The novel anti-neuroblastoma agent PF403, inhibits proliferation and invasion in vitro and in brain xenografts

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Abstract. Neuroblastoma is the most common cancer in infants and the fourth most common cancer in children. Our previous study showed that PF403 had a potent antitumor ability. In the present study, we evaluated the anti-neuroblastoma property of PF403 and investigated the underlying mechanisms. MTT assay, colony formation assay and flow cytometry assay were used to assess cytotoxicity of PF403 on SH-SY5Y cells. Transwell assay was chosen to estimate the anti-invasion ability of PF403 on neuroblastoma cells. The protein expression was detected by western blot analysis. The SH-SY5Y brain xenograft model was used to assess in vivo antitumor activity of PF403. PF403-mediated SH-SY5Y cell death was found to be dose- and time-dependent, and PF403 was able to limit invasion and metastasis of neuroblastoma cells. MRI and pathology analysis proved that the pro-drug of PF403, CAT3, inhibited SH-SY5Y cells in vivo. PF403 decreased expression of phosphorylated FAK, MMP-2 and MMP-9 proteins, and downregulated the activity of PI3K/AKT and Raf/ERK pathways, followed by regulation of the proteins expression of Bcl-2 family, activated caspase-3, -9 and PARP and initiation of apoptosis of neuroblastoma cells. PF403 exerted cytotoxicity against SH-SY5Y neuroblastoma cell both in vitro and in vivo, and inhibited its invasion ability, suggesting PF403 has potential as a new anticancer drug for the treatment of neuroblastoma.

Introduction

Neuroblastoma (NB) is a common childhood malignant tumor of neural crest origin, generated in the paravertebral sympathetic ganglia and the adrenal medulla. NB is also the most common extracranial solid tumor in children (>7% of tumors in patients younger than 15 years) and a major cause of neoplastic death in infancy (15% of deaths in pediatric oncology) (1). Current surgery and radiotherapy in conjunction with chemotherapy has greatly improved survival rates for the patients with low-risk and intermediate-risk NB. However, high-risk patients still have an overall survival rate of <40% despite intensive therapy (2). Thus, it is urgent to develop new drugs for the treatment of high-risk NB.

Phenanthroindolizidine and phenanthroquinolizine alkaloids are family of plant-derived alkaloids composed of more than sixty compounds (3). The biological properties of these alkaloids include cancer cell growth inhibition (in vitro and in vivo), anti-inflammatory activity, anti-ameobicidal and anti-viral activity (4-6). Our previous studies show that (+)-13a-(S)-Deoxytylophorinidine (CAT) (Fig. 1A) had potent antitumor activity both in vivo and in vitro (7, 8). Furthermore, 3-O-Desmethyl-13a-(S)-deoxytylophorinine (PF403) (Fig. 1B), one of CAT in vivo metabolites, showed stronger proliferation inhibitory activity than CAT on several tumor cell lines (9).

In the present study, we investigated the anti-neuroblastoma potential of PF403 in human NB cell line and in an in vivo NB mouse model. Since our previous studies on pharmacokinetic had proved poor bioavailability of PF403, the pro-drug of PF403, CAT3, was chosen to study anti-neuroblastoma activity in vivo (8). Both MRI and pathology analysis verified the anti-neuroblastoma ability of PF403 in situ. Then mechanisms of PF403 on anti-neuroblastoma were studied. Collectively, our results for the first time addressed multiple mechanisms associated with PF403-mediated neuroblastoma cell death and suggested the possible application of PF403 for neuroblastoma therapy.

Materials and methods

Cells and chemicals. SH-SY5Y cell line was obtained from the cell center of CAMS&PUMC. The cells were maintained...
in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen) in a humidified incubator containing 5% CO₂ at 37°C. PF403 and CAT₃ were obtained from the research group of Professor Shi-shan Yu (Institute of Materia Medica, China).

Cell viability assay. SH-SY5Y cells were seeded in a 96-well plate. After 24 h, the cells were treated with different concentrations of PF403. After 48, 72 and 96 h of incubation, cells were incubated with 1 mg/ml of MTT (Sigma, St. Louis, MO, USA) for 4 h at 37°C in a CO₂ incubator. Mitochondrial reduction of MTT to formazan was measured by absorbance at 570 nm with reference wavelength at 450 nm. IC₅₀ values were calculated and expressed as mean ± standard deviation of three independent experiments.

Colony formation assay. The cells were seeded at a density of 200 cells/ml into 6-well culture plates for 24 h, then washed with phosphate-buffered saline (PBS) and cultured by DMEM with a series concentration of PF403 added. Colonies were allowed to grow for 14 days. After removing the medium, each well was carefully washed twice with PBS. The cells were fixed in methanol for 15 min and then stained with crystal violet for 20 min. Finally, positive colony formation (>50 cells per colony) was counted.

