Overexpression of the orphan receptor Nur77 and its translocation induced by PCH4 may inhibit malignant glioma cell growth and induce cell apoptosis

Li-Fu Chang[1#], Po-Cheng Lin[1#], Li-Ing Ho[2], Po-Yen Liu[3], Wan-Chen Wu[4], I-Ping Chiang[5], Hui-Wen Chang[5], Shinn-Zong Lin[6], Yeu-Chern Harn[7], Horng-Jyh Harn[5]*, and Tzyy-Wen Chiou[1]*

[1]Department of Life Science and Graduate Institute of Biotechnology, National Dong Hwa University, Hualien, Taiwan, R.O.C.

[2]Department of Respiratory Care, Veterans General Hospital-Taipei, Taipei, Taiwan, R.O.C.

[3]Graduate Institute of Chinese Medical Science, China Medical University, Taichung, Taiwan, R.O.C.

[4]Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan, R.O.C.

[5]Department of Pathology, China Medical University Hospital, Taichung, Taiwan, R.O.C.

[6]Center for Neuropsychiatry, China Medical University and Hospital and Beigang Hospital, Taichung and Yun-Lin, Taiwan, R.O.C.

[7]Graduate Institute of Networking and Multimedia, National Taiwan University, Taipei, Taiwan, R.O.C.

The authors contribute equally in this study.

*Corresponding authors:

Dr. Tzyy-Wen Chiou, Ph.D.

Department of Life Science and Graduate Institute of Biotechnology,

National Dong Hwa University

No. 1, Sec. 2, Da Hsueh Rd., Shoufeng, Hualien, Taiwan, Republic of China.

Tel: 886-3-8633638

Fax: 886-3-8630262

E-mail address: twchiou@mail.ndhu.edu.tw

Dr. Horng-Jyh Harn

Pathology Department

China Medical University & Hospital

2 Yuh-Der Road, Taichung, Taiwan, 40447, ROC

Tel: 886-4-22052121 ext. 2661; Fax: 886-4-22052121 ext. 2566

E-mail: dukeharn@www.cmuh.org.tw

Running title: A new compound for malignant glioma therapy

Abstract

Background

In previous study, n-butylidenephthalide (BP), a natural compound from *Angelica sinensis*, has anti-glioblastoma multiform (GBM) cell effects. In this study, we modified BP structure to increase anti-GBM cell effects. The anti-GBM cell effects of one derivative of BP, (Z)-*N*-(2-(dimethylamino)ethyl)-2-(3-((3-oxoisobenzofuran-1(3H)-ylidene)methyl)phenoxy)acetamide (PCH4) were tested in vitro and in vivo.

Methods

MTT assay and PI/Annexin V assay were performed to evaluate the anti-GBM effects of PCH4. The Nur77 expression and translocation were assayed by RT-PCR and western blot. The Nur77 siRNA was used to down-regulate the Nur77 expression. The JNK inhibitor (SP600125) was used to block the JNK pathway

Results

The anti-GBM effect of PCH4 is four times more than BP. The IC_{50} of PCH4 on DBTRG-05MG cells was 50 μg/ml. Nur77 expression and translocation from the nucleus to the cytoplasm were important in PCH4-induced apoptosis. Furthermore, the down-regulation of PCH4-induced Nur77 expression by Nur77 siRNA reduced PCH4-induced apoptosis. In addition, PCH4-induced apoptosis was associated with the JNK pathway. The JNK inhibitor, SP600125, inhibited Nur77 mRNA expression and reduced PCH4-induced apoptosis.

Conclusions

In conclusion, PCH4, a derivative of BP, induced Nur77-mediated apoptosis via the JNK pathway and this mechanism, which is different from that of BP, may explain the increase in the antitumor effects on GBM.

