Andrographis paniculata methanol extract suppresses the phosphorylation of ETV6-NTRK3

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Abstract. ETS variant transcription factor 6 (ETV6)-neurotrophic receptor tyrosine kinase 3 (NTRK3) (EN) fusions are typically found in rare diseases, such as primary renal fibrosarcoma (only six cases have been reported), secretory carcinoma of the breast and salivary gland (1 case), and AML (4 cases). Few cases have been reported, and expression of the EN gene fusion requires additional clinical data and fundamental research to be supported. The aim of the present study was to determine the inhibitory effect of *Andrographis paniculata* methanol extract (MeAP) on EN-related cell lines, IMS-M2 and BaF3/EN, as well as evaluate the mechanism of action. Vero cells were used as control cells. Trypan blue staining and MTT were used to evaluate the inhibitory effect of MeAP on tested cells. Western blotting and immunoprecipitation were used to detect the activation of EN after MeAP treatment. The IC_{50} values of MeAP were found to be 12.38±0.57 μg/ml (IMS-M2) and 13.06±0.49 μg/ml (BaF3/EN). MeAP was observed to inhibit cell proliferation in a time, dose, and cell density-dependent manner. The IC_{50} value for MeAP in Vero cells was markedly higher, at 109.97±4.24 (μg/ml), indicating a much less sensitive effect. Furthermore, MeAP treatment inhibited EN phosphorylation and induced apoptosis in these cells. Collectively, the present study demonstrated that MeAP has an oncogenic effect on EN fusion-positive cell lines, in particular.

Introduction

The medicinal plant *Andrographis paniculata* (Burm. f.) Wall. ex Nees (*A. paniculata*) is indigenous to Southeast Asia, China, North America, and the West Indies (1). This herb is widely used in herbal remedies to treat malaria, viral hepatitis and liver cancer (2). Numerous natural compounds have been identified in *A. paniculata*, including andrographolide and its derivatives. These compounds are well-known for their biological activities, including anti-SARS CoV-2 activity (3,4). Anticancer studies on this plant have begun a good many years ago, using B16F0 melanoma syngenic and HT-29 xenograft models (2003), colon cancer cell line HT-29 (2004), human lymphocytes and albino mice (2014), the Caco-2 model (2017), and acute myeloid leukemia (AML) cell line U937 (2018) (5-9). The anticancer effect of *A. paniculata* has been demonstrated in a variety of ways, including cell cycle arrest, induction of apoptosis, anti-angiogenic behavior, and suppression of IL-6 expression (10). Andrographolide was reported to inhibit PI3K/AKT signaling in the A549 cell model (11).

The neurotrophic receptor tyrosine kinase *(NTRK)1, NTRK2*, and *NTRK3* genes code for members of the tropomyosin receptor kinase (Trk) family, an upstream molecule in the PI3K/AKT signaling pathway (12). Native Trk must be stimulated by an extracellular signal, such as nerve growth factor, brain-derived growth factor, or neurotrophin 3, in order to become active (12). However, due to the fusion of *NTRK3* and ETS variant transcription factor 6 (*ETV6)*, ETV6-NTRK3 (EN) can self-phosphorylate (13). The *EN* fusion gene has been identified in congenital fibrosarcoma (14), secretory breast carcinoma (15), AML (16,17), mammary analog secretory carcinoma of the salivary gland (18), chronic eosinophilic leukemia (19) congenital mesoblastic nephroma (20), and thyroid cancer associated with ^{131}I radiation exposure (21). Due to the already low prevalence of NTRK fusions in most tumours (<1%) EN cases are even more uncommon (22). To date, TRK inhibitors are a viable treatment option for patients whose tumours test positive for the EN fusion. Both larotrectinib and entrectinib, which target the NTRK fusion protein, have shown promising results in recent clinical trials against locally advanced and metastatic solid tumours (23,24). EN fusions are typically found in rare diseases, such as primary renal fibrosarcoma (only six cases have been reported), secretory carcinoma of the breast and salivary gland (1 case), and AML (4 cases). Few cases have been reported, and thus EN gene fusion expression requires additional clinical data and fundamental research to be supported.

The purpose of the present study was to ascertain the inhibitory effect of *A. paniculata* methanol extract (MeAP) on EN-associated cell lines (IMS-M2 and BaF3/EN) as well as to identify the mechanism of action.

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Materials and methods

Materials and plant extraction preparation. *Andrographis paniculata* (Burm. f.) Wall. ex Nees (*A. paniculata*) was collected in August 2017 and verified by Dr Dang Van My (Traditional Medicine Center, Tinh Bien, Vietnam) (voucher no. BNAG-2017-0115). The leaves were cleaned and dried at 40˚C in a dry oven after collection. Dry samples were blended into a fine powder and mixed with methanol (1:10 w/v). The mixture was spun at room temperature for 4 days before filtering with Whatman filter paper. The obtained extract was evaporated at 40˚C under vacuum. Subsequently, the crude extract continued to be placed in the drying oven at 40˚C for solvent evaporation and elevated drying. The extraction efficacy was 4.18%. The 200-mg/ml stock solution was produced by weighing and dissolving the dry crude extract in dimethylsulfoxide (DMSO). The stock solution was stored at -20˚C.

