Phenanthrene-Based Tylophorine-1 (PBT-1) Inhibits Lung Cancer Cell Growth through the Akt and NF-κB Pathways

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Tylophorine and related natural compounds exhibit potent antitumor activities. We previously showed that PBT-1, a synthetic C9-substituted phenanthrene-based tylophorine (PBT) derivative, significantly inhibits growth of various cancer cells. In this study, we further explored the mechanisms and potential of PBT-1 as an anticancer agent. PBT-1 dose-dependently suppressed colony formation and induced cell cycle G2/M arrest and apoptosis. DNA microarray and pathway analysis showed that PBT-1 activated the apoptosis pathway and mitogen-activated protein kinase signaling. In contrast, PBT-1 suppressed the nuclear factor kappaB (NF-κB) pathway and focal adhesion. We further confirmed that PBT-1 suppressed Akt activation accelerated RelA degradation via IκB kinase-α and down-regulated NF-κB target gene expression. The reciprocal recruitment of RelA and RelB on COX-2 promoter region led to down-regulation of transcriptional activity. We conclude that PBT-1 induces cell cycle G2/M arrest and apoptosis by inactivating Akt and by inhibiting the NF-κB signaling pathway. PBT-1 may be a good drug candidate for anticancer chemotherapy.

Introduction

Natural products have long been major sources of anticancer and other drugs. Several plants of the *Tylophora* genus have been used medicinally as anti-inflammatory, antiarthritis, and antiamebic agents in East Asia.[1] The phenanthroindolizine alkaloid tylophorine and its analogues are found primarily in plants of the Asclepiadaceae family, including members of the *Tylophora* genus.[2] Although tylocrebrine, a natural product related to tylophorine, failed in anticancer clinical trials in 1966 because of toxicity to the central nervous system (CNS), certain cytotoxic analogues were reevaluated for antitumor potential by the National Cancer Institute (NCI) using a 60-tumor cell line panel. As we reported previously, a series of novel polar water-soluble synthetic phenanthrene-based tylophorine derivatives (PBTs[a]) exhibit potent cytotoxic activity against the A549 human lung cancer cell line.[3] These compounds may have little or no CNS toxicity because their increased polarity should prevent them from penetrating the blood−brain barrier. Compound PBT-1 showed modest in vivo antitumor activity against human A549 xenografts in nude mice and potent in vitro cytotoxic activity.[4]

Tylophorine analogues exhibit potent growth-inhibitory activity against a broad range of human cancer cells,[5−10] and this antitumor activity results from the irreversible inhibition of protein synthesis at the elongation stage of the translation cycle.[11−13] Several key metabolic enzymes, including thymidylate synthase[9] and dihydrofolate reductase, have been reported as biological targets of tylophorine alkaloids.[8] Tylophorine derivatives also inhibit activator protein-1-mediated, CRE-mediated, and nuclear factor κB (NF-κB) mediated transcription.[14,15] These discoveries illustrate the potential of tylophorine derivatives as a new class of antitumor drugs. However, the comprehensive evaluation of the antitumor activity of tylophorines has not been reported, and the mechanism responsible for the inhibitory effects on cancer cell growth is largely unknown.

We used cDNA microarray analysis to investigate the antitumor activity of tylophorine analogues against human lung cancer cells and analyzed the effect of PBT-1 on gene expression. On the basis of the microarray data, we explored the possible signaling pathways that may inhibit cell growth and induce apoptosis. The effects of tylophorine analogues on key signaling pathways were also investigated.

Results

Effect of PBT Series Compounds on Cancer Cell Cytotoxicity. Our previous data showed that novel 9-substituted 2,3-methylenedioxy-6-methoxy-PBTs exhibit potent cytotoxic activity against certain human cancer cell lines, including a multidrug-resistant variant.[4] However, the biological functions of these PBTs have not been explored. To identify the pharmacophores relating to the high potency of the new PBT series, 10 compounds were examined. Their structures are shown in Figure 1A. The inhibitory effect of the drugs was calculated as the percentage of viable drug-treated cells compared with the percentage of viable cells in the untreated control. The median inhibitory concentration (IC_{50}) value was defined as the concentration of drug that inhibited cell growth by 50%. The CL1-0 lung cancer cell line was exposed to increasing concentrations of compounds for 48 h. PBT-1, PBT-2, PBT-9, and PBT-10 exhibited dose-dependent growth inhibition, with IC_{50} values of 0.81, 0.44, 0.37, and 0.46 μg/mL, respectively, at 48 h (Figure 1B). The IC_{50} values of the six remaining compounds were >2 μg/mL; PBT-3, PBT-7, and PBT-8 exhibited greater cytotoxic activity than PBT-4, PBT-5, and

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[a] Abbreviations: PBTs, phenanthrene-based tylophorine derivatives.

