Protein kinase Cα-mediated cytotoxic activity of ineupatorolide B from *Inula cappa* DC. in HeLa cells

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Abstract. There is an ongoing search for plant-derived sesquiterpenes, particularly for those with anticancer activity against human cancer cells. The sesquiterpene ineupatorolide B (InB), isolated from the *Inula cappa*, showed potent growth-inhibitory activity against HeLa cells but less activity against MM1-CB melanoma cells. Staining by terminal deoxynucleotidyl transferase dUTP nick-end labeling method revealed that this activity was, at least in part, due to the induction of apoptosis. The activities of major transcription factors were examined by using a luciferase reporter assay. The results showed that the transactivation ability of nuclear factor of activated T-cell (NFAT) was enhanced. The activation of NFAT by InB was largely suppressed by preincubation with protein kinase C (PKC) inhibitors such as staurosporine and K252a. Western blot analysis revealed that the the levels of phosphorylated PKCα, but not other subtypes, increased after treatment with InB. Knockdown of PKCα using siRNA attenuated the cytotoxic activity of InB. Thus, InB may exhibit growth-inhibitory activity through the activation of PKCα, followed by an increase in NFAT transactivation ability.

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Introduction

Plants are believed to contain chemical compounds that inhibit the proliferation of cancer-derived cells *in vitro*, and many attempts have been made to isolate anticancer drugs from plants. For example, the diterpene paclitaxel is a well-known anti-proliferative agent isolated from *Taxus brevifolia* (1). Various groups have conducted research on other diterpene-containing species, with the aim of finding more effective agents for the treatment of cancer (2,3).

Inula cappa is a subshrub of the genus *Inula*. Its roots and/or whole plants have been used as medicines because of their pharmacological effects such as antitussive activity, promoting the expulsion of phlegm, promoting blood circulation to restore menstrual flow and wound healing (4). It is generally used as a folk medicine by the Zhuang minority in the districts of Wenshan and Xichou in Yunnan Province, China, for its anti-inflammatory and detumescence effects. *Inula cappa*, named 'Na Han' by the Dai nationality, was one of the primary ingredients of the ethnic medicine formula 'Ya Jiao Ha Dun San', which was efficiently used for the treatment of rheumatoid arthritis, laryngotracheitis, irregular menstrual periods and abdominal pain. To date, the chemical constituents such as sesquiterpenoids, triterpenoids, steroids, anthraquinones, flavonoids, balmy compounds, amides, and organic acids have been obtained from the roots of *Inula cappa* (5). Among these, the triterpenoids and steroids are the most predominant constituents.

In the present study, the biological activity of the sesquiterpene ineupatorolide B (InB) was examined using MTT assay, TUNEL assay, luciferase reporter assay and western blotting using human cancer cell lines. These results suggested the involvement of protein kinase Cα (PKCα) in the cytotoxic acitivity of InB.

Materials and methods

Agents. The structures of two sesquiterpenoids, InB and eupatolide (Eup), have been analyzed using one-dimensional (1-D) and two-dimensional (2-D) nuclear magnetic resonance (NMR) spectral data and previously studied (6,7). The

compounds were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Cells and culture conditions. The following human cancer derived cell lines were used: HeLa (cervical cancer), HOC-21 (ovarian adenocarcinoma), T-98 (glioblastoma), U251SP (glioblastoma), A549 (lung carcinoma), QG-56 (lung carcinoma), PC-6 (lung carcinoma), HLE (hepatoma), and MM1-CB (melanoma) (8). The cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) calf serum (Thermo Fisher Scientific, Waltham, MA, USA) or in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd.) supplemented with 10% fetal bovine serum supplemented with antibiotics [100 μg/ml of streptomycin and 100 U/ml of penicillin G (both from Meiji Seika Kaisha, Ltd., Tokyo, Japan)] at 37˚C in a humidified atmosphere containing 5% CO_2.