Key words: glioblastoma multiform, the derivative of n-butylidenephthalide, (Z)-*N*-(2-(dimethylamino)ethyl)-2-(3-((3-oxoisobenzofuran-1(3H)-ylidene)methyl)phenoxy)acetamide, PCH4, apoptosis, JNK pathway

Introduction

Glioblastoma multiform (GBM) is the most aggressive type of CNS gliomas, accounting for 53.8% of all CNS gliomas cases (Porter et al., 2010). In our previous study, n-butylidenephthalide (BP), which is isolated from the chloroform extract of *Angelica sinensis*, has antitumor effects in vitro and in vivo (Tsai et al., 2006). In vitro, GBM cells treated with BP undergo cell cycle arrest at the G_0/G_1 phase and Nur77-mediated apoptosis, which occurs via the protein kinase C (PKC) pathway (Tsai et al., 2006; Lin et al., 2008a). In hepatocellular carcinoma cells, BP also induces apoptosis by inhibiting protein kinase B (AKT) and the activation of the cAMP response element binding protein (CREB) pathway (Chen et al., 2008a). In addition, Nur77-mediated apoptosis is associated with c-Jun N-terminal kinases (Han et al., 2006).

The transcription factor Nur77 is a member of the nuclear hormone receptor family and plays a role in apoptosis (Liang et al., 2007; Lee et al., 2009; Yang et al., 2009). In T cells, the expression of Nur77 is induced by T cell receptor activation and increases T cell apoptosis (Li et al., 2006). In addition, in prostate cancer cells, GBM cells, hepatocellular carcinomas, and lung cncer cells, Nur77 expression also induces apoptosis (Li et al., 1998; Chintharlapalli et al., 2005; Chen et al., 2008a; Lin et al., 2008b). The apoptotic effects of Nur77 were discovered during the study of CD437, a retinoid-related molecule that induces apoptosis (Li et al., 1998).

Apoptosis induced by Nur77 is due to its translocation from the nucleus to the mitochondria through the activation of the JNK, PKC, and CREB pathways or the inhibition of the AKT pathway. Nur77 then interacts with Bcl-2 to form a pro-apoptosis complex. Finally, cytochrome c is released from the mitochondria and apoptosis occurs (Moll et al., 2006; Chen et al., 2008b; Lin et al., 2008a). In addition, activation of the ERK pathway also plays an important role in cadmium-induced Nur77 expression and apoptosis in lung cancer A549 cells (Shin et al., 2004).

To synthesize the most effective and least toxic derivative of BP, we examined the relationship between the structure and the corresponding activity. Among 32 synthetic derivatives, the antitumor activity of PCH4 is four times than BP and PCH4 is more soluble. Thus, we examined the role of Nur77 in the antitumor effects of PCH4.

The aim of this study was to elucidate the relationship between the structure and the corresponding activity of one synthetic derivative of BP. The synthetic derivative of BP, PCH4, had the most powerful antitumor effect of the 32 compounds, and thus, we assessed the role and mechanism of Nur77-induced apoptosis after GBM cells were treated with PCH4.

Materials and Methods

Cell line and cell culture

The human GBM cell lines DBTRG-05MG and GBM 8401 were obtained from the

Bioresources Collection and Research Center in the Food Industry Research and Development Institute (BCRC, Hsin Chu, Taiwan). The cells were maintained in RPMI-1640 medium (Gibco, California, USA) containing 10% FBS, 0.01 M HEPES, and 1 mM sodium pyruvate in a 37ºC incubator containing 5% CO_2.

Chemicals and Reagents

Derivatives of BP were dissolved in DMSO at a concentration of 50 mg/ml and stored at -20ºC. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in phosphate buffer at a concentration of 5 mg/ml and stored at 4ºC. Carmustine, MTT, DMSO, and cadmium acetate were purchased from Sigma (St. Louis, USA).

MTT assay

In this study, cell viability was determined by an MTT assay as previously described (Lin et al., 2008b). Briefly DBTRG-05MG or GBM 8401 cells were seeded at 3×10^3 cells/well for 24 hrs. Cells were then treated with different concentrations of PCH4 (12.5, 25, 50, 75, 100, 125, 150 μg/ml) for 24 hr. Finally, the purple crystals were dissolved in DMSO, and the absorbance was detected with an ELISA Reader at an absorption wavelength of 570 nm.

Apoptosis assay

After treatment with the various chemicals, the cells were harvested and resuspended in binding buffer (Becton, Dickinson and Company, NJ, USA). The PCH4 treated DBTRG-05MG cells were harvested and labeled with 10 mg/ml Annexin V-FITC and 20 mg/ml propidium iodide (PI) (Becton, Dickinson and Company, NJ, USA). After labeling, cells were analyzed with flow cytometry (FACScan; BD Biosciences).