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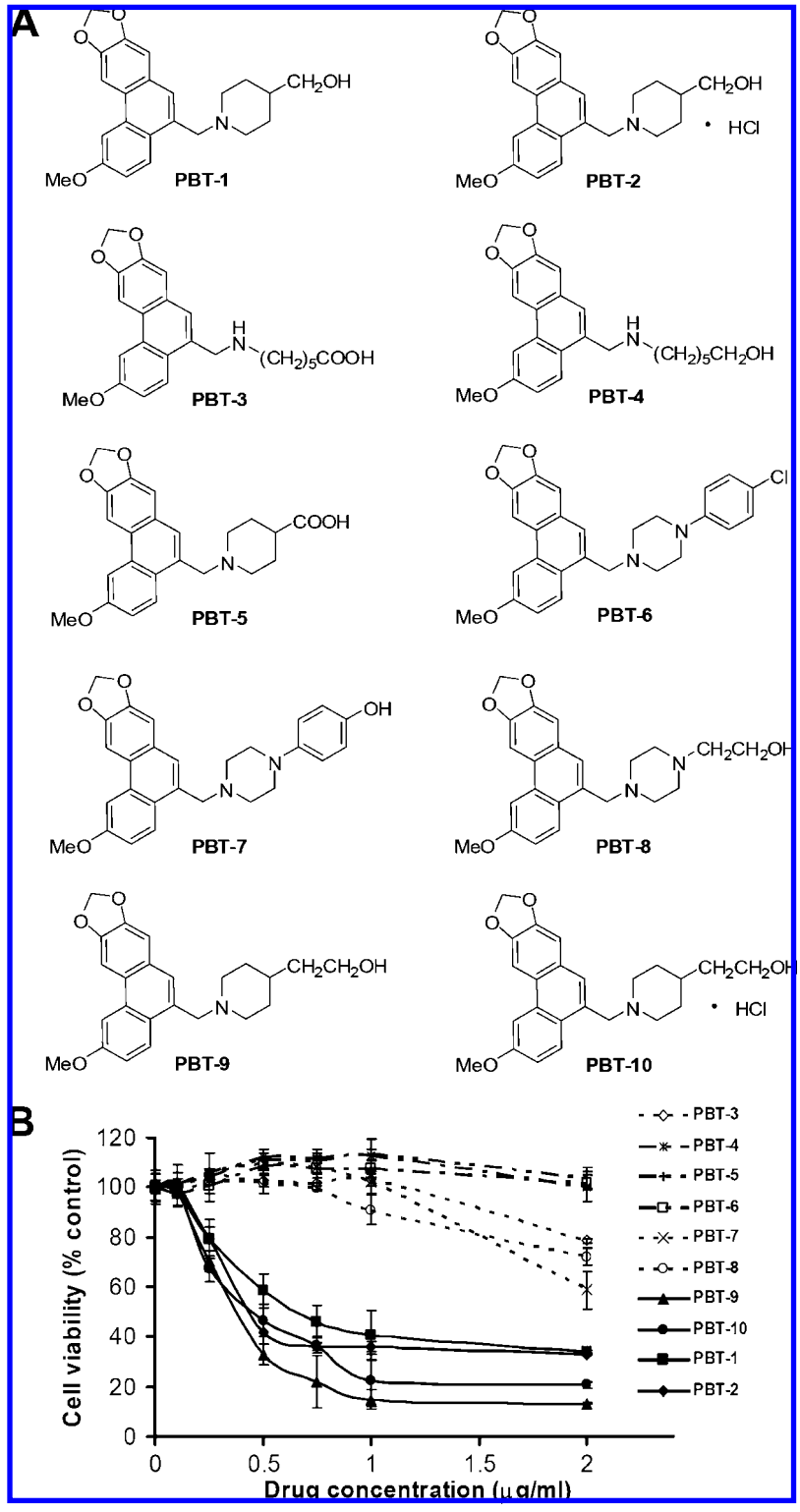

Figure 1. Structure and cytotoxic activity of PBT-series compounds in lung cancer cells: (A) chemical structures of PBT compounds; (B) CL1-0 cells were treated with various PBT compounds as indicated for 48 h. The viable cells were detected with an MTS assay. PBT-1, PBT-2, PBT-9, and PBT-10 exhibited dose-dependent growth inhibition, and the IC_{50} values were 0.81, 0.44, 0.37, and 0.46 μg/mL, respectively.

PBT-6. These results indicate that the structural features contributing to the greatest cytotoxic effects on lung cancer cells are a pendent pyridine/pyridinium ring with a hydroxymethyl or hydroxyethyl substituent, as found in PBT-1, PBT-2, PBT-9, and PBT-10.

PBT-1 Can Suppress Lung Cancer Cell Growth. To explore the antitumor potential of PBTs, CL1-0, CL1-5, H460, PC-9, and A549 lung cancer cells as well as BEAS2B, immortalized normal bronchial epithelial cells were treated with the highly potent compound PBT-1, and cell viability was determined by the MTS assay. The resulting IC_{50} values for PBT-1 were around 0.2−0.4 μg/mL against these lung cancer cells, but there was no significant inhibition on BEAS2B cells (Figure 2A). With increasing concentration of the drug, CL1-0 cells treated with PBT-1 showed cell shrinkage and rounding, followed by broken membranes (Figure 2B). The inhibitory