Measurement of cell viability. Cell viability was estimated using the MTT assay, as previously described (9,10). In brief, logarithmically proliferating cells were seeded ($1x10^4$ cells/well) in 96-well plates (Asahi Glass, Tokyo, Japan) with the medium containing the test compounds at the indicated doses and then cultured for 2 days. After the culture period, the activity of mitochondrial succinic dehydrogenase was measured by further incubation of the cells with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) for 4 h. After this incubation period, the absorbance of each well was measured at 570 nm with a reference wavelength at 655 nm. Cell survival was calculated from this absorbance and presented as the percentage of surviving cells.

TUNEL assay. We conducted *in situ* labeling of the fragmented DNA using the TUNEL method (11,12). In brief, HeLa cells were cultured in the presence of InB at 10 μM for 12 h or 24 h. After culturing, we stained the fragmented DNA in the cells using an apoptosis *in situ* detection kit (Wako Pure Chemical Industries, Ltd.) in accordance with the manufacturer's instructions.

Luciferase assay. The firefly luciferase reporter plasmid, PG13-Luc (13), was provided by Dr Bert Vogelstein (Howard Hughes Medical Institute). The plasmids, pGL3-p21-Luc (14) and pGL3-Bax-Luc (15) were provided by Dr Mian Wu (University of Science and Technology of China). The plasmid pGV-B2 hNoxa-Luc (16) was provided by Dr Nobuyuki Tanaka (Nippon Medical School). SRE-Luc (serum-responsive element), IgK-Luc (NF-κB), nuclear factor of activated T-cell (NFAT)-Luc, CRE-Luc (cAMP-responsive element), and control *Renilla* luciferase reporter SV40-Rluc were purchased from Promega Corp. (Madison, WI, USA).

HeLa cells seeded in 24-well plates were then transfected with test genes along with the firefly luciferase and *Renilla* luciferase reporter plasmids using Lipofectamine-Plus (Thermo Fisher Scientific). Two days after the transfection, firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase Assay system (Promega Corp.) and a luminescencer (Atto, Tokyo, Japan). Subsequently, the firefly luciferase activities were normalized to the *Renilla* luciferase control activities, as previously described (17). The inhibitory compounds used were previously described (10,18).

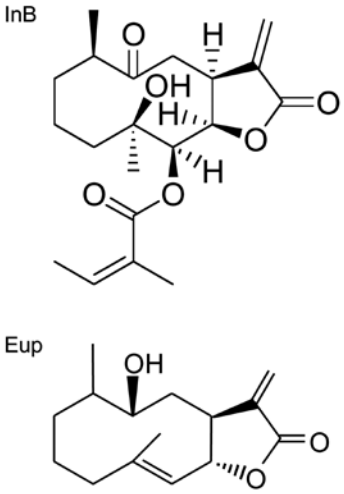

Figure 1. Chemical structures of ineupatorolide B (InB) and eupatolide (Eup).

Western blot analysis. Western blotting analysis was carried out as previously described (19,20). After treatment with InB, the cells were washed with phosphate-buffered saline and then lyzed by incubation in a sodium dodecyl sulfate (SDS) sample buffer at 100˚C for 3 min. The whole cell lysate was then subjected to SDS-polyacrylamide gel electrophoresis, followed by western blotting using the specific antibodies targeting PKCα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phospho-specific PKCα (p-PKCα) and PKCδ (p-PKCδ) (both from Cell Signaling Technology, Inc., Danvers, MA, USA), PKCε (p-PKCε; Santa Cruz Biotechnology), and PKCθ (p-PKCθ) and PKCλ (p-PKCλ) (both from Cell Signaling Technology, Inc.), and β-actin (Santa Cruz Biotechnology, Inc.).

Knockdown experiments. PKC subtype-specific siRNAs and a non-targeted negative control siRNA were obtained from Qiagen (Hilden, Germany). HeLa cells were transfected with siRNA using RNAiFect transfection reagent (Qiagen). Two days after the transfection, the cells were re-plated for the MTT assay. The subtype-specific knockdown of PKC was confirmed by western blotting.