RNA extraction

For analysis of mRNA expression, RT-PCR was used. DBTRG-05MG and GBM 8401 cells were treated with 50 μg/ml of PCH4 for 0.5, 1, 3, or 6 hr. Total RNA was extracted and purified using an RNeasy mini kit (Qiagen, California, USA). RNA quality was confirmed with electrophoresis.

RT-PCR

cDNAs were produced from 1 μg of total RNA using a Reverse-iT first strand synthesis kit (ABgene, Epsom, UK). The cDNA was amplified with Nur77, Nurr1, NOR1, and GAPDH primers using polymerase chain reaction. The primers are as follows: Nur77; (Forward) 5′-CGACCCCCTGACCCCTGAGTT-3′ and (Reverse) 5′-GCCCTCAAGGTGTTGGAGAAGT-3′.

Nurr1; (Forward) 5′-CGACATTTCTGCCTTCTCC-3′ and (Reverse)

5′-GGTAAAGTGTCCAGGAAAAG-3′ NOR1; (Forward) 5′-TCTGCCTTCCAAACCAAAG-3′ and (Reverse) 5′-TGATGGAAAGTCTGAGGAC-3′. glyceraldehyde-3-phosphate dehydrogenase; (Forward) 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and (Reverse) 5′-CATGTGGGCCATGAGGTCCACCAC-3′. The PCR conditions were: initial denaturation at 95ºC for 10 min, 35 cycles of denaturation at 95ºC for 30 sec, annealing at 60ºC for 30 sec, extension at 72ºC for 1 min, and a final extension step at 72ºC for 10 min. The PCR products were then analyzed on a 2% agarose gel.

Cell fractionation

Nuclear, cytoplasmic, and mitochondrial fractions were separated using a mitochondrial fractionation kit (Active Motif, California, USA). GBM 8401 cells were treated with 50 μg/ml PCH4 for 6, 12, 24, or 48 hr. The cells were detached with 0.05% trypsin, washed once with PBS, and resuspended in cold 1× cytosolic buffer for 15 min on ice. Cells were homogenized with a homogenizer on ice for 30-50 strokes and passed through a 22-gauge needle. The nuclei were recovered by centrifugation at 800 × *g* for 20 min at 4ºC. After the centrifugation, the supernatant were mitochondria and cytoplasm fractions. The nuclear pellet was lysed in lysis buffer (Intron biotechnology, joongang induspia, korea) on ice for 30 min. After the mitochondria and cytoplasm supernatant was centrifuged at 10000 × *g* for 20 min at 4ºC, the

mitochondrial and cytoplasmic fractions were separated. The mitochondria fraction was the pellet and the supernatant was the cytoplasmic fraction. Finally, the mitochondrial fraction was lysed in mitochondria buffer (Active Motif, California, USA).

Western blot analysis

Total cell protein was extracted using PRO-PREPTM protein extraction solution (INtRON,). Briefly, the cell pellets were lysed with protein extraction solution and incubated at -20ºC for 20 min. Then, the cell lysates were centrifuged at 15000 × g for 5 min, and total protein was collected. The protein concentration was measured using a protein assay kit (Strong Biotech, Taipei, Taiwan). 20 μg of total protein were separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. Non-specific binding was blocked with 5% skim milk. Proteins of interest were detected with primary antibodies to Nur77, phospho-ERK, ERK, phospho-p38, p38, phospho-SAPK/JNK, SAPK/JNK, phospho-AKT, AKT, phospho-PKC, cytochrome c, and β-actin (Cell signaling, Danvers, USA). The primary antibodies were detected with horseradish peroxidase–conjugated anti-mouse, anti-rabbit, or anti-goat as appropriate for 1 hr at 25ºC. Bound HRP–conjugated secondary antibody was visualized with an enhanced chemiluminescence (ECL) Plus system.

RNA interference

siRNA specific for Nur77 (5′-CAGUCCAGCCAUGCUCCUCUU-3′) was purchased from Santa Cruze. GBM 8401 cells were transfected with siRNA (25, 50, and 100 nM) using lipofectamine. After 48 hr, the expression of Nur77 was measured using RT-PCR.

