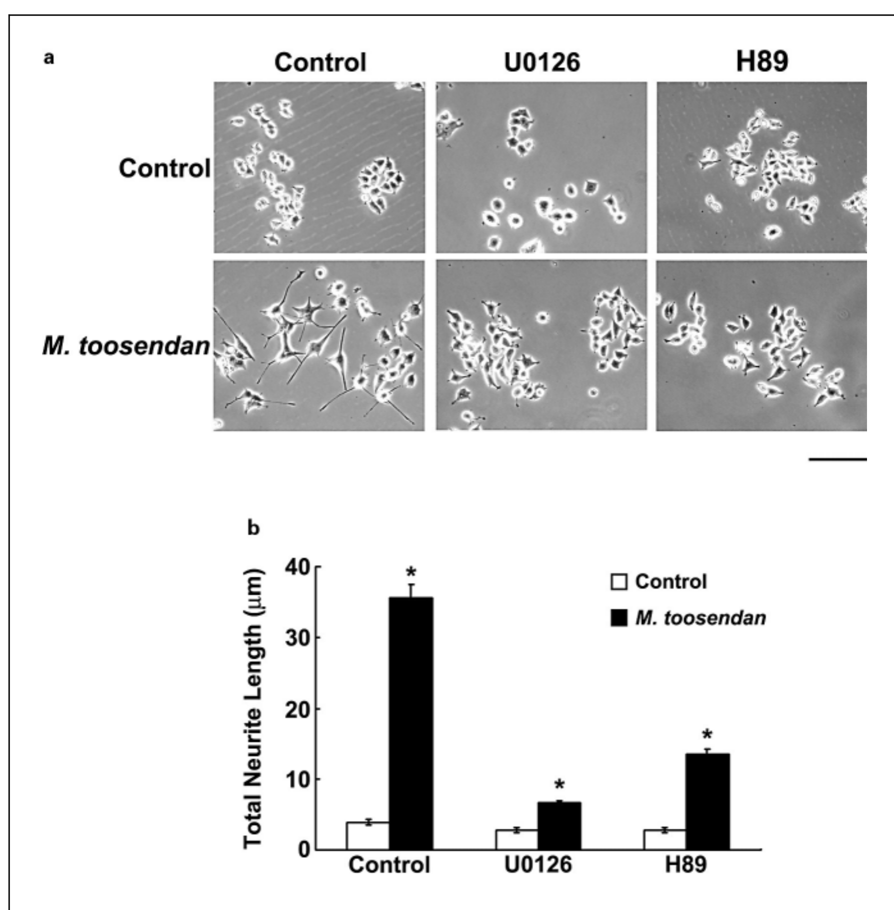

Fig. 5. The effects of U0126 and H89 on *Melia toosendan*-induced neurite outgrowth in PC12 cells. **a** Phase-contrast photomicrographs of day-3 cultures showing the inhibitory effects of U0126 and H89 on *M. toosendan*-induced neurite outgrowth. Scale bar: 50 μm. **b** Neurite outgrowth was quantified as described in Materials and Methods. Results represent the mean value expressed in neurite length per cell ± SEM (n = 5 dishes). * $p < 0.01$ compared with DMSO controls.

response to *M. toosendan* (fig. 4d–f). While pretreatment with H89 (10 μ*M*) for 1 h inhibited the ERK1/2 activation induced by *M. toosendan* (fig. 4d), the other two inhibitors at effective concentrations that blocked PKC [21] and PI-3 kinase [22] failed to attenuate the effect of *M. toosendan* on ERK1/2. These results suggested that ERK1/2 phosphorylation induced by *M. toosendan* was dependent on PKA activation but independent of PKC and PI-3 kinase pathways.