Statistical analysis. Statistical analyses were performed using the Student's t-test with StatView software (version 4.5; Abacus Concepts, Berkeley, CA, USA), as previously described (19).

Results

Growth-inhibitory activity of InB. Among the many compounds found in *Inula cappa*, we selected two bioactive compounds, InB and Eup. The chemical structures of InB and Eup are shown in Fig. 1. The cytotoxic potential of InB and Eup was examined using the MTT assay after culture in the presence of each compound for 2 days. A representative result is shown in Fig. 2. The IC_{50} concentrations of InB for HeLa, A549, and MM1-CB cells were 4.8, 10.8 and 23.2 μM, respectively. The IC_{50} values for the other cells examined thus far are shown in

Table I. IC_{50} values of InB and Eup.

	Cell lines								
Agents	HeLa	HOC-21	T-98	U251SP	A549	QG-56	PC-6	HLE	MM1-CB
InB	4.8	7.9	7.1	13.8	10.8	4.4	17.7	21.6	23.2
Eup	64.4	46.5	67.7	>100	70.9	>100	>100	30.2	26.0

The half-maximal inhibitory concentrations (IC_{50}, μM) of ineupatorolide B (InB) and eupatolide (Eup) toward the indicated cell lines were calculated based on the results of an MTT assay.

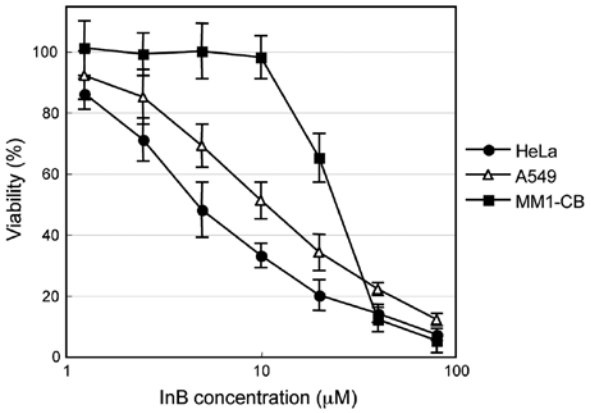

Figure 2. Comparison of the cell viabilities of human cancer cell lines treated with ineupatorolide B (InB). Cervical cancer HeLa, lung carcinoma A549, and melanoma MM1-CB cells were used. The cells were treated with the indicated agents for 2 days, followed by estimation of cell viability using the MTT assay, as described in the Materials and methods. Data are presented as means ± standard deviation (SD) of three independent experiments.

Table I. InB exhibited a more potent growth-inhibitory activity than Eup for all cell lines examined. We further examined the cytotoxic activity of InB against HeLa cells, which were among the most sensitive to InB.

Induction of apoptosis by InB. To examine whether the cytotoxic activity of InB was due to the induction of apoptosis, we performed TUNEL staining, which can detect the typical DNA fragmentation accompanied by apoptosis. TUNEL-positive cell numbers increased as early as 12 h after the addition of InB, and >80% of the cells were stained after 24 h (Fig. 3). These results suggest that the growth-inhibitory activity of InB was, at least in part, due to the induction of apoptosis.

Activation of p53 and NFAT reporter plasmids by InB. The transactivation ability of several major transcription factors was examined using a luciferase reporter assay. Among the reporters for p53 (PG13-Luc, p21-Luc, Bax-Luc, and hNoxa-Luc), serum-responsive factor (SRE-Luc), NF-κB (IgK-Luc), NFAT (NFAT-Luc) and CREB (CRE-Luc), the reporters PG13-Luc, Bax-Luc, and NFAT-Luc were activated by InB in a dose-dependent manner (Fig. 4A). The activation of NFAT-Luc by InB in HeLa cells was greater than that observed in InB-resistant MM1-CB cells whereas the activation of PG13-Luc and Bax-Luc was similar between these cells (data not shown). Thus, the elevation of NFAT