Transfection of the luciferase reporter system

GBM 8401 cells were co-transfected with 4 μg of plasmid consisting of the Nur77 promoter linked to luciferase and 0.4 μg of plasmid pRL-TK using lipofectamine 2000. After 48 hr, the transfected cells were treated with 60 μg/ml PCH4 or 1 μM CD437 for 6 hr. Promoter activity was assayed using a dual luciferase assay kit (promega, WI, USA). Luciferase activity was normalized to renilla luciferase expressed by the plasmid pRL-TK.

Results

PCH4 inhibits the growth of GBM cells in vitro and induces apoptosis

The MTT assay revealed that PCH4 inhibited the growth of DBTRG-05MG and GBM 8401 cells in a dose-dependent manner in vitro. The IC_{50} of PCH4 on DBTRG-05MG and GBM 8401 cells was 50 μg/ml and 65 μg/ml, respectively, after 24 hr of PCH4 treatment (Fig. 1). Cells treated with PCH4 exhibited shrinkage and fragmentation of chromosomes. The apoptotic effects of PCH4 were evaluated with PI/Annexin V staining and flow cytometry. (Fig. 2). When DBTRG-05MG cells were treated with different concentration of PCH4 for 24 hrs, the percentage of apoptotic cells were increased with the increased concentration of PCH4. At 75 μg/ml or 100 μg/ml of PCH4, the percentages of apoptotic cells were 33.7% or

83.9% respectively.

PCH4 induces mRNA expression of Nur77, Nurr1, and NOR1

We examined mRNA expression of Nur77 in PCH4-treated DBTRG-05MG cells by using RT-PCR. Nur7 was induced by treatment with PCH4. Nur77 was clearly upregulated 3 hr and 30 min, respectively, after PCH4 treatment. NOR1 was upregulated slightly 3 hr after PCH4 treatment (Fig. 3). Because Nur77 is implicated in apoptosis and growth inhibition, upregulation of Nur77 mRNA may be associated with PCH4-induced apoptosis.

PCH4 induces apoptosis and Nur77 expression in DBTRG-05MG cells via the JNK pathway

To determine whether MAPK, JNK, or CREB play a role in PCH4-induced apoptosis, DBTRG-05MG cells were treated with 75 μg/ml of PCH4 for 0, 15, 30, 60, or 180 min and then analyzed using western blotting. We found that pJNK and pERK were upregulated following PCH4 treatment but the expression of pPKC, pAKT, and AKT were not changed (Fig. 4).

Next, we used a JNK inhibitor (SP600125) or an ERK inhibitor (U0126) to examine whether the PCH4-induced Nur77 expression occurred via the JNK or ERK pathways. To confirm that SP600125 inhibited the JNK pathway, we performed western blotting for PCH4-induced pJNK and pcJun, the downstream mediator of pJNK, in the presence of

SP600125 or U0126. When we used SP600125 in PCH4-treated DBTRG cells, pJNK and pcJun were downregulated in a dose-dependent manner (Fig. 4b). In addition, PCH4-induced Nur77 expression was decreased when cells were pre-treated with SP600125 (Fig. 4c). Further, PCH4-induced apoptosis was decreased when PCH4-treated DBTRG cells were pre-treated with 10-50 μM SP600125 (Fig. 4d). However, PCH4-induced apoptosis was not blocked when we used U0126 (data not shown).

PCH4-induced Nur77 migrates from the nucleus to the cytoplasm

We examined whether Nur77 migrated from the nucleus to the cytoplasm. Localization of Nur77 was observed by immunofluorescence microscopy. In control cells, Nur77 was mainly located in the nucleus. In PCH4-treated cells, Nur77 translocated from the nucleus to the cytoplasm (Fig. 5a). To confirm the translocation of Nur77, cytosolic and nuclear fractions were examined with western blot analysis. Nur77 was principally located in the nuclear fraction before PCH4 treatment (Fig. 5b). However, Nur77 was progressively increased in the cytoplasmic fraction during PCH4 treatment (Fig. 5b).

PCH4-induced apoptosis in DBTRG cells is triggered by Nur77 expression

To determine whether Nur77 is crucial in PCH4-induced apoptosis, Nur77 siRNA was used to suppress Nur77 expression. Nur77 expression was downregulated by Nur77 siRNA in a

dose-dependent manner (Fig. 6a). Next, to determine the role of Nur77 in PCH4-induced apoptosis, we used PI and Annexin V staining to identify apoptotic cells after treatment with Nur77 siRNA and PCH4. Fewer apoptotic cells were observed after PCH4 treatment of DBTRG cells transfected with Nur77 siRNA, and these effects were time dependent. When 100 nM Nur77 siRNA was used, the percent of PCH4-induced apoptotic cells was significantly decreased from 36% to 16% ($p<0.05$) (Fig. 6b). Thus, PCH4 induced apoptosis via Nur77 in GBM 8401 cells.