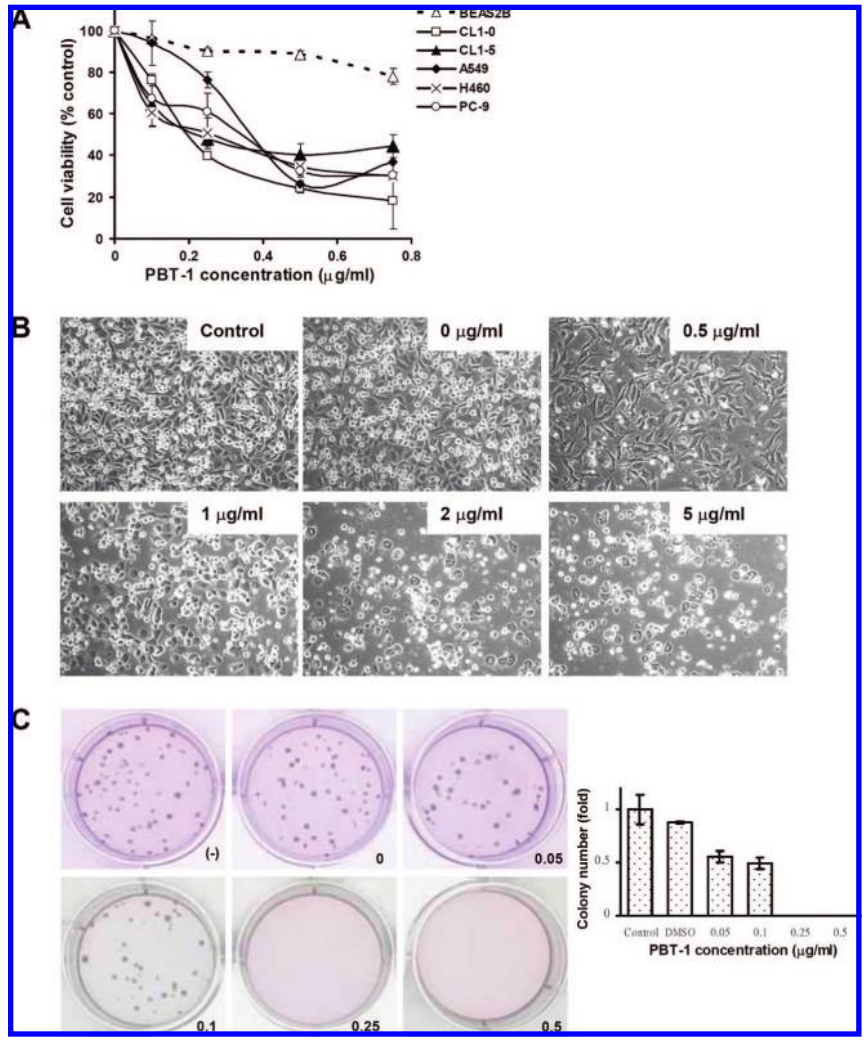

Figure 2. Growth inhibition and loss of clonogenicity in lung cancer cell lines. (A) Various cells were treated with an increasing concentration of PBT-1 of 0, 0.1, 0.25, 0.5, and 0.75 μg/mL for 48 h. The viable cells were detected with an MTS assay. (B) The changes in cell morphology were observed microscopically. The number of viable cells of CL1-5 decreased markedly at a dose of 1 μg/mL of PBT-1. (C) Five hundred cells were plated in soft agar as illustrated with different concentrations of PBT-1 of 0, 0.05, 0.1, 0.25, and 0.5 μg/mL and then incubated for 3 weeks. The cells were fixed with 4% paraformaldehyde and stained with 0.01% crystal violet for 16 h. The inhibitory effect of PBT-1 on the colony formation of CL1-0 cells was determined as the percentage of visible colony numbers in the drug-treated groups compared with the untreated control groups. The data are expressed as the mean $\pm$ SD.

effect of PBT-1 on the colony formation of CL1-0 cells was calculated as the percentage of visible colonies of the drug-treated groups compared with the percentage in the untreated control groups. Treatment with PBT-1 also suppressed colony formation in a dose-dependent manner. The growth of CL1-0 cells was suppressed fully when the concentration of PBT-1 was >0.25 μg/mL.

PBT-1 Induces Cell Cycle Arrest in the G2/M Phase and Activates Apoptotic Proteins. To understand the mechanism of PBT-1's effect on cell growth, we used DNA flow cytometry to investigate changes in the cell cycle in CL1-0 cells treated with PBT-1. Treatment with PBT-1 time-dependently decreased the proportion of cells in the G0/G1-phase and increased the proportion of cells in the G2/M-phase up to 12 h (Figure 3A). Interestingly, a sub-G1 peak and an S-phase peak appeared after 16 h of PBT-1 treatment. To confirm this cell cycle analysis, we measured the effects of PBT-1 on regulatory proteins of cell cycle progression. Western blot analysis indicated that treatment with PBT-1 caused cyclin B1 and cyclin D1 protein accumulation in a dose-dependent manner but had no effect on cyclin A, cyclin E, p21, or p27 protein levels (Figure 3B). In addition, annexin V and Western blot analyses indicated that treatment with PBT-1 induced apoptosis of lung cancer cells (Figure 3C and Figure 3D).

Gene Expression Profiles of Lung Cancer Cells after PBT-1 Treatment. To elucidate the molecular mechanism of PBT-1's induction of cell apoptosis, we used a cDNA microarray with Gene Spring software to analyze the changes in gene expression after PBT-1 treatment. In total, 1437 putative genes showed a statistically significant 2-fold difference in expression in CL1-0 lung cancer cells after 24 h of PBT-1 treatment (0.5 μg/mL) compared with untreated control cells. Of these 1437 genes, 883 were up-regulated and 554 were down-regulated. The genes were up-regulated or down-regulated to a similar extent. The up-regulated genes are those involved in signal pathways such as mitogen-activated protein kinase (MAPK); the apoptotic pathway was activated markedly after treatment with PBT-1. In contrast, focal adhesion and metabolic pathway genes were down-regulated significantly by treatment with PBT-1. Some of these PBT-1-induced genes are listed and categorized by their putative functions in Table 1. Protein kinase A, CASP8, and FADD-like apoptosis regulator, tumor necrosis factor receptor 10b, MAPK kinase kinase 14, and NF-κB inhibitor-α