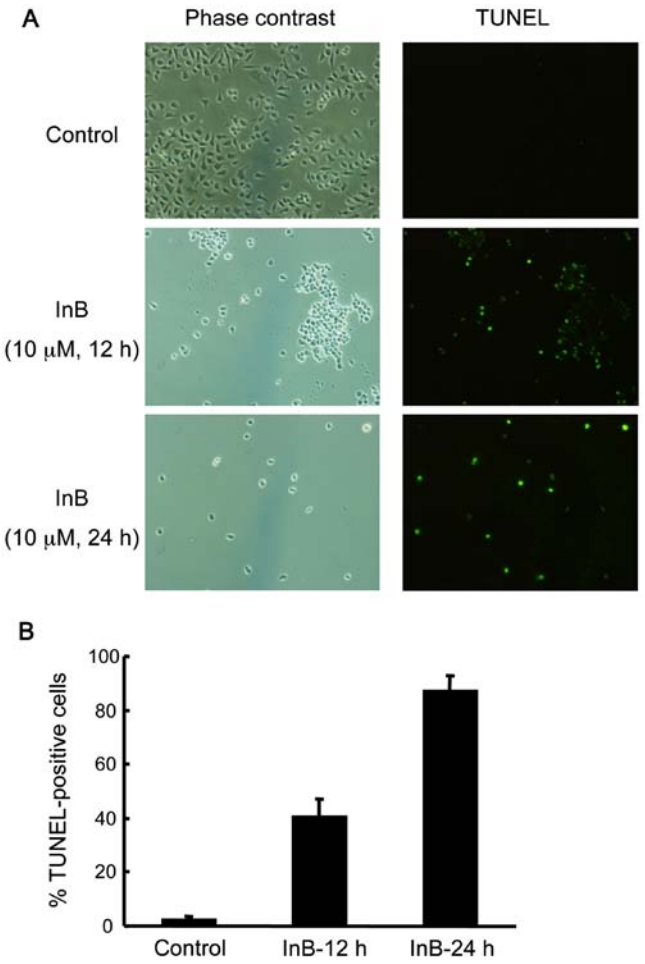

Figure 3. Induction of apoptosis by ineupatorolide B (InB). (A) HeLa cells were cultured for 12 or 24 h in the presence of 10 μM InB. The nuclei of the cells were stained using the TUNEL method, as described in the Materials and methods. Phase contrast (left) and TUNEL-stained cells (right) are shown. (B) Relative percentages of TUNEL-positive cells are shown. Error bars represent SD.

transcriptional activity may be associated with the cytotoxic activity of InB.

PKC inhibitor suppression of the InB-induced NFAT activation. We then examined the effects of various inhibitors or activators on NFAT reporter activity. Preincubation with PKC inhibitors such as staurosporine and K252a suppressed the InB-induced activation of NFAT (Fig. 4B), suggesting the involvement of PKC. Wortmannin and LY290004, both