The inhibitor of EN, PKC412 (Sigma-Aldrich; Merck KGaA) was dissolved in DMSO and was used as a positive reagent control (13). Control cells were grown with the same amount of carrier DMSO as in the highest reagent concentration. In all of the experiments, the amount of DMSO was kept below 0.1% to keep it from killing cells.

Cell lines and culture conditions. In the present study, the *EN*-positive human AML cell line, IMS-M2, provided by Professor Yuko Sato (University of Tokyo, Tokyo, Japan), the stable transfection with *EN*, BaF3/EN (established by HTC), and the Vero (ATCC-CCL-81™) cells were used (13,25). Vero cells were used as negative control cells to specify the selective effect of MeAP on cells with EN. The cells were cultured at 37˚C in a humidified incubator with 5% CO_2 in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (both from Sigma-Aldrich; Merck KGaA).

Cell viability. The cells (1x10^5 cells/ml) were seeded in 6-well plates with or without MeAP treatment. Following treatment of the cells, 10 μl of cell suspension and 10 μl of 0.4% Trypan blue (product no. T8154; Sigma Aldrich; Merck KGaA) were mixed together. The cells were manually counted with a hemocytometer (26). After 48 h of incubation, the cytotoxicity of MeAP was determined dose-dependently, and the half maximal inhibitory concentration (IC_{50}) was then calculated.

For dose and cell-density dependent tests, cells were treated with or without MeAP (12.5 μg/ml) for 48 h. For the time-dependent test, cells were treated with or without MeAP (12.5 μg/ml) at 24, 48, 72 and 96 h.

The cytotoxic activity of MeAP was determined using the 3-(4,5-dimethylthiazol)-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. DMSO (100 μl) was used to used to dissolve the purple formazan and the absorbance was read at 570 nm using a universal microplate reader (27) for the remaining adherent cell lines. The methods cited were modified to accommodate the research conditions of the laboratory.

To identify the selective effects of MeAP on leukemia cells, the Selective Index (SI) values were calculated. SI values >3 were considered to be highly selective for cells of interest (28).

Morphological changes in MeAP-treated cells. IMS-M2 and BaF3/EN cells were seeded overnight at a density of 1x10^5 cells/well in six-well culture plates. The cells were then treated with varying concentrations of MeAP (6.25, 12.5, 25, and 50 μg/ml) or PKC412 (60 nM) and maintained at 37˚C and 5% CO_2 for 72 h. The untreated cells served as the control. To detect the morphological changes in the cells, an inverted light microscope was used at a magnification of x10.

Western blot analysis. The IMS-M2 and BaF3/EN cells were plated at a density of 1x10^5 cells/ml on a 10-cm dish with varying concentrations of MeAP (12.5, 25, or 50 μg/ml). After the indicated time points (4, 8 or 24 h) of incubation, the cells were removed and washed twice with PBS (-) (TBR Technology Corporation). The cells were then lysed in ice-cold protein lysis buffer (10 mM disodium diphosphate, 50 mM sodium fluoride, 5 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM phenylmethylsulfonyl fluoride, 0.01% Triton X-100, 150 mM sodium chloride, and 75 μg/ml aprotinin) (29). After centrifuging the cells at 15,000 x g for 10 min (4˚C), total protein cell lysates were obtained. Protein samples (20 μg, as measured by the BCA protein assay kit) were loaded onto wells, and the proteins were resolved on a 12.5% polyacrylamide gel electrophoresis, and then electroblotted onto a Hypond-P membrane (Amersham; Cytiva). The membrane was then blocked at room temperature for 1 h with 5% skim milk buffer. Following a wash, primary antibodies were used to probe the membrane, and ECL was used to detect antibody binding (Amersham; Cytiva). Anti-TrkC (C-14) (1:500; cat. no. sc-11; Santa Cruz Biotechnology, Inc.), anti-actin (1:1,000; cat. no. A2066; Sigma-Aldrich; Merck KGaA), anti-phosphotyrosine 4G10 (1:1,000; cat. no. 05-321MG; Upstate Biotechnology, Inc.; Merck KGaA), caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.) and anti-PARP (1:1,000; cat. no. 016-16831; FUJIFILM Wako Pure Chemical Corporation) were used as primary antibodies. The primary antibodies were incubated at room temperature for 1 h, or overnight at 4˚C. The membranes were then washed twice for 15 min each time and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature (1:1,000; anti-mouse IgG HRP (cat. no. sc-2031) or anti-rabbit IgG HRP (cat. no. sc-2317; both from Santa Cruz Biotechnology, Inc.).

Immunoprecipitation (IP). IMS-M2 or BaF3/EN cells were treated with MeAP (12.5, 25 or 50 g/ml) for 8 h and then harvested for IP. The cells were lysed as described above. A total of 500 mg of total cell lysates were immunoprecipitated overnight at 4˚C with anti-TrkC (C-14) (1:500; cat. no. sc-11; Santa Cruz Biotechnology, Inc.). Subsequently, Protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biosciences; Cytiva) was added and all the procedures were carried out in accordance with the manufacturer's instructions. The immunoprecipitates were washed with Tris-buffered saline with Tween-20 three times. Using SDS-PAGE and western blotting, the bound proteins were separated and analysed.