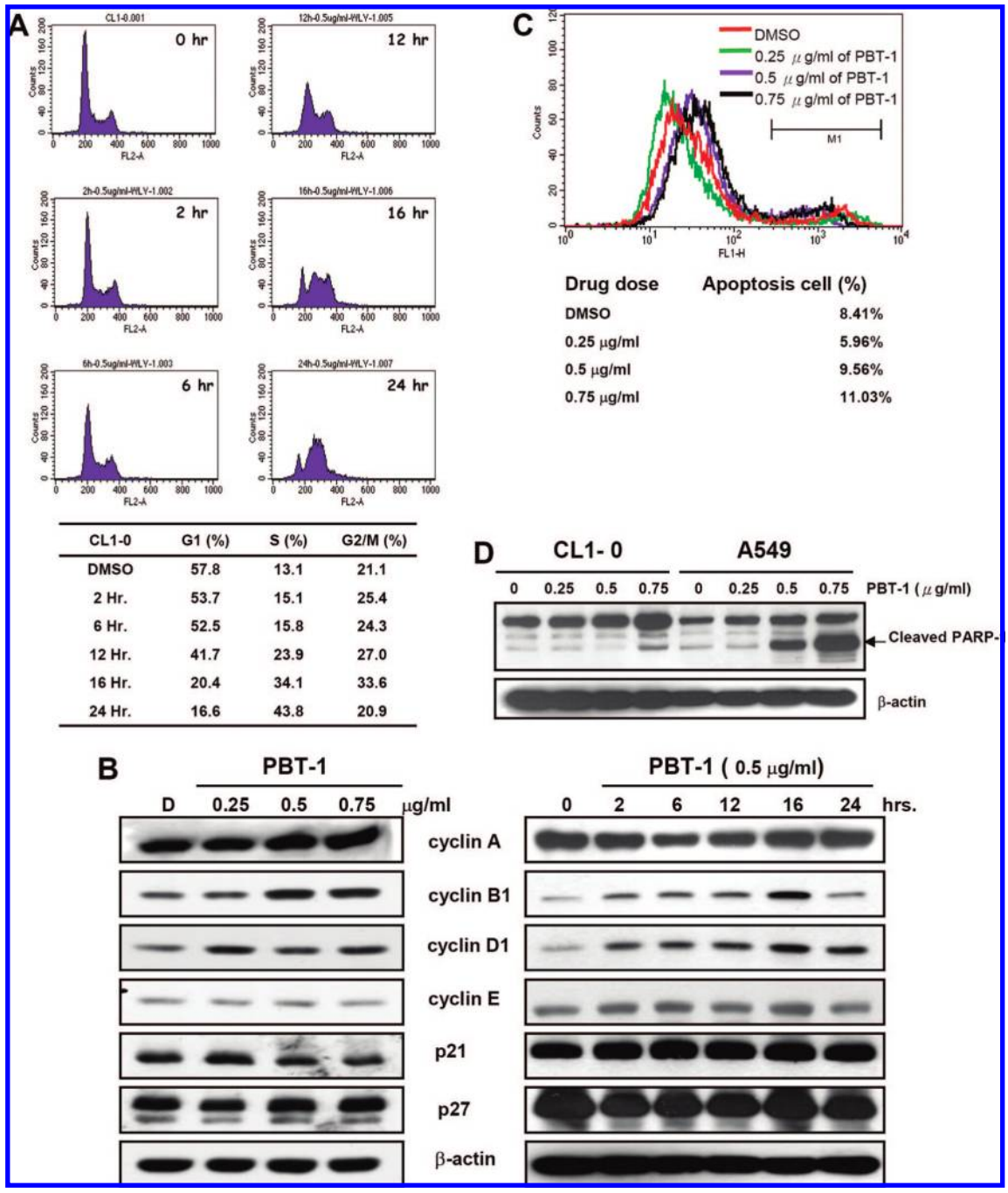

Figure 3. PBT-1 induces cell cycle arrest in G2-M phase and activates apoptotic proteins. (A) CL1-0 cells/dish (2×10^5) were seeded onto each 60 mm dish and incubated for 24 h. Various concentrations of PBT-1 were added to the culture medium and incubated for an additional 2, 6, 12, 16, and 24 h. Cells were then harvested and analyzed by flow cytometry. The cell cycle phase distribution was determined using CellQuest software. (B) CL1-0 cells were treated with increasing concentrations of PBT-1 for 24 h or 0.5 μg/mL for the time indicated. The expression levels of cell cycle regulatory proteins were analyzed by Western blotting. (C) CL1-0 cells were treated with increasing concentrations of PBT-1 for 24 h, stained with Fluor 488 annexin V, and PI and analyzed by flow cytometry. The percentage of apoptotic cells is shown in the following table. (D) The expression levels of PARP-1 were analyzed by Western blotting, with actin expression as the internal control.

were up-regulated significantly (2-fold) by PBT-1 treatment. The microarray data were also confirmed by real-time RT-PCR.

PBT-1 Can Activate Cell Apoptosis Pathways. To predict the putative signaling pathway involved in PBT-1 stimulation, we used the KEGG and BIOCARTA pathway databases to analyze the differentially expressed profiles of PBT-1-induced genes to fit the transduction-signaling map. Two major pathways were up-regulated by PBT-1: the cell cycle regulatory pathway and the apoptotic pathway. Cell cycle analysis showed that PBT-1 increased the expression of cdc25a (2.9-fold) and cyclin D1 (2.1-fold) and decreased the expression of cyclin A (0.33-fold) (Figure 4A). The results for the apoptosis pathway showed that PDK1 and Akt were down-regulated (0.26- and 0.48-fold), and IκB were up-regulated (2.2-fold). These data suggest that PBT-1 suppresses the NF-κB signaling pathway by suppressing Akt-IKK activation (Figure 4B).

PBT-1 Inhibits Akt Phosphorylation and NF-κB Activation. To confirm whether PBT-1 can suppress the activation of both Akt and NF-κB, we used Western blot analysis and an NF-κB reporter assay to examine the changes in Akt and NF-κB activity after PBT-1 treatment. Western blot analysis confirmed that Akt phosphorylation decreased and IκB protein

Table 1. Affected Genes Related to Apoptosis Pathway

UniGene	symbol	microarray, fold change, mean ± SD	molecular function	real-time PCR, fold change, mean ± SD	primers
NM_002730	PRKACA	5.38 ± 2.11	kinase	1.39 ± 0.31	TCAAACTGATCCAAGTGGGC GCCCTGAGAACAGGACTGAG
NM_003879	CFLAR	3.01 ± 0.68	signal transducer	2.80 ± 0.33	TCAGAATCCTTTCCAGTGGG TCCAGGCTTTCGGTTTCTTT
AL353950	PPP3CA	2.53 ± 0.42	phosphatase	5.31 ± 0.61	TCCCTCCTTCATAAGATGCG CAATTGATCCCAAGTTGTCG
U72398	BCL2L1	2.70 ± 0.28	protein binding	1.83 ± 0.17	GGGGTAAACTGGGGTCGCATT ACCTGCGGTTGAAGCGTTC
BC001281	TNFRSF10B	2.53 ± 0.43	signaling molecular	1.71 ± 0.21	AAGACCCTTGTGCTCGTTGT AGGTGGACACAATCCCTCTG
NM_003954	MAP3K14	2.25	kinase	2.94 ± 0.61	CCCTTCTCTCACAGCTCCAT ATGGAGGACAAGCAGACTGG
AI078167	NFKBIA	2.23	protein binding	6.8 ± 0.89	CCATGGTCAGTGCCTTTTCT GTCAAGGAGCTGCAGGAGAT
AF346607	IRAK1	2.1	kinase	2.23 ± 0.23	GGTGCTTCTCAAAGCCACTC ACACGGACACCTTCAGCTTT
U57843	PIK3CD	2.03	kinase	1.69 ± 0.25	GGCATCCTGCGTTGTTACTT GACGATAAGGAGTCAGGCC
N32526	AKT3	0.48	kinase	1.61 ± 0.15	CACTGAAAAGTTGTTGAGGGG AGGTTGGGTTCAGAAGAGGG
AU146532	PDK1	0.26	kinase	0.56 ± 0.07	GGAGGTCTCAACACGAGGTC GTTCATGTCACGCTGGGTAA