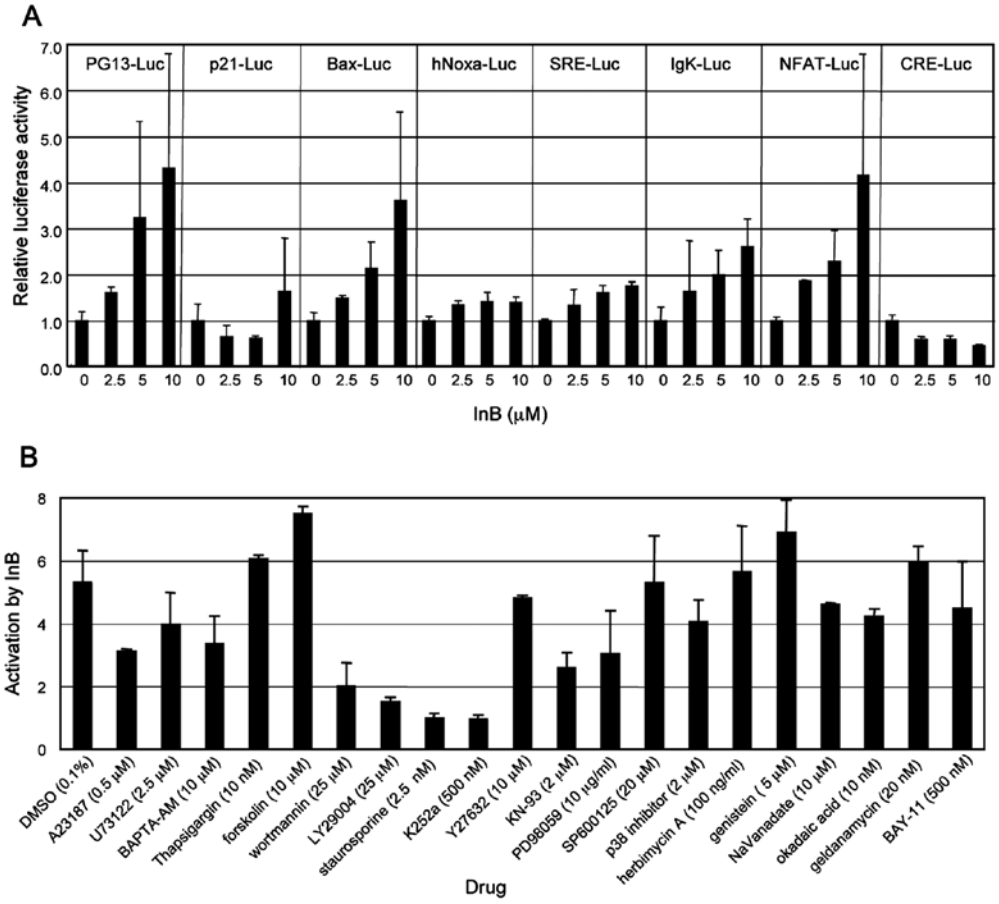

Figure 4. Activation of p53 and NFAT by ineupatorolide B (InB). (A) HeLa cells were co-transfected with firefly reporter plasmids such as pG13-Luc, p21-Luc, Bax-Luc, hNoxa-Luc, SRE-Luc, IgK-Luc, NFAT-Luc and CRE-Luc, and the *Renilla* reporter plasmid, SV40-Rluc, and then cultured for 48 h. The cells were then treated with InB at concentrations of 0, 2.5, 5 or 10 μM for 24 h and harvested. The firefly and *Renilla* luciferase activities in the cell extracts were sequentially measured. The activity of firefly luciferase was normalized using *Renilla* luciferase activity. The error bars represent SD. (B) Effects of inhibitory compounds on the activation of NFAT by InB. The cells were treated as described above, except that they were pretreated with various drugs at the concentrations indicated for 1 h before the addition of InB. The relative activation of luciferase activity in cells treated with InB at a concentration of 10 μM vs. that of cells without InB treatment is shown.