PCH-4-induced Nur77 expression occurs via the AP-1 binding site in the Nur77 promoter

Sequence analysis of the Nur77 promoter suggested that this gene may contain cis-acting elements and an AP-1 binding site, which may play an important role in PCH4-induced apoptosis. To determine the role of the AP-1 binding site in PCH-4-induced Nur77 expression, plasmids containing mutations in nucleotides -496/+67 in the Nur77 promoter and a wild-type control were constructed (Fig. 7a). The promoters were placed upstream of luciferase cDNA. These constructs were transfected into DBTRG cells, which were treated with vehicle or PCH4. The plasmid pRL-TK was used as an internal control for adjusting transfection efficiency. Transfection of the wild-type plasmid (pNur77 496/+67) and PCH4 treatment resulted in a 2.5-fold induction of luciferase activity (Fig. 7b). To examine the role of the AP-1 binding site in PCH4-induced Nur77 expression, we transfected a plasmid with a

mutated AP-1 site. Significantly lower luciferase activity was observed following PCH4 treatment (~91%) (Fig. 6b). This result suggested that AP-1 may play an important role in PCH4-induced Nur77 expression.

PCH4 inhibits the growth of xenografted DBTRG cells in nude mice associated with Nurr77 expression

To evaluate the anti-malignant glioma activity of PCH4 in vivo, 2×10^6 DBTRG cells were injected subcutaneously into the dorsal subcutaneous tissue of nude mice. After the tumor size reached about 80~120 mm^3, mice were randomly divided into three groups: control, PCH4 50 mg/kg, and PCH4 100 mg/kg (n = 5 per group). Control mice were subcutaneously injected with vehicle (DMSO) for five successive days. Mice in the PCH4 50 mg/kg group and PCH4 100 mg/kg group were subcutaneously injected with the indicated dose of PCH4 for five successive days. Tumor growth was inhibited in the PCH4 50 mg/kg group and the PCH4 100 mg/kg group (Fig. 8a). Human DBTRG tumors treated with 50 mg/kg or 100 mg/kg PCH4 showed an upregulation of Nur77 and caspase 3 expression 5 days after treatment (Fig. 8b). In addition, western blot analysis showed that Nur77 and cleaved caspase 3 protein were increased in tumors following PCH4 treatment (Fig. 8c).

Discussion

Alkylating agents such as carmustine (BCNU) and temozolomide are clinically available for malignant glioma therapy. Their mechanism of action against malignant glioma is DNA methylation on guanine base. Because the mechanism is non-specific, these drugs are cytotoxic to normal cells. In addition, the usage of these alkylating drugs is sometimes limited due to the drug resistance resulting from the expression of O^6-methylguanine methyltransferase (MGMT) in gliomas (Sharma et al., 2009). Although it is previously reported that the MGMT expression of 30-60% gliomas is lowered by epigenetic silence and is consequently sensitive to the alkylating drugs (Weller et al., 2010), there is still a considerable portion of gliomas which can not be effectively treated by these alkylating drugs. The development of a new targeted drug is therefore urgent.

Traditional Chinese herbs, which contain many unique and biomedically powerful compounds, are a rich source of therapeutic candidates. Recent examples include paclitaxel and camptothecin. n-butylidenephthalide, which is extracted from *A. sinensis*, is a natural compound that has been investigated for its antitumor effect on GBM cells both in vitro and in vivo. However, synthesis of a rationally designed n-butylidenephthalide derivative is necessary to increase the cytotoxic effects on GBM cells.