increased in a dose-dependent manner when CL1-0 cells were exposed to increasing PBT-1 concentration (Figure 5A). The reporter assay showed that NF-κB transcriptional activity was induced by stimulation with TNF-α and that PBT-1 dose-dependently suppressed TNF-α-induced NF-κB transcriptional activity in CL1-0 cells (Figure 5B). Western blot analysis showed that phosphor-Akt increased after TNF-α treatment but that the TNF-α-induced activation of Akt was inhibited by PBT-1 in both CL1-0 and A549 cells (Figure 5C). In addition, RelA and IκB protein decreased when the cells were co-treated with TNF-α and PBT-1. The expression level of COX-2, a gene known to be downstream of NF-κB, was high in cells treated with TNF-α, but its expression decreased in cells exposed to PBT-1. Interestingly, the activity of IKKα, but not IKKβ, increased after treatment with PBT-1 (Figure 5D). The chromatin IP assay showed a decreased binding affinity of RelA and NF-κB1 (p50) to the NF-κB response element on the COX-2 promoter in A540 cells. In contrast, the binding activity of Re1B was higher after exposure to PBT-1 co-treated with TNF-α than after exposure to TNF-α alone, but the binding activity of Re1B decreased when exposed to PBT-1 at >0.25 μg/mL (Figure 5E).

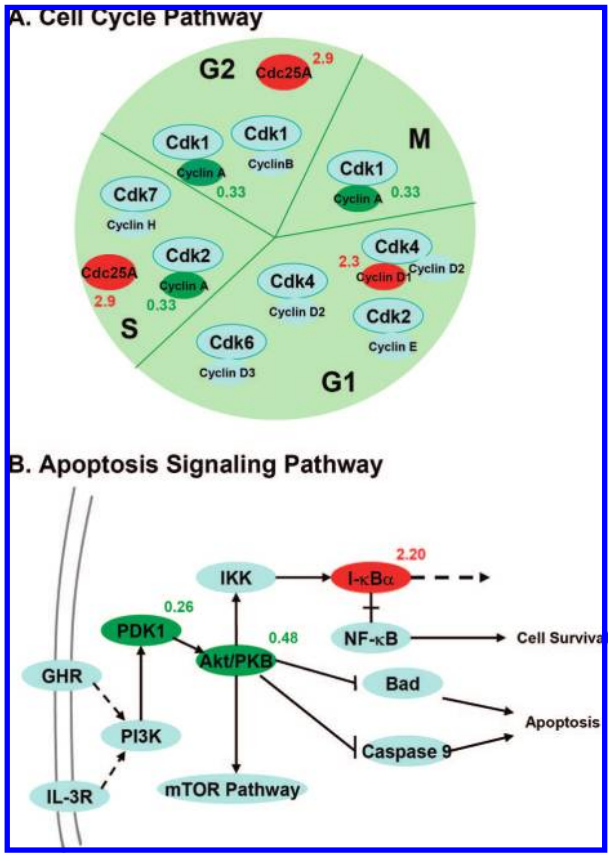

Figure 4. PBT-1 induced genes in different pathways according to the KEGG and BIOCARTA pathway databases. PBT-1-induced genes are colored red, and the red numbers close to the genes indicate the fold increase. PBT-1-suppressed genes are colored green. (A) Cell cycle pathway. (B) Apoptosis pathway. The hypothetical PBT-1-regulated signaling pathways were modified from the KEGG and BIOCARTA databases.

Discussion

In this study, we demonstrated that the PBT analogue PBT-1 has potent cytotoxicity against various lung cancer cells and can induce cell cycle G2/M arrest and apoptosis. The microarray data showed that PBT-1 increases the expression of genes belonging to certain signaling pathways, such as MAPK and apoptosis, and dramatically suppresses the expression of genes for metabolic enzyme. These data indicate that PBT-1 treatment can regulate these signaling pathways. We showed further that the key signaling pathways affected in lung cancer cell survival involve inactivation of Akt and NF-κB. Our data suggest that PBT-1 affects cell growth by inhibiting the NF-κB signaling pathway. This suggests that PBT-1 is a good candidate for anticancer therapy.

Tylophorine compounds comprise a new class of anticancer agents because of their novel chemical structure combined with the unique spectrum of activity compared with current antitumor drugs, based on NCI tumor cell panel studies. Because a related compound, tylocrebrine, failed in clinical trials because of CNS toxicity, investigators have since tried to develop derivatives that are not toxic to the CNS. We designed and synthesized a series of novel 9-substituted 2,3-methylenedioxy-6-methoxy-PBTs, and one analogue (PBT-1) showed in vivo activity in a