Statistical analysis. Data were compiled from three independent experiments and are presented as the mean ± SEM. Data were compared using a paired Student's t-test or a one-way

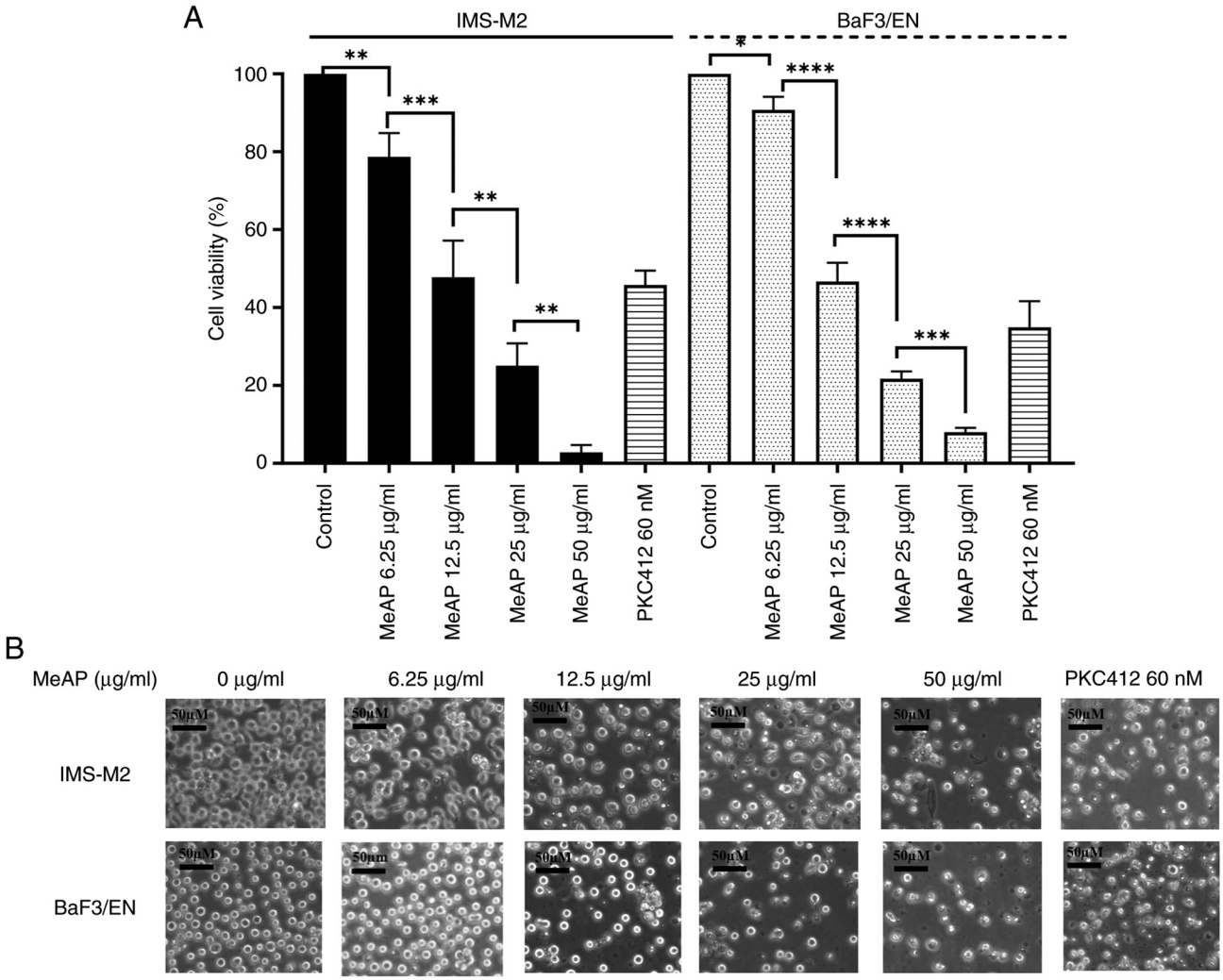


Figure 1. Cell viability inhibition effect of MeAP in a dose-dependent manner. MeAP was added to IMS-M2, and Ba/F3-CFS cells at various concentrations (6.25 to 50 μg/ml) for 48 h, and cell viability assays were performed as described in Materials and methods, with PKC412 at 60 nM used as a positive control. (A) The results are expressed as a percentage of cells dying from MeAP treatment in comparison to untreated cells (handled with DMSO at the same concentration as plant extracts). (B) Changes in the morphology of cells are observed using a phase contrast inverted microscope. Triplicate experiments were conducted. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. MeAP, *Andrographis paniculata* methanol extract.

ANOVA with Tukey's post hoc test in GraphPad Prism version 8.3.0. (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of MeAP on EN-positive cells in a dose-dependent manner. The inhibitory potential of MeAP was determined by assessing its cytotoxic effects on IMS-M2 and BaF3/EN cells at varying concentrations. MeAP (0, 6.25, 12.5, 25 and 50 μg/ml) was added to the 6-well plates and results were collected after 48 h. PKC412 at 60 nM (13) was used as a positive control. MeAP was found to inhibit the viability of these cells (Fig. 1A). The ANOVA method was used to determine whether there were any statistically significant differences in the toxic tendency of the extract across these cell lines. The IC_{50} values for IMS-M2 and BaF3/EN were 12.38±0.57 μg/ml and 13.06±0.49 μg/ml, respectively.