Inhibition of ERK1/2 and PKA Attenuated the Effects of M. toosendan *on PC12 Cell Differentiation*

To investigate whether ERKs and the PKA pathway mediated the effects of *M. toosendan* on neurite outgrowth, U0126 and H89 were used in PC12 cell differentiation experiments. When PC12 cells were treated with U0126 1 h prior to the addition of *M. toosendan* extracts, neurite outgrowth of PC12 cells induced by *M. toosendan* was inhibited (fig. 5). Similarly, H89 treatment also inhibited *M. toosendan*-induced neurite outgrowth of PC12 cells (fig. 5). Taken together, the effects of *M. toosendan* on PC12 cell differentiation were dependent on the activation of the PKA/ERK-signaling pathway.

M. toosendan-*Induced Neuronal Differentiation and PKA and ERK1/2 Phosphorylation Was Attenuated in A126–1B2 Cells*

The cell line A126–1B2 was isolated from a mutagenized population of PC12 cells on the basis of selection with a toxic concentration of dbcAMP [23]. We have confirmed that the level of type II PKA in A126–1B2 cells was reduced by more than 50% when compared to wild-type PC12 cells. While A126–1B2 cells responded to NGF with neurite formation in 2 days (data not shown), they failed to respond to the addition of *M. toosendan* (fig. 6a). The inability of A126–1B2 cells to extend neurites in response to *M. toosendan* suggests that PKA is required for the neuronal differentiation induced by *M. toosendan*.

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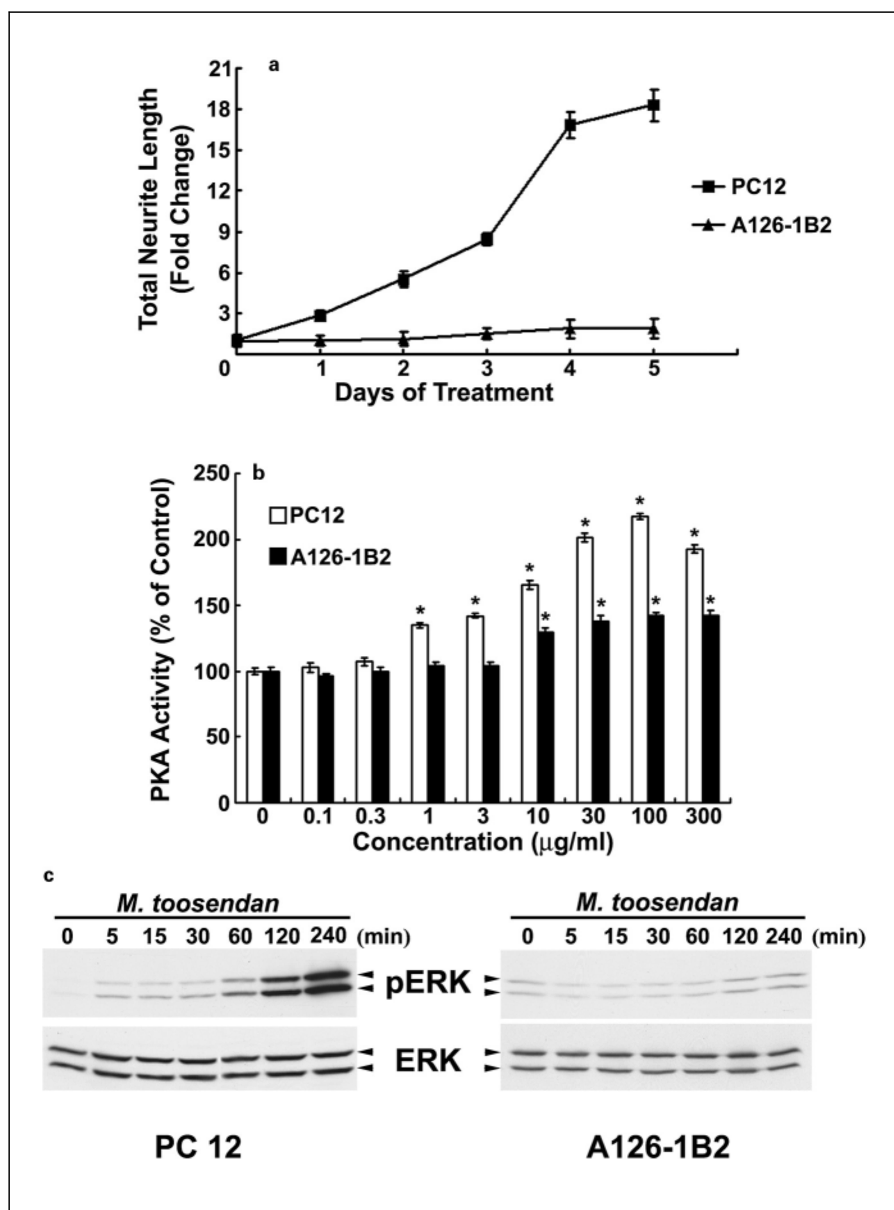