Nur77 is a unique orphan member of the nuclear receptor family and is the most potent proapoptotic member of the nuclear receptor superfamily. In a previous study, we showed that

n-butylidenephthalide induces cell apoptosis in GBM cells via targeting of Nur77 to mitochondria (Lin et al., 2008b). This phenomenon was also investigated in other types of cancer cells including lung, prostate, ovary, colon, and stomach tumor cells (Liu et al., 1994; Uemura et al., 1995; Stocco et al., 2002; Shin et al., 2004). We showed that apoptosis of GBM cells induced by n-butylidenephthalide requires Nur77 expression and that Nur77 translocates from the nucleus to the cytoplasm where it interacts with Bcl-2. This interaction results in a conformational change in Bcl-2 that converts it into a cytotoxic molecule. These findings suggest that n-butylidenephthalide derivatives that induce cytoplasmic localization of Nur77 and its interaction with Bcl-2 may preferentially kill cancer cells.

In this study, we synthesized several new n-butylidenephthalide derivatives (Table 1) to increase the solubility of the drug, increase the cytotoxicity on GBM cells, and identify compounds that modulate the Nur77-mediated apoptotic pathway. Among these derivatives, we found that the n-butylidenephthalide derivative, PCH4, was more soluble than n-butylidenephthalide. PCH4 inhibited proliferation and induced apoptosis of GBM cells. In addition, Nur77 was involved in the PCH4-induced apoptosis. Therefore, PCH4 may be a new potential targeting drug in malignant glioma therapy.

In other studies, Nur77 was shown to be an oncogenic survival factor expressed in the nucleus of cancer cells. However, following apoptotic stimulation, Nur77 translocates from the nucleus to the cytoplasm where it binds to Bcl-2, triggering cytochrome c release and

apoptosis (Li et al., 2000).

In our study, we found that PCH4-induced Nur77 expression was upregulated in a time-dependent manner using an RT-PCR assay. GBM cells underwent apoptosis after PCH4 treatment. We next examined PCH4-induced expression of Nur77 and the translocation of Nur77 in apoptotic GBM cells (Figs. 2 and 4). PCH4-induced Nur77 expression was increased 1 hr after PCH4 treatment and translocation of the Nur77 protein was observed at 6 hr.

We compared PCH4 and BP induced-Nur77 expression, which was previously studied. BP-induced Nur77 expression was highest at 3 hr and was decreased at 6 hr. PCH4-induced Nur77 expression was also highest at 3 hr, but it was still high at 6 hr, unlike BP-induced Nur77 expression. Because Nur77 was associated with tumor cell apoptosis, we hypothesize that PCH4 was more effective than BP because of the prolonged Nur77 expression at 6 hr. In the animal study, we used PCH4 to treat nude mice with a GBM xenograft and the anti-GBM activity of PCH4 is dose dependent. In addition, we also observed that the Nur77 and cleaved caspase3 expression were accompanied with increasing dosage of PCH4. Interestingly, the tumor size in PCH4-treated animals did not increase substantially during the first 12 days. However, the tumor size began to increase after first 12 days. This phenomenon may be due to the metabolism time of PCH4. Thus prolonging PCH4 treatment may be required in further investigation.

Because Nur77 was associated with tumor cell apoptosis, we decreased Nur77 expression using Nur77 siRNA. When we used 100 nM Nur77 siRNA to downregulate Nur77 expression, PCH4-induced apoptosis was reduced by 78.1%. This result demonstrates that Nur77 is a major pathway in PCH4-induced GBM cell apoptosis.

The induction of Nur77 expression has been studied in several cell types. Nur77 expression was rapidly induced by nerve growth factor in PC12 cells via calcium ions (Milbrandt, 1988), by cadmium in human lung cancer cell lines via extracellular signal-regulated kinase and protein kinase A (Shin et al., 2004), and by PGF2a and butaprost in human embryonic kidney 293/EBMA cells via protein kinase C (Liang et al., 2004). In addition, a recent report indicated that activation of JNK or inhibition of the AKT pathway induces the translocation of Nur77 from the nucleus to the cytoplasm in other cancer cells (Han et al. 2006). Thus, regulation of Nur77 expression may involve a variety of intracellular signaling pathways that depend on different stimuli.

In this study, we found that PCH4-induced apoptosis was also associated with the JNK pathway. When GBM cells were treated with PCH4, we found that components of the JNK pathway were upregulated at 15 min. After the JNK pathway was upregulated, Nur77 expression was increased at 1 hr and then GBM cells underwent apoptosis. When we used a JNK inhibitor (SP600125), PCH4-induced apoptosis was reduced by about 60%, and Nur77 expression was also downregulated. These results suggested that the JNK pathway is a major

signaling pathway in PCH4-induced apoptosis. In our previous study (Lin et al., 2008b), BP induced Nur77 expression through the PKC pathway, but in this study, we found that PCH4 induced Nur77 expression through the JNK pathway. PCH4 may bind to a different receptor than BP and lead to Nur77 expression that lasts longer than the expression that occurs through the PKC pathway.