Morphological assessment was conducted 48 h after treatment (Fig. 1B). The observations indicated that the cells grew normally in the untreated group, with normal morphology such as round shape, uniform size, intact membrane and nucleus (IMS-M2 and BaF3/EN). In comparison to the control group, when the extract concentration was increased, the number and size of the treated cells significantly decreased. The shape of the cells was altered and cells began to shrink. By contrast to organelle dilation caused by early membrane permeability, cell shrinkage is a frequent and prominent morphological property of the apoptotic cell death process (30,31). The observational experiment revealed that the assessed cell lines exhibited apoptotic tendencies.

Effect of MeAP on AML cells in time-dependent manner. For up to 96 h, IMS-M2 and BaF3/EN cells were examined for viability with or without MeAP (12.5 μg/ml) treatment. At five time points (0, 24, 48, 72 and 96 h), the number of viable cells was observed and calculated. As revealed in Fig. 2, MeAP was able to inhibit the viability of cells in time-dependent manner. After 24 h of exposure to the extract, the cells grew very slowly and the number of cells was not significantly altered (Fig. 2, left panels). Between 48 and 72 h, the number of viable cells began to markedly decline.

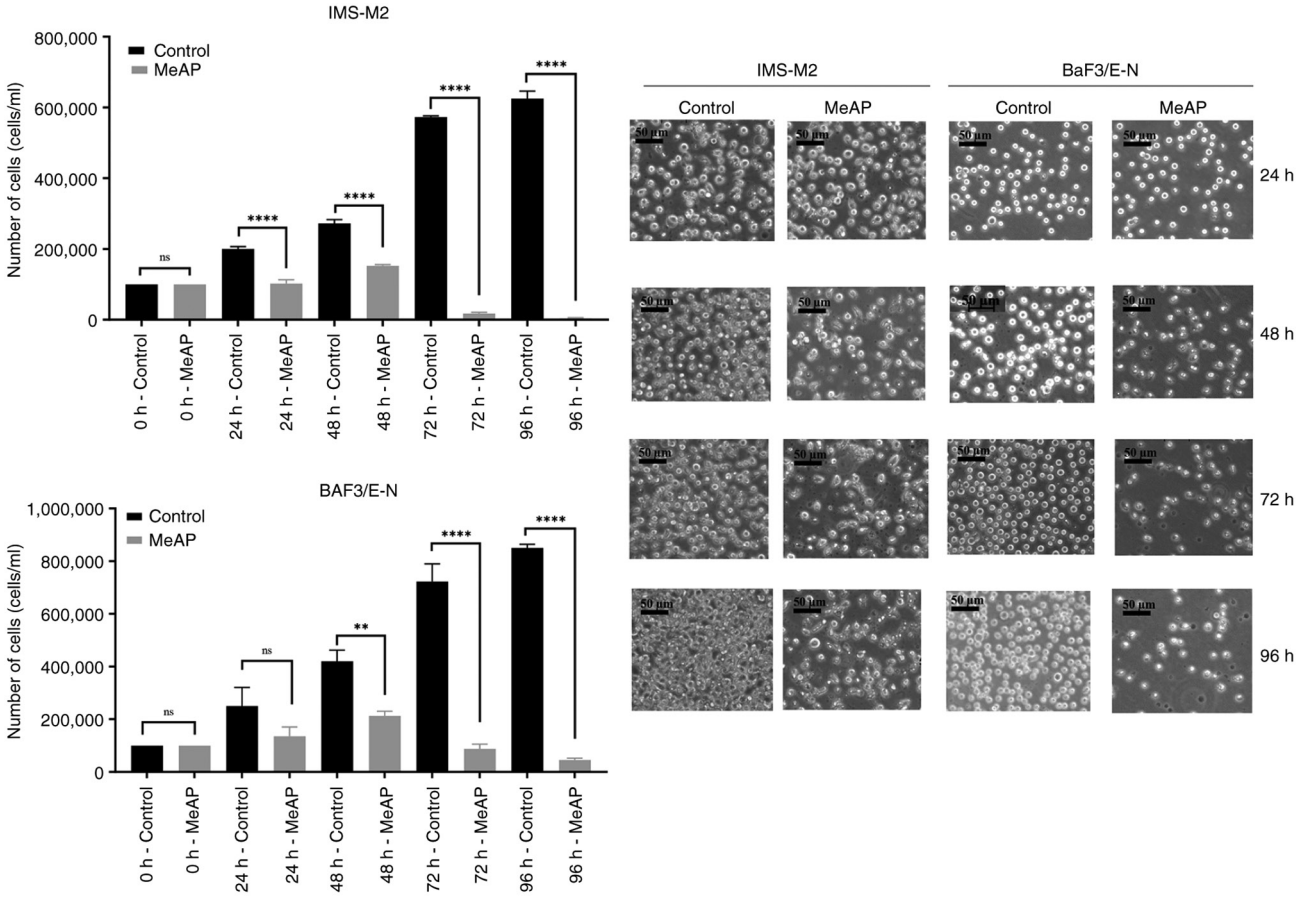


Figure 2. Cell viability inhibition effect of the extract in a time-dependent manner. MeAP (12.5 μg/ml) was added to IMS-M2 and BaF3/EN cells for various time points (0 to 4 days). Cell viability assays were performed and cells were observed under phase contrast inverted microscope as described in Materials and methods. **P<0.01 and ****P<0.0001. MeAP, *Andrographis paniculata* methanol extract; ns, not significant.