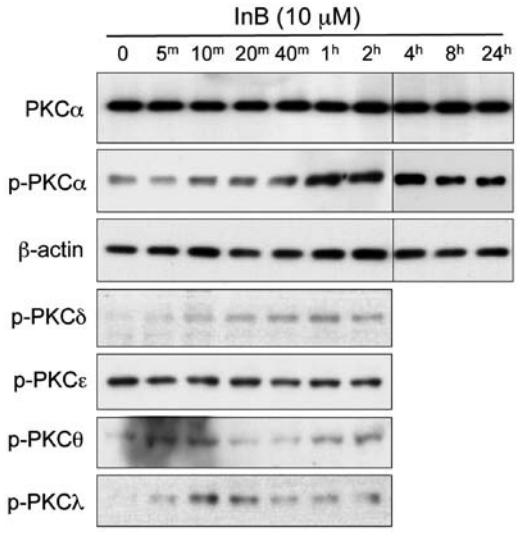

Figure 5. Activation of PKC by ineupatorolide B (InB). HeLa cells were treated with 10 μM InB for 0, 5 min (5^m), 10 min (10^m), 20 min (20^m), 40 min (40^m), 1 h (1^h), 2 h (2^h), 4 h (4^h), 8 h (8^h) and 24 h (24^h), and then a cell extract was prepared. Western blotting was performed using anti-PKC, phospho-specific anti-PKCα, phospho-specific anti-PKCδ, phospho-specific anti-PKCε, phospho-specific anti-PKCθ, phospho-specific anti-PKCλ, and control anti-β-actin antibodies.

of which are inhibitors of phosphatidylinositol 3-kinase, also attenuated the NFAT activation, but less effectively. Pretreatment with the other substances tested had almost no effect on InB-induced activation of NFAT. These substances included A23187 (calcium ionophore), U73122 (phospholipase C inhibitor), BAPTA-AM (intracellular calcium chelator), forskolin (adenylate cyclase activator), Y27632 (ROCK inhibitor), KN-93 (CaMK inhibitor), PD98059 (MEK inhibitor), SP600125 (JNK inhibitor), p38 inhibitor (p38 MAPK inhibitor), herbimycin A (Src kinase inhibitor), genistein (tyrosine kinase inhibitor), vanadate (tyrosine phosphatase inhibitor), okadaic acid (PP2A and PP1 inhibitor), geldanamycin (HSP90 inhibitor) and BAY-11 (NF-κB inhibitor) (10).

Activation of PKCα by InB. Activation of PKC is accompanied by phosphorylation, which can be detected by western blotting using a phospho-specific antibody. The phosphorylation level of PKCα, but not of other PKC subtypes such as PKCδ, PKCε, PKCθ, and PKCλ, increased after treatment with InB (Fig. 5). This was not caused by an increase in the levels of protein expression because the levels of total PKCα were not altered during the incubation time of up to 24 h. An initial increase of

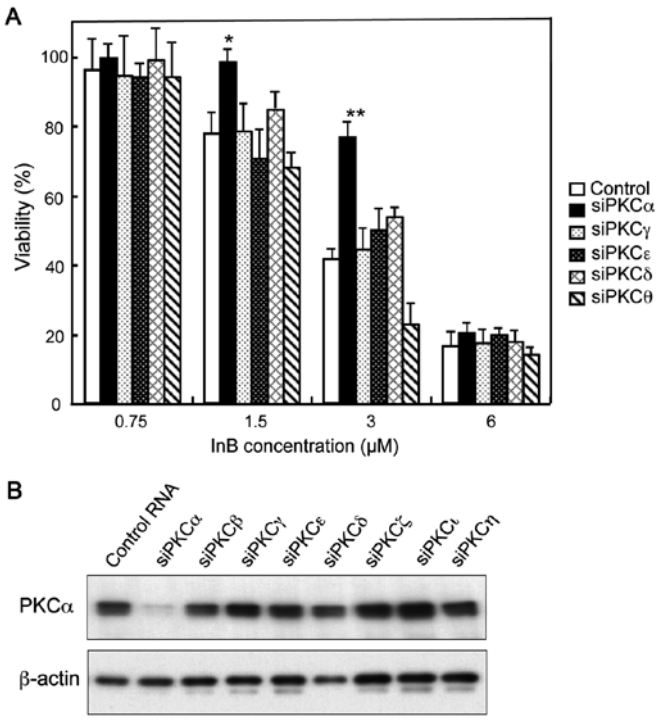

Figure 6. Knockdown of PKCα attenuates the cytotoxic effects of ineupatorolide B (InB). (A) PKC subtype-specific siRNA or control siRNA was transfected into HeLa cells and incubated for 2 days. Cells were then replated, and the viability was examined using the MTT assay. Relative viability versus that without InB is shown. Error bars represent SD. $^{*}P<0.05$; $^{**}P<0.01$ vs. control siRNA-transfected cells by Student's t-test. (B) Subtype-specific knockdown of PKC was confirmed by western blotting using pan-PKC and anti-β-actin antibodies.

phosophorylated PKCα was observed as early as 10 min after the addition of InB, and the level increased to a maximum 4 h after the addition of InB. Thereafter, the phosphorylation level gradually decreased; however, it maintained a higher level than the basal level in non-treated cells 24 h after InB addition.