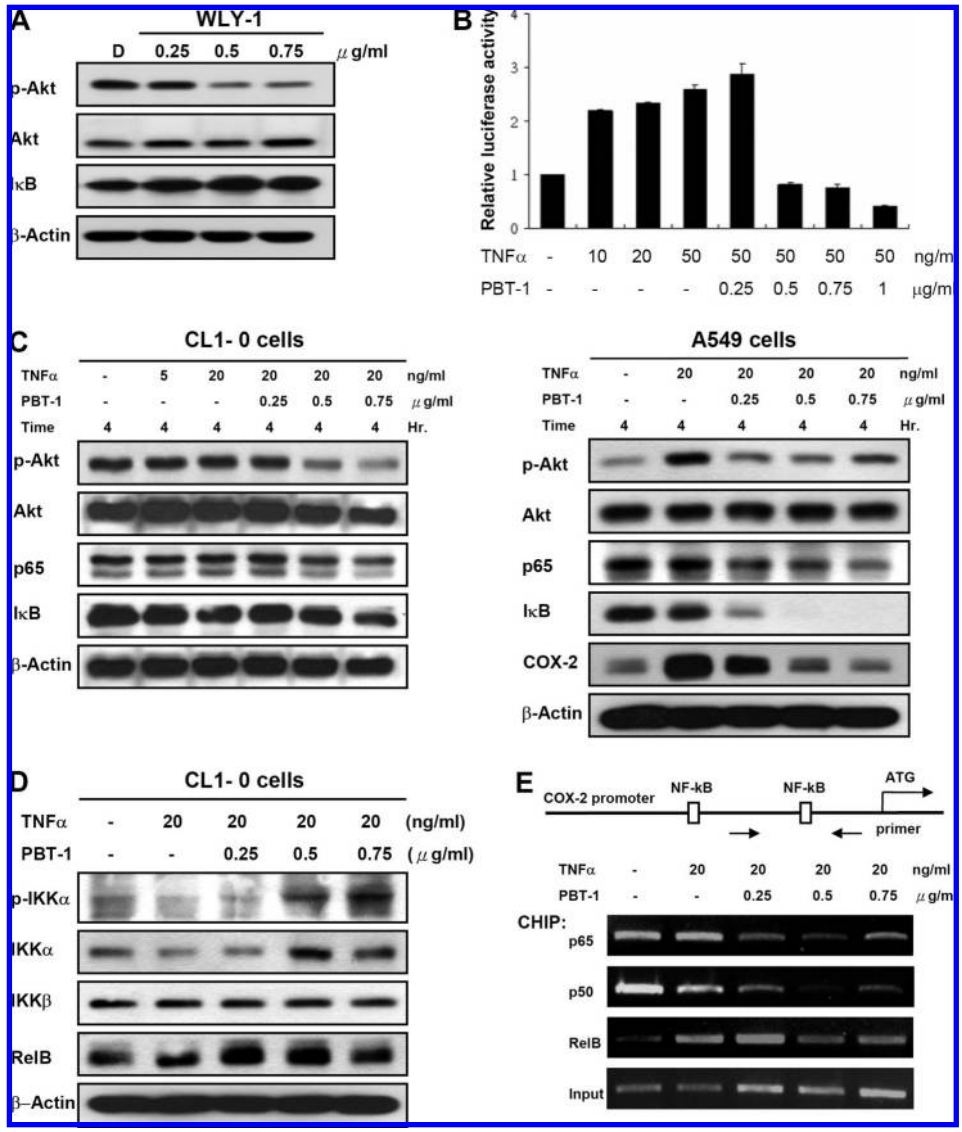

Figure 5. PBT-1 inhibition of the NF-κB signaling pathway correlates with suppression of Akt activity. (A) CL1-0 cells were treated with increasing concentrations of PBT-1 for 24 h. The activity and expression levels of Akt and IκB were analyzed by Western blotting. (B) CL1-0 cells were transiently transfected with pNF-κB-Luc vector and pSV-β-galactosidase vector as the internal control for 20 h. The medium was then changed to serum-free medium without or with increasing concentrations of PBT-1 as indicated for 1 h, and the cells were stimulated with various concentrations of TNF-α as indicated for 4 h. The luciferase and β-galactosidase activities were measured using the Luciferase Assay Kit and Galacto-Light Plus system. One representative experiment ($n = 3$, independent transfection/treatment) is shown. All data are presented as the mean $\pm$ SD (C, D). CL 1−0 and A549 cells were serum starved overnight, pretreated with various concentrations of PBT-1 as indicated, and then stimulated with TNF-α as indicated for 4 h. The cell extract was probed with specific antibodies as indicated. A representative Western blot is shown. (E) Chromatin immunoprecipitation (ChIP) assays were performed on untreated control A549 cells and on cells treated with PBT-1 and TNF-α as indicated for 4 h. The extracted chromatin was immunoprecipitated with anti-RelA, anti-p50, and anti-RelB antibodies as indicated. The recovered DNA was amplified by PCR using primers covering the region of the COX2 promoter from nt −359 to +106. Control amplifications were performed on preimmunoprecipitated ("input") chromatin. A representative ChIP is shown.

murine model without overt toxicity to the animals.[3,4] The biological function and molecular mechanism responsible for the effect of PBT-1 on cell cytotoxicity merit investigation. Interestingly, CL1-0 and CL1-5 cells, which were both isolated from the same original clinical sample, exhibited the same sensitivity (IC_{50}) to PBT-1, even though they have different intensities of invasive ability. This suggests that the key mechanism responsible for PBT-1's ability to suppress cell growth is involved in tumorigenesis but not in the invasion machinery.

To elucidate the mechanism of action of PBT-1, we examined its effect on cell cycle progression. We found that PBT-1 induced G2/M arrest, which was accompanied by accumulation of cyclin B1 (Figure 2) and activation of the MAPK signaling pathway, as predicted by our microarray data (Table 1). Interestingly, paclitaxel also induces cyclin B1 accumulation,[16] cell cycle G2/M arrest, and apoptosis through an MAPK-dependent pathway.[17] Thus, PBT-1 and paclitaxel may activate the same death-signaling pathway to induce cell apoptosis. Gao et al. found a dose-dependent S-phase accumulation at 24 h in human nasopharyngeal carcinoma KB cells treated with another tylophorine analogue class (DCBs) developed by their laboratory.[15] They also demonstrated that in human pancreatic duct carcinoma cells, the percentage of G2/M-phase cells increased gradually with time of exposure to a tylophorine analogue, whereas the percentage of S-phase cells increased initially and then decreased with exposure time.[18] We found that PBT-1 treatment increased the percentage of G2/M-phase cells at 12 h,

but this was followed by a decreased percentage of G2/M-phase cells and a corresponding increased percentage of S-phase cells after 16 h. These contrasting effects may relate to the variant behavior of cell types and differences in the particular tylophorine analogues. However, our microarray data also suggest that these compounds decrease cell growth by interrupting the regulatory proteins in cell cycle progression (Figure 4B).