After 72 h of treatment, only 15% of the cells remained viable, and decreased to ~0% 24 h later. The cell density decreased as the culture time was extended. Fig. 2, right panel shows how cell morphology changed after MeAP co-cultured, with cell shrinkage, cell debris, and membrane blebbing.

Effect of MeAP on AML cells in a cell density-dependent manner. Statistical analysis of the effect of MeAP (12.5 μg/ml) revealed statistical differences between the control and experimental groups at the same cell density, as well as differences between the groups (Fig. 3). Experiments with various densities ($P<0.0001$; $<\alpha=0.05$) were performed. When treated with MeAP, almost all cells died at a density of 10^4 cells/ml. When the cell density increased from $5x10^4$ to $5x10^5$ cells/ml, the percentage of viable cells increased from 8.72 to 21.86%. The percentage of viable cells in the experimental group was 23.84±0.58% at a density of 10^6 cells/ml. The percentage of cells in the MeAP-treated group was significantly lower than that in the control group, decreasing from 100 to 76.15% at a density of 10^4-10^6 cells/ml. In summary, the inhibitory effect of MeAP on IMS-M2 and BaF3/EN cell viability at the IC_{50} concentrations was cell density-dependent.

Selective index. As illustrated in Fig. 4, MeAP exerted a significantly less sensitive effect on Vero cells, with an IC_{50} value of 109.97±4.24 (μg/ml). It was also demonstrated that MeAP had an SI value >3, which indicated that it had marked cytotoxic potential and was very efficient at killing leukemia cells (Fig. 4, right panel).

MeAP inhibits the activity of EN protein. Although it is unknown how EN leads to cancer, the fusion protein has been considered as a significant target for cancer treatment (9,32). Additionally, previous research using an EN-expressing cell model demonstrated that PKC412 is an effective inhibitor of EN-associated leukemia (13).

It was hypothesized that the viability inhibition observed in both cell lines is due to the fusion protein's phosphorylation being inhibited. To elucidate the mechanism of MeAP-mediated viability inhibition in IMS-M2 and BaF3/EN cells, the phosphorylation status of EN in these cells was examined after treatment with or without MeAP. To determine the status of EN tyrosine phosphorylation, the total protein of EN was immunoprecipitated with TrkC antibody and immunoblotted with 4G10 antibody. As predicted, MeAP treatment inhibited the phosphorylation of EN in a dose-dependent manner, but total EN protein levels were unaffected (Fig. 5).

MeAP induces apoptosis in IMS-M2 and BaF3/EN cells. Cell viability inhibition of IMS-M2 and BaF3/EN cells following MeAP treatment may result in changes in the shape and size of cells, as illustrated in Figs. 1-3. Next, it was examined