Fig. 6. *Melia toosendan* did not induce neurite outgrowth nor ERK1/2 phosphorylation in PKA-deficient PC12 cells. **a** Neurite outgrowth was quantified as described in Materials and Methods. Results represent the fold change relative to its solvent control ± SEM (n = 3). **b** The PKA activities in wild-type and PKA-deficient A126–1B2 PC12 cells following treatment with *M. toosendan* (0.1–300 μg/ml) for 1 h were measured by PKA activity assay as described in Materials and Methods. The experiments were repeated three times with similar results. Data represented the result from a typical experiment, and are expressed as percent of PKA activity in untreated DMSO control (* $p < 0.0.1$). **c** The profile of ERK1/2 phosphorylation induced by NGF or *M. toosendan* in wild-type and PKA-deficient PC12 cells, A126–1B2. PC12 cells (left panels) or A126–1B2 (right panels) were serum-starved for 4 h, and then treated with NGF (10 ng/ml, upper panels) or *M. toosendan* (100 μg/ml, lower panels) for various time periods as indicated. Phosphorylation of ERK1/2 was detected by anti-phospho-ERK1/2 antibody (pERK). The expression of ERK1/2 protein and loading of samples were examined by immunoblotting with anti-ERK1/2 antibody (ERK). The experiment was repeated three times with similar results.

To determine whether *M. toosendan* induced PKA activity, PC12 and A126–1B2 cells were treated with *M. toosendan* (0.1–300 μg/ml) for 1 h, and PKA activity was determined by quantifying the phosphorylated substrate as described in Materials and Methods. Treatment of PC12 cells with *M. toosendan* (100 μg/ml) increased the PKA activity by ~2-fold over the basal level (fig. 6b). This increase in PKA activity was significantly attenuated in A126–1B2 cells (fig. 6b). The requirement of PKA in *M. toosendan*-induced activation of ERK1/2 was also examined in A126–1B2 cells. These cells and wild-type PC12 cells were treated with 10 ng/ml NGF or 100 μg/ml *M. toosendan* for various time periods. In A126–1B2 cells, *M. toosendan* activation of ERK1/2 was reduced to near basal level when compared with wild-type cells (fig. 6c). Western blot analysis demonstrated that the decrease in ERK1/2 activation after *M. toosendan* stimulation in A126–1B2 cells was not due to the change in protein expression of ERK1/2 (fig. 6c).

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Discussion

In the present study, we have provided evidence that incubation of PC12 cells with a crude extract of the fruits of *M. toosendan* results in enhanced neuronal differentiation. Intriguingly, the extent of neurite outgrowth induced by *M. toosendan* is greater than that observed with NGF treatment. Based on the results of specific kinase inhibitors and PKA-deficient PC12 cells, we conclude that the effects of *M. toosendan* on neuronal differentiation are mediated by PKA and sustained ERK1/2 activation. Taken together, this study provides the first demonstration that *M. toosendan* promotes neuronal differentiation of PC12 cells via the activation of PKA and ERKs. These findings provide new insights into the understanding of the actions of *M. toosendan* on neuronal cells.