There are potential *cis*-acting elements in the Nur77 promoter region. A region from -496 to -334 was identified that contains enhancers that are responsive to prostaglandin $F_{2\alpha}$ and butaprost via the PKC pathway (Liang et al., 2004). The major PKC signal response element in the Nur77 promoter is an AP-1-like element (Kim et al., 2005), whereas a portion of the Nur77 promoter (-496 to -67) containing four AP-1 motifs has also been reported (Uemura et al., 1995). In this study, we examined whether the AP-1 motif is involved in PCH4-induced apoptosis, and studied the transcriptional mechanisms of PCH4-induced Nur77 mRNA expression. We used a Nur77 promoter (-496 to +67) containing these four AP-1 motifs, which was subcloned into a luciferase reporter plasmid. Following transfection with a plasmid containing the wild-type Nur77 promoter, PCH4 treatment resulted in a 2.5-fold increase in luciferase activity, compared with vehicle control (Fig. 7). To investigate whether these AP-1 motifs are functional during PCH4 treatment, two AP-1 motifs (-197 to -192; -179 to -173) were mutated in the Nur77 promoter using the protocol of Kim et al. Mutated AP-1 motifs resulted in a significant decrease in PCH4-induced Nur77 promoter activity. This result

suggests that PCH4 treatment led to an increase in Nur77 expression through AP-1 motifs.

In summary, the mechanisms of the antitumor activity of PCH4 were studied. We found that PCH4 induced Nur77-mediated apoptosis in GBM cells. The JNK signaling pathway was implicated in the regulation of PCH4-induced apoptosis via AP-1 motifs in the Nur77 promoter. These results suggest that Nur77 may be a target gene for PCH4 and that this drug may be useful for targeting malignant glioma in the clinic.

Acknowledgements

This work was supported by a grant from National Science Council of the Republic of China (96-2320-B-039-044-MY3).

References

Chen YL, Jian MH, Lin CC, Kang JC, Chen SP, Lin PC, Hung PJ, Chen JR, Chang WL, Lin SZ, Harn HJ (2008a) The induction of orphan nuclear receptor Nur77 expression by n-butylenephthalide as pharmaceuticals on hepatocellular carcinoma cell therapy. Mol Pharmacol 74:1046-1058.

Chen YL, Jian MH, Lin CC, Kang JC, Chen SP, Lin PC, Hung PJ, Chen JR, Chang WL, Lin SZ, Harn HJ (2008b) The induction of orphan nuclear receptor Nur77 expression by n-butylenephthalide as pharmaceuticals on hepatocellular carcinoma (HCC) cells therapy. Molecular pharmacology.

Chintharlapalli S, Burghardt R, Papineni S, Ramaiah S, Yoon K, Safe S (2005) Activation of Nur77 by selected 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes induces apoptosis through nuclear pathways. J Biol Chem 280:24903-24914.

Han YH, Cao X, Lin B, Lin F, Kolluri SK, Stebbins J, Reed JC, Dawson MI, Zhang XK (2006) Regulation of Nur77 nuclear export by c-Jun N-terminal kinase and Akt. Oncogene 25:2974-2986.

Kim H, Lee JE, Kim BY, Cho EJ, Kim ST, Youn HD (2005) Menin represses JunD transcriptional activity in protein kinase C theta-mediated Nur77 expression. Exp Mol Med 37:466-475.

Lee SO, Chintharlapalli S, Liu S, Papineni S, Cho SD, Yoon K, Safe S (2009) p21 expression is induced by activation of nuclear nerve growth factor-induced Balpha (Nur77) in pancreatic cancer cells. Mol Cancer Res 7:1169-1178.

Li H, Kolluri SK, Gu J, Dawson MI, Cao X, Hobbs PD, Lin B, Chen G, Lu J, Lin F, Xie Z, Fontana JA, Reed JC, Zhang X (2000) Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. Science (New York, NY 289:1159-1164.