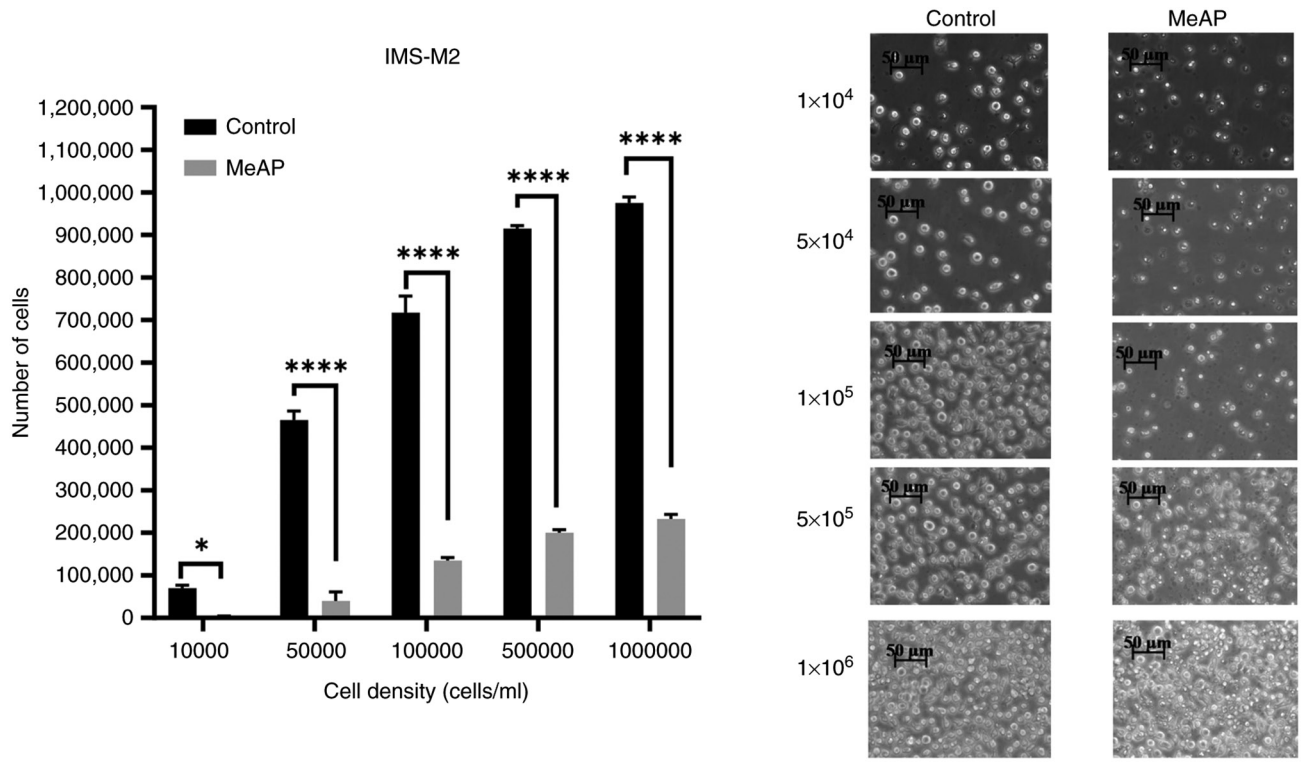
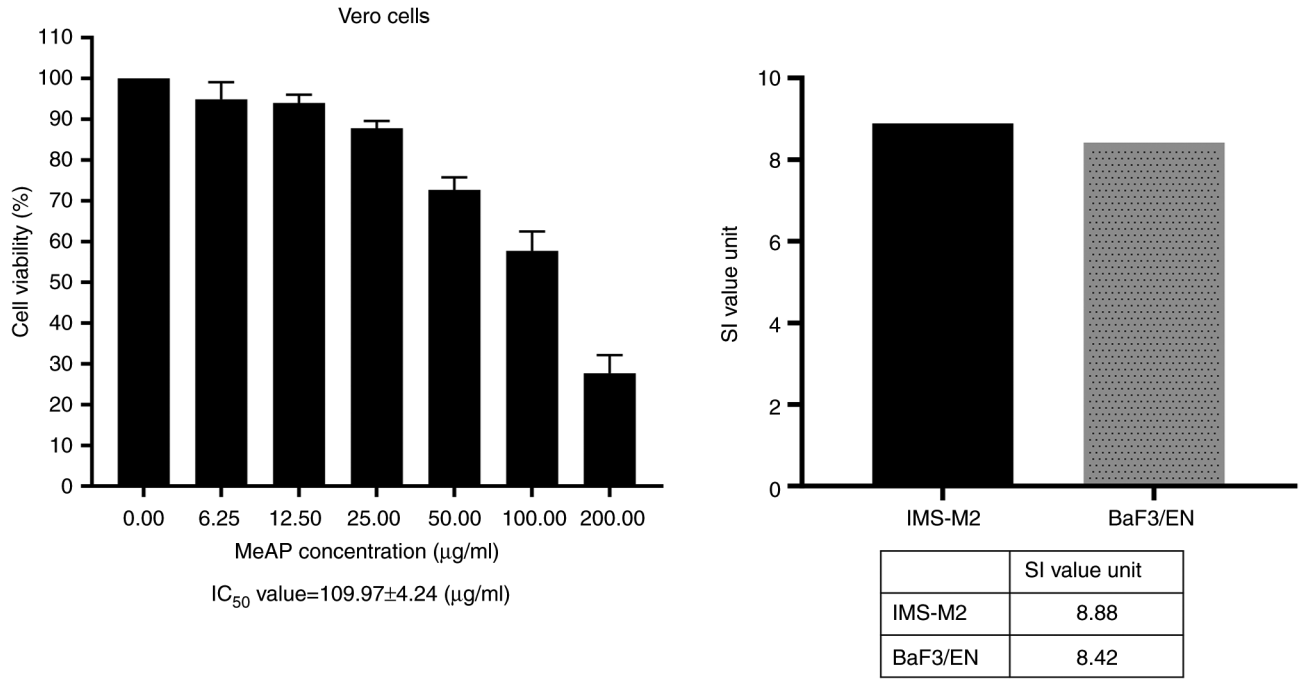


Figure 3. Inhibitory effects of MeAP on the viability of IMS-M2 and BaF3/EN cells are density-dependent. Cells (density, 10^4-10^6 cells/ml) were cultured together with MeAP (12.5 μg/ml) for 48 h and cell viability assays and cell morphology was determined as described in Materials and methods. *P<0.05 and ****P<0.0001. MeAP, *Andrographis paniculata* methanol extract.