NF-κB is a key transcription factor for the inflammatory response,[19] tumor progression,[20] and drug resistance.[21,22] Suppression of NF-κB activity leads to induction of an apoptotic response.[23] Thus, NF-κB is an important therapeutic target in cancer treatment.[24] Using a cDNA microarray technique, we found that treatment of cells with PBT-1 led to down-regulation of Akt and up-regulation of IκB. These results suggest two possible mechanisms: (1) that there is a link between Akt inactivation and IκB accumulation and (2) that NF-κB activity may be suppressed by PBT-1 treatment because of IκB accumulation. IKK regulates IκB degradation by phosphorylation, and IKK activity is regulated by Akt.[25,26] Thus, suppression of Akt by PBT-1 treatment could explain why IκB can escape protein degradation and accumulate in the cytoplasm.

The abundant dephosphorylated IκB can bind NF-κB to block its translocation from the cytoplasm to nucleus so that downstream genes of NF-κB, such as COX-2, cannot be expressed. Contrary to expectation, we found that PBT-1 can up-regulate but cannot suppress the expression of IKKα and its phosphorylation. Lawrence et al. demonstrated that IKKα can suppress NF-κB activity by accelerating both the turnover of NF-κB subunits RelA and c-Rel and their removal from proinflammatory gene promoters, resulting in the resolution of inflammation in macrophages.[27] These results are consistent with our finding that RelA degradation correlated with the increasing expression of IKKα upon treatment with PBT-1. Interestingly, our data showed that RelB, a downstream gene of IKKα, was also activated by PBT-1 treatment (Figure 5). Other studies demonstrated that RelB plays a role in endotoxin (lipopolysaccharide) induced tolerance and anti-inflammatory effects through the reciprocal recruitment of RelA and RelB to NF-κB target gene promoters, resulting in the down-regulation of NF-κB target genes.[28,29] Taken together, these results indicate that PBT-1 may activate an alternative NF-κB pathway to inhibit the canonical NF-κB pathway induced by proinflammatory cytokines by activating IKKα.[30] This novel finding provides a unique perspective on the traditional anti-inflammatory and antiarthritis uses of *Tylophora* alkaloids, namely, that these compounds can remit the symptoms of asthma and inflammation by activating the IKKα-RelB pathway to tolerate the immune response induced by environmental stimulation, as well as autoimmune diseases. However, this hypothesis needs to be further studied.

Akt (protein kinase B) has been identified as a downstream target of growth factor receptor activation, such as by insulin-like growth factor, epidermal growth factor, basic fibroblast growth factor, insulin, interleukin-6, and macrophage colony stimulating factor.[31−33] Akt controls vital cellular functions such as cell survival, cell cycle progression, and glucose metabolism. The Akt gene is overexpressed and is constitutively active in many human cancers.[34−39] Bad, a proapoptotic member of the Bcl-2 family, is a substrate of Akt, which can phosphorylate Bad significantly at Ser^{136} and then inhibit its proapoptotic functions.[40,41] Inactivation of Akt is considered an attractive approach for chemotherapy. Recent studies have shown that inhibition of Akt alone or in combination with other cancer chemotherapeutics can reduce the threshold of apoptosis and kill cancer cells.[42,43] We found that PBT-1 can suppress inducible activation of Akt in lung cancer cells. Intriguingly, the inactivation of Akt by PBT-1 treatment correlated with the down-regulation of COX-2 but not with IKK activity. This suggests that PBT-1, a novel Akt inhibitor, can regulate the Akt-dependent regulation of NF-κB through other mediators, such as mTOR, which regulates IKK activity.[44] The mechanism for this action remains unclear. Taken together, our data show that PBT-1 may be a highly promising anticancer drug that suppresses cancer cell growth by inhibiting Akt and NF-κB activity. However, the detailed molecular mechanism of this PBT-1-related suppression of Akt activation and NF-κB requires further investigation.

In conclusion, we have shown that PBT-1 can induce cell cycle G2/M arrest and apoptosis by interrupting the regulatory proteins in cell cycle progression and by inhibiting the NF-κB signaling pathway by inactivating Akt. This new class of tylophorine compounds has a unique mode of action that differs from that of other known antitumor compounds. PBT-1 or a future analogue will be a good candidate for a new agent for anticancer therapy.

Experimental Section

Cell Lines. The human lung adenocarcinoma cell lines CL1-0 and CL1-5 were established in our laboratory[45,46] and with A549 cells (American Type Culture Collection CCL-185) were grown in normal RPMI 1640 culture medium (GIBCO-Life Technologies, Inc., Gaithersburg, MD) or Dulbecco's modified Eagle's medium (GIBCO-Life Technologies), supplemented with 1.5 g/L of $NaHCO_3$, 4.5 g/L glucose, and 10% fetal bovine serum (FBS, GIBCO-Life Technologies). Tumor necrosis factor-α (TNF-α) was purchased from Peprotech (Rocky Hill, NJ). H460, PC-9, and BEAS2B cells were gifts from Dr. Ker-Chau Li, Dr. Chih-Hsin Yang, and Dr. Reen Wu, respectively.

Compounds. Ten tested phenanthrene-based derivatives (PBT-1-PBT-10) were synthesized by Dr. KH Lee's laboratory.[3,4]

Cell Proliferation Assay. Cell proliferation was measured by using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). Cells were transferred in four replicates to a 96-well plate at a concentration of 2×10^3 cells/well in 100 μL of complete RPMI 1640 with or without the drugs. The cells were incubated at 37 °C in 5% CO_2 for 48 h, the viability of the cells was analyzed after the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) reagent to the cultured cells, and the cells were lysed according to the manufacturer's instructions. Absorbance was determined using Emax microplate reader (Molecular Devices, Union City, CA) at a wavelength of 490 nm.

Colony Formation Assay. Cells (5×10^2/well) were plated in six-well plates. After 24 h, cells were exposed to serial dilutions of the drugs for the times indicated. Then 500 or 1000 viable cells were plated in triplicate in six-well plates, grown for 10−14 days, fixed, and stained with 0.5% methylene blue in 50% ethanol for 1 h. The plates were washed and dried, and the colonies were counted to obtain a cloning efficiency for each drug concentration.