Li QX, Ke N, Sundaram R, Wong-Staal F (2006) NR4A1, 2, 3--an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. Histology and histopathology 21:533-540.

Li Y, Lin B, Agadir A, Liu R, Dawson MI, Reed JC, Fontana JA, Bost F, Hobbs PD, Zheng Y, Chen GQ, Shroot B, Mercola D, Zhang XK (1998) Molecular determinants of AHPN (CD437)-induced growth arrest and apoptosis in human lung cancer cell lines. Molecular and cellular biology 18:4719-4731.

Liang B, Song X, Liu G, Li R, Xie J, Xiao L, Du M, Zhang Q, Xu X, Gan X, Huang D (2007) Involvement of TR3/Nur77 translocation to the endoplasmic reticulum in ER stress-induced apoptosis. Exp Cell Res 313:2833-2844.

Liang Y, Li C, Guzman VM, Chang WW, Evinger AJ, Pablo JV, Woodward DF (2004) Upregulation of orphan nuclear receptor Nur77 following PGF(2alpha), Bimatoprost, and Butaprost treatments. Essential role of a protein kinase C pathway involved in EP(2) receptor activated Nur77 gene transcription. British journal of pharmacology 142:737-748.

Lin PC, Chen YL, Chiu SC, Yu YL, Chen SP, Chien MH, Chen KY, Chang WL, Lin SZ, Chiou TW, Harn HJ (2008a) Orphan nuclear receptor, Nurr-77 was a possible target gene of butylidenephthalide chemotherapy on glioblastoma multiform brain tumor. Journal of neurochemistry.

Lin PC, Chen YL, Chiu SC, Yu YL, Chen SP, Chien MH, Chen KY, Chang WL, Lin SZ, Chiou TW, Harn HJ (2008b) Orphan nuclear receptor, Nurr-77 was a possible target gene of butylidenephthalide chemotherapy on glioblastoma multiform brain tumor. J Neurochem 106:1017-1026.

Liu ZG, Smith SW, McLaughlin KA, Schwartz LM, Osborne BA (1994) Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. Nature 367:281-284.

Milbrandt J (1988) Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron 1:183-188.

Moll UM, Marchenko N, Zhang XK (2006) p53 and Nur77/TR3 - transcription factors that directly target mitochondria for cell death induction. Oncogene 25:4725-4743.

Porter KR, McCarthy BJ, Freels S, Kim Y, Davis FG (2010) Prevalence estimates for primary

brain tumors in the United States by age, gender, behavior, and histology. Neuro Oncol 12:520-527.

Sharma S, Salehi F, Scheithauer BW, Rotondo F, Syro LV, Kovacs K (2009) Role of MGMT in tumor development, progression, diagnosis, treatment and prognosis. Anticancer Res 29:3759-3768.

Shin HJ, Lee BH, Yeo MG, Oh SH, Park JD, Park KK, Chung JH, Moon CK, Lee MO (2004) Induction of orphan nuclear receptor Nur77 gene expression and its role in cadmium-induced apoptosis in lung. Carcinogenesis 25:1467-1475.

Stocco CO, Lau LF, Gibori G (2002) A calcium/calmodulin-dependent activation of ERK1/2 mediates JunD phosphorylation and induction of nur77 and 20alpha-hsd genes by prostaglandin F2alpha in ovarian cells. The Journal of biological chemistry 277:3293-3302.

Tsai NM, Chen YL, Lee CC, Lin PC, Cheng YL, Chang WL, Lin SZ, Harn HJ (2006) The natural compound n-butylidenephthalide derived from Angelica sinensis inhibits malignant brain tumor growth in vitro and in vivo. J Neurochem 99:1251-1262.

Uemura H, Mizokami A, Chang C (1995) Identification of a new enhancer in the promoter region of human TR3 orphan receptor gene. A member of steroid receptor superfamily. The Journal of biological chemistry 270:5427-5433.

Weller M, Stupp R, Reifenberger G, Brandes AA, van den Bent MJ, Wick W, Hegi ME (2010) MGMT promoter methylation in malignant gliomas: ready for personalized medicine? Nat Rev Neurol 6:39-51.

Yang H, Bushue N, Bu P, Yvonne Wan YJ (2009) Induction and intracellular localization of Nur77 dictate fenretinide-induced apoptosis of human liver cancer cells. Biochem Pharmacol.