	SI value unit
IMS-M2	8.88
BaF3/EN	8.42

Figure 4. IC_{50} and SI values of MeAP in Vero cells. Vero cells were treated with MeAP at various concentrations for 48 h, and the viability of cells was determined as described in Materials and methods (left panel). The SI value for MeAP was calculated by dividing the IC_{50} value of Vero cells by the IC_{50} value of leukemia cells. MeAP had SI values >3, indicating that it has a high level of cytotoxicity and high selectivity for leukemia cells (right panel). IC_{50}, half maximal inhibitory concentration; SI, selective index; MeAP, *Andrographis paniculata* methanol extract.

whether MeAP treatment could affect the expression of apoptotic markers. These cells were treated with MeAP at a concentration of 50 μg/ml for up to 24 h. The results indicated that MeAP treatment activated the caspase cascade. PARP and caspase-3 molecules were turned into cleaved forms in the cells that were exposed to MeAP (50 μg/ml), which indicated

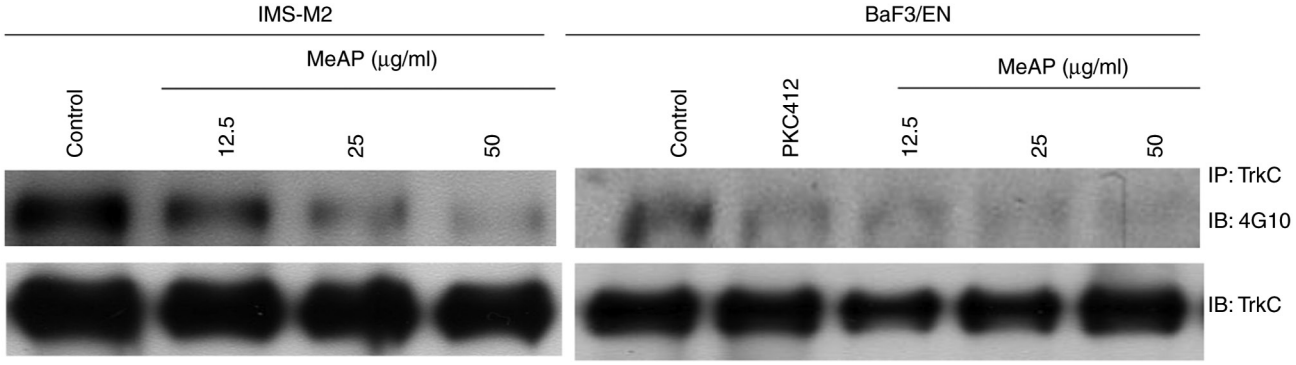


Figure 5. Effect of MeAP on EN activity inhibition. MeAP was co-cultured with IMS-M2 or BaF3/EN cells at the indicated concentrations. After 8 h, total cell lysates were prepared and western blot analysis with the indicated antibody was performed. PKC412 at 60 nM was used as a positive control. MeAP, *Andrographis paniculata* methanol extract.

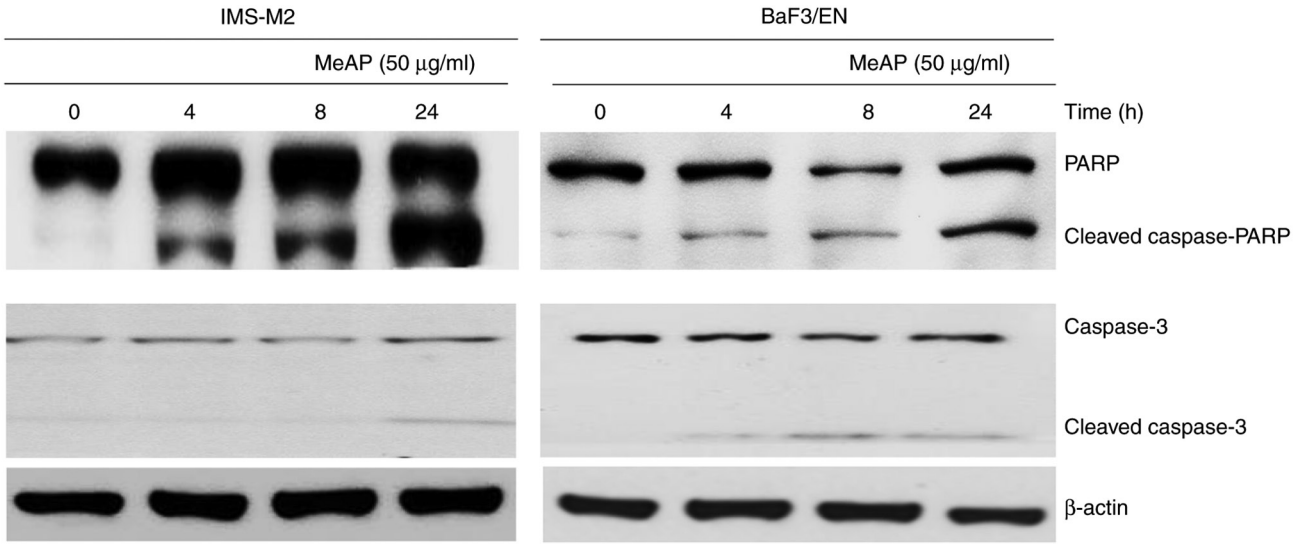


Figure 6. MeAP induces apoptosis in IMS-M2 and BaF3/EN cells. Cells were co-cultured with MeAP (50 μg/ml) for up to 24 h. Western blot analysis was performed on cell lysates using caspase-3 and PARP antibodies. The results indicated that treatment with MeAP extract induced caspase-3 and PARP cleavage. MeAP, *Andrographis paniculata* methanol extract.

that apoptosis occurred. These findings indicated that MeAP induced apoptosis in IMS-M2 and BaF3/E-N cells (Fig. 6).