Knockdown of PKCα attenuates the cytotoxic activity of InB. We then examined the effects of siRNAs targeting various PKC subtypes on cellular survival in the presence of InB. Knockdown of PKCα but not PKCγ, PKCε, PKCδ, or PKCθ attenuated the cytotoxic activity caused by the addition of InB at concentrations of up to 3 μM (Fig. 6A). The expected knockdown of PKCα was confirmed by western blotting, which showed an almost complete reduction of PKCα protein with no apparent effects on the other PKC subtypes (Fig. 6B). The effects of siRNAs targeting PKCβ, PKCγ, PKCζ, PKCι, and PKCη showed no apparent difference as compared with those of control siRNAs (data not shown). Consequently, it is suggested that InB can exhibit cytotoxic effects via the activation of PKCα.

Discussion

Inula cappa contains many bioactive compounds (6,7,21,22), among which sesquiterpenoids may play a main role. In this study, the effects of InB, one of sesquiterpenoids, on the proliferation and survival of tumor cell lines were examined. Compared with A549 lung carcinoma and MM-CB melanoma cells, we found that HeLa cervical cancer cells were highly sensitive to InB than (Fig. 2). The decrease of the cell survival capacity after treatment with InB may mainly be attributable to the induction of apoptosis (Fig. 3). Luciferase reporter assay were performed to analyze the mechanism of action of InB. The results showed the involvement of p53 and NFAT/Ca^{2+} in the signaling pathway (Fig. 4A). Because the activation of NFAT by InB was reduced in InB-resistant MM1-CB cells, we further examined the NFAT signaling pathway. Using various inhibitors (Fig. 4B) and siRNAs (Fig. 6), we found the involvement of PKCα in the cytotoxic effects caused by InB.

The involvement of NFAT in apoptosis remains obscure; however, a recent report verified the induction of apoptosis by NFATc3 (23). InB-induced transactivation ability of NFAT was inhibited by PKC inhibitors such as staurosporine and K252a (Fig. 4B). This is consistent with a recent report which stated that the staurosporine analogue GF109203X, reduced NFAT activity in osteoclast progenitor cells (24).

The activation of PKCα was observed as early as 10 min after the addition of InB (Fig. 5). This alteration appeared to be the initial event induced by InB, and therefore, it is probable that PKCα is the direct target of InB. The tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (25) is a well-known diacylglycerol-like PKC-activating compound. What could be the difference between tumor-promoting TPA and cytotoxic InB? TPA is highly active because of its direct activation of PKC at nM concentration, whereas InB can cause effects at μM concentrations. TPA can activate the conventional and novel subtypes of PKC such as PKCα, β, γ, δ, ε, η and θ, however, InB activated PKCα exclusively (Fig. 5). After treatment with TPA, the levels of the total protein amount of PKC were rapidly downregulated to undetectable levels within 24 h (26). Conversely, phosphorylated PKCα was still detectable and the total amount of PKCα was not altered after a 24-h treatment period with InB (Fig. 5). Thus, the specific and continuous activation of PKCα by InB may account for the cytotoxic effects leading to apoptosis.

In the reporter assay, p53 was also activated by InB, although the activation of p53 in InB-sensitive HeLa cells was similar to that in InB-resistant MM1-CB cells. Because p53 plays a main role in the induction of apoptosis, adenoviral wild-type p53 has been used for gene therapy in esophageal squamous cell carcinoma (27). InB as a p53-activating compound is a promising and novel anticancer drug.

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