The molecular mechanism underlying NGF-induced neuronal differentiation has been intensely studied in PC12 cells [18]. It is well documented that activation of Ras/Raf/MEK/ERK pathway is critical in mediating the neuronal effects of NGF [14]. In particular, there is compelling evidence that sustained ERK1/2 signaling is required in the neuronal differentiation of PC12 cells induced by NGF [14, 24]. In this light, it is noteworthy that *M. toosendan*, like NGF, also triggers prolonged activation of ERK1/2. Since *M. toosendan* fails to activate the high-affinity NGF receptor, TrkA, and its effect is not attenuated in the presence of K252a, it is unlikely that the neuroactivity is due to the presence of NGF in the herbal extract. Our unpublished data using Western blot analysis also showed that treatment of PC12 cells with *M. toosendan* did not induce phosphorylation of Raf-1, suggesting that the activation of ERK1/2 by *M. toosendan* is not mediated by Ras/Raf-1. The ERK1/2 phosphorylation triggered by *M. toosendan* treatment of PC12 cells appears to be dependent on PKA/MEK activation and provides another potential mechanism by which the MEK/ERK pathway becomes activated, subsequently leading to the induction of neurite outgrowth. While other MAP kinases, such as p38 MAPK, also play a role in PC12 cell differentiation [25], we found that inhibition of p38 MAPK did not affect *M. toosendan*-induced PC12 cell differentiation (unpublished data). Taken together, based on the ability of *M. toosendan* to induce PKA activity and the results obtained with specific kinase inhibitors and PKA-deficient PC12 cells, our data suggest that the PKA/ERK pathway mediates the neuronal effects of *M. toosendan* in PC12 cells. It is noteworthy that the dependence of *M. toosendan*-induced neuronal differentiation on PKA activation contrasts the lack of PKA requirement by NGF or retinoic acid in the induction of neurite outgrowth [26, 27].

The PKA pathway has been reported to play an important role in regulating gene expression during the growth and differentiation of PC12 cells via activation of ERKs [28, 29]. Both B-Raf and Raf-1 can independently lead to ERK phosphorylation via activation of MEK in PC12 cells. Previous studies have shown that cAMP elevation activates ERK1/2 through the PKA-dependent activation of Rap1, which selectively stimulates B-Raf and inhibits Raf-1 [30, 31]. Therefore, our finding suggests that the *M. toosendan*-induced ERK activation likely involves the PKA/Rap1/B-Raf pathway. Most of the transcriptional actions of PKA in PC12 cells are mediated by accumulation of cAMP [32]. However, we found that treatment of PC12 cells *M. toosendan* in PC12 cell did not stimulate the production of cAMP (unpublished observations). While it is likely that the active ingredient(s) in *M. toosendan* might act as cAMP analog(s) and activate PKA, one cannot rule out the possibility that the accumulation of cAMP might be below the detectable level. Moreover, since treatment of PC12 cells with NGF or dibutyryl cAMP has been reported to induce immediate early genes, such as c-fos and egr-1 [18], it would be of interest to investigate the expression of these genes in PC12 cells following *M. toosendan* treatment.

In the present study, we have demonstrated the presence of neuroactive compounds in *M. toosendan* that promotes neuronal differentiation. We report here that incubation with *M. toosendan* results in the reduction of PC12 cell growth as well as induction of neurite outgrowth in PC12 cells. The effects of *M. toosendan* on neuronal differentiation are mediated by PKA and sustained activation of ERKs. Our findings not only suggest that *M. toosendan* embraces a rich source of neuroactive compounds that induce the neuronal differentiation, but also reveals the potential signaling molecules involved in its action. Further studies aimed at the purification and identification of active components in *M. toosendan* are in progress in our laboratory.

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