Discussion

NTRK1, *NTRK2*, and *NTRK3* encode the tyrosine kinases TrkA, TrkB, and TrkC, respectively. In a variety of tumor types, oncogenic gene fusions involving members of the NTRK family have been identified and appear to result in constitutive Trk kinase activity (33). Orally administered larotrectinib is a highly selective inhibitor that inhibits all three Trk protein isoforms at nanomolar concentrations (34). Significant tumor regressions have been reported with larotrectinib in a child with congenital fibrosarcoma harboring an *EN* fusion and an adult with a soft tissue sarcoma harboring an *LMNA-NTRK1* fusion (35,36). Larotrectinib has also demonstrated preliminary activity in a number of other cancer types with *NTRK* fusion positivity (23). In July 2016, the FDA designated larotrectinib as a breakthrough therapy for the treatment of unresectable or metastatic solid tumors with *NTRK* fusions.

The *EN* fusion gene is expressed at low levels in AML, however it exerts a potent transforming effect on numerous cell lines, including hematopoietic cells, and transformed cells can induce tumors in nude mice (13,37). Previous research documented two cases of *EN*-AML, one with AML M2 and severe myelofibrosis at the time of diagnosis, as well as rapidly spreading leukemia cells to multiple organs, and the other with primary myelofibrosis and progression to AML M7. The *EN* fusion gene may be involved in the pathogenesis of acute myeloid leukemia (37).

Due to the rarity of EN-positive cases in the world (22), the established cell lines were also rare and not commercially available. Extreme difficulty exists in obtaining cell lines with EN fusion. In the present study, two EN-positive cells were utilised, one from a human (IMS-M2 cell line) and the other from transfected mouse BaF3 cells (BaF3/EN). It was demonstrated that BaF3/EN cell growth was dependent on EN-signalling (13).

Because the number of cases is so low, there is almost no literature on the use of medicinal herbs to treat diseases caused by the fusion gene *EN*. This may be the first study to

mention the use of medicinal herbs to prevent leukemia cell proliferation caused by the *EN* fusion gene.

Currently, reports on the ability of inhibition of the proliferation of cancer cells in *A. paniculata* have primarily focused on andrographolide compounds, which are found in high concentrations in leaves (38). Indeed, this compound was shown to induce cell cycle arrest and mitochondrial-mediated apoptosis in the leukemia cell line HL-60 with an IC_{50} of 14.01 μg/ml after a 24-h experiment (39). Other research has demonstrated that the IC_{50} for this compound's toxicity to MCF-7 breast cancer cell lines is 500 g/ml. Furthermore, scientists have investigated the effects of this compound on many other cancer cell lines and discovered a positive inhibitory effect on the non-small lung cancer cell line A549, as well as on the nasopharyngeal cancer cell lines (HSC-2, HSC-3, and HSC-4) (40,41).

In addition, MeAP has been demonstrated to inhibit the proliferation of colon cancer (HT-29 cells) (5), human alveolar basal epithelial cell line (A-549), human breast adenocarcinoma cell line (MCF-7), human embryonic kidney (HEK), human cervical cancer cell line (HeLa), and human invasive ductal carcinoma cell line (BT-544), as demonstrated in previous study (42). Furthermore, The GC-MS chromatogram of MeAP extracts displayed 21 peaks, indicating the presence of 21 distinct phytochemical compounds. It was determined that 2(5H)-furanone (14.73%), quinic acid (QA; 17.32%), and phytol (11.43%) were the most abundant phytochemicals (42).

In the present study, the IC_{50} values of MeAP were determined to be 12.38±0.57 μg/ml (IMS-M2) and 13.06±0.49 μg/ml (BaF3/EN). The SI value helps to evaluate the specificity of an extract for certain cells. A high SI value indicates a more selective extract. An SI value >3 units indicates the general toxicity of a compound (28). The results in the present study revealed that MeAP had an SI value of >3, which indicated that it exerted marked cytotoxic potential and was very effective at killing leukemia cells that had *EN*.

Moreover, MeAP treatment inhibited EN phosphorylation and induced apoptosis in these cells. Therefore, it was concluded that the inhibitory effect of MeAP on AML cell lines harboring the *EN* fusion gene was dependent on dose, time, and cell density. MeAP can stop the viability of EN-carrying AML cells by blocking the phosphorylation of EN, which causes the cells to die.

The limitation of the present study is that no knockout or overexpression cell line with its parental cell line was used, such as IMS-M2 vs. IMS-M2 EN knockout or BaF3 vs. BaF3/EN, to provide solid evidence for the selective activity of MeAP. Future research should be conducted to address this issue.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HTC and BTKL conceived and designed the study. VNT, NTQ and HTC performed the experiments; VNT, NTQ, BTKL and HTC acquired and analyzed the data, as well as wrote and revised the manuscript critically for important intellectual content. BTKL and HTC confirm the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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