Cell Cycle Analysis. A total of 2×10^5 CL1-0 cells/dish were seeded onto each 60 mm dish and incubated for 24 h. Various concentrations of PBT-1 were added to the culture media, and the cells were incubated for an additional 2, 6, 12, 16, and 24 h. Cells were harvested and fixed in cold 70% ethanol at 4 °C for 16 h and incubated with 20 μg/mL RNase A at 37 °C for 30 min and then with 50 μg/mL propidium iodide (PI) at 4 °C for 30 min. Samples were analyzed immediately by flow cytometry (BD Biosciences, San Jose, CA). The cell cycle phase distribution was determined using CellQuest software (BD Biosciences).

Western Blot Analysis. Equal amounts (50 μg) of cell lysate were separated by 10% SDS−PAGE and transferred to a polyvinylidene membrane (Millipore, Billerica, MA). The membrane was probed with antibodies directed against cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin B1, cyclin D1, cyclin E,

p21, p27, RelA, p-Akt, Akt, IκB kinase α (IKKα), p-IKKα/β, and α-actin (all from Sigma, St Louis, MO). Antibodies were diluted in TBS (pH 7.5) containing 0.05% (v/v) Tween-20 and 5% (w/v) dried milk. Blots were incubated with the appropriate horseradish peroxidase conjugated secondary antibodies (Amersham Biosciences, Uppsala, Sweden). Bound antibodies were visualized by electrochemical luminescence staining with autoradiographic detection using Kodak X-Omat Blue film (PerkinElmer Life Science, Boston, MA).

Apoptosis Assay. Control or treated cells were resuspended in annexin-binding buffer, stained with Alexa Fluor 488 annexin V or PI,[42] and incubated at room temperature for 15 min. The cells stained only with annexin V were used as the positive control to set the apoptotic window, and the cells stained only with PI were used as the positive control to set the necrotic window. Double-stained, formaldehyde-treated cells were mainly necrotic. The acquired data were analyzed using CellQuest software (BD Biosciences).

DNA Microarray Analysis. The detailed protocol for the human DNA microarray analysis has been reported in our previous studies.[47,48] Total RNA was extracted from the CL1-0 cells incubated with or without the drugs using RNAzol B solution (Life Tech, Gaithersburg, MD), and the mRNA was extracted using an mRNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Five micrograms of mRNA from each sample was used in each array. The microarray images were scanned, digitized, and analyzed using a flatbed scanner (PowerLook 3000, UMAX, Taipei, Taiwan) and GenePix 3.0 software (Axon Instruments, Union City, CA). When designing the microarray experiments, we adhered to the guidelines of the Microarray Gene Expression Data Society (www.mged.org/Workgroups/MIAME/miame_checklist.html).

Identification of Pathways Using the KEGG and BioCarta Databases. Gene identification was performed to determine which biochemical pathways were altered during treatment with PBT-1. Having identified genes on the basis of the cDNA microarray data, we were also interested in determining whether any of these genes were part of the same pathway. Accordingly, we searched the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/pathway.html) and the BioCarta (http://www.biocarta.com/genes/allpathways.asp) biochemical pathway database using the genes selected from cDNA microarray analysis described previously.[49]

Real-Time Quantitative RT-Polymerase Chain Reaction. To validate the microarray data, real-time quantitative RT-PCR was performed according to our previous described method.[48] Primers were performed as listed in Table 1. All reactions were carried in 20 μL volumes containing 10 μL of iTaqTM Fast SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA). GAPDH was performed as internal control.

Transient Transfection and Luciferase Activity. Adherent cells (1×10^5) cells in six-well plates were transiently transfected with 0.2 μg of a pNf-κB-Luc vector (Stratagene, La Jolla, CA) and 1 μg of pSV-β-galactosidase vector dissolved in 10 μL of lipofectamine (Invitrogen, Carlsbad, CA) as the internal control. The plasmids were transfected according to the manufacturer's instructions. After 20 h, the medium was changed to serum-free medium, the cells were pretreated with increasing concentrations of PBT-1 as indicated for 1 h, and the cells were stimulated with various concentrations of TNF-α as indicated for 4 h. Cell extracts were harvested using 250 μL of lysis buffer (Tropix, Inc., Bedford, MA) per well. To measure the luciferase and β-galactosidase activities, cell extracts (20 μL each) were assayed separately using the Luciferase Assay Kit and Galacto-Light Plus system (Tropix, Inc.), respectively. Luciferase activity was measured and analyzed using an FB12 luminometer (Zylux Corporation, Oak Ridge, TN).

Chromatin Immunoprecipitation (ChIP) Assay. The chromatin immunoprecipitation assay has been described previously.[50] Briefly, A549 cells were cross-linked with 1% formaldehyde and the reaction was stopped with 0.125 M glycine. The cells were solvated in 250 μL of cell lysis buffer. The pelleted nuclei were suspended in 150 μL of nuclei lysis buffer and diluted with immunoprecipitation (IP) dilution buffer. The samples were sonicated at 12 W for 45 s. The DNA−protein supernatant was reacted with anti-RelA (Santa Cruz), anti-p50, and anti-RelB antibodies at 4 °C overnight. The immunoprecipitated complexes were collected with protein A/G Sepharose beads (Sigma) and then washed sequentially with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and Tris-EDTA buffer. The precipitates were eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$). To reverse the cross-linking, 5 M NaCl and RNase were added, and the samples were incubated at 65 °C overnight. The extracted DNA was analyzed by polymerase chain reaction (PCR) using primers spanning the proximal (nt +87 to +106) or distal (nt −340 to −359) regions of human COX-2.[51] After 35 cycles of amplification, the PCR products were run on a 2% agarose gel and visualized with ethidium bromide staining.

Statistical Analysis. Our previous studies provide detailed descriptions and discussion of the issues involved in generating the microarray data, data normalization, and statistical analysis and interpretation.[47,52] Genes that were up-regulated or down-regulated in response to PBT-1 treatment were identified and used for pathway analysis. An up-regulated gene had to show a 2-fold increase in the cDNA microarray. These genes were analyzed further by our in-house data-mining tool based on KEGG and BIOCARTA pathway databases (http://biochip.nchu.edu.tw/SpecificDB/mouse.html.)

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