Flow cytometry assay. Cells were treated with a different of concentrations of PF403. After 72 h of treatment, cells were fixed with 70% ethanol, stained with propidium iodide (PI), and were analyzed by flow cytometry. The data were analyzed with CellQuest Pro software (BD Biosciences, San Jose, CA, USA). The experiments were repeated at least three times.

Invasion assay. Matrigel (10 µl) (BD Biosciences), a reconstituted basement membrane, was pre-coated on the upper compartment of Millicell (Millipore, Billerica, MA, USA) containing 8-µm pores. Approximately 600 µl of DMDM with 10% FBS was added to the lower compartment; 200 µl of the completed medium containing 2x10⁵ SH-SY5Y cells were seeded to the upper compartment. PF403 was added to the upper compartment at a series of concentrations. After culturing for 24 h at 37°C in a humidified 5% CO₂, cells migrated to the lower compartment were stained and counted under the microscope.

Pharmacokinetic analyses. Animals (Vital River Laboratories, Beijing, China) were randomly assigned to ten groups (n=3/group) that were given a single dose of CAT₃, (10 mg/kg) orally. Mice were decapitated following 5, 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h. Brain structures were dissected on dry ice. Brain tissue homogenate with PF403 (5, 10, 25, 100, 250, 500, 1,000 and 2,500 ng/ml) of blank group was used as standard solution. High performance liquid chromatography (HPLC) system Agilent 1100 Series (Agilent Technologies, Santa Clara, CA, USA), equipped with a binary pump, an autosampler and degasser. Separation was performed using a Zorbax C18 column (2.1x100 mm, 3.5 µm). The column was thermostatted at 25°C. The optimized mobile phase solvents were: 0.1% formic acid/water =35/65, CAT₃ and its metabolites were separated at a flow rate of 0.2 ml/min with a linear gradient. Mass spectral data were obtained in positive electrospray mode. High purity nitrogen used as a sheath gas, was generated with a nitrogen generator. The m/z of PF403 was 350.1-281. Levels of CAT₃ and its metabolites were calculated using the calibration standard curves, constructed by linear regression analysis of peak area vs. concentration curves. CAT₃ and PF403 (2 µl) incubated with hepatic microsome, plasma, artificial intestinal or artificial gastric juice at 37°C for 15 or 20 min. LC/MS analyzed concentration of CAT₃ and PF403.

Animals and surgical technique. Five-week-old female Balb/c nude (nu/nu) mice (Vital River Laboratories) were housed in standard facilities and given free access to water and rodent food. All animal experiments in the present study were conducted according to the guidelines of the Law for The Care and Welfare of Animals in China and approved by the Animal Experiment Committee of Institute of Materia Medica. For intracranial injection, mice were anesthetized with pentobarbital sodium (i.p) in a dosage of 50 mg/kg, placed in a stereotaxic restrain, and a small surgical incision was made in the skin covering the skull 2 mm to the right of the bregma. Cell suspensions (5 µl) containing ~5x10⁵ SH-SY5Y cells were slowly (~30 sec) injected intracranially at 2.5 mm below the skull surface using a 26-gauge needle. Immediately thereafter the incision was closed with surgical adhesive and the animals were monitored until conscious and returned to their cages. Animals were randomly assigned to control, TMZ treated group (120 mg/kg/d), CAT₃ low-dose group (6 mg/kg/d) and CAT₃ high-dose group (12 mg/kg/d) (n=3). From the 15th day of the operation, mice in CAT₃ low-dose groups and CAT₃ high-dose groups were treated of CAT₃, orally; and mice in TMZ group were treated of TMZ orally.

Imaging analysis. All mice experienced high resolution MR (Bruker, Bremen, Germany) scanning weekly after operation. The MR images (Siemens, Munich, Germany) were acquired using a 1.5 tesla clinical unit. The slice thickness for coronal and transverse scan was 0.5 mm. The mice were anesthetized with intraperitoneal injection of pentobarbital sodium solution in a dosage of 50 mg/kg. After coronal and transverse T2 weighted images had been obtained, coronal plane enhanced T1 weighted images were acquired by an intravenous injection of dimeglumine gadopentetate (Magnevist, Bayer Schering Parma, Germany) at a dose of 2 ml/kg body weight via caudal vein.
Histopathological analysis. Paraffin-embedded mouse whole brain. The thickness of sections was 5 µm. Serial sections were also stained with H&E. The stained images of each section were acquired using a microscope (Nikon, Tokyo, Japan). H&E staining was used in diagnosis, and analysis of the pathology changes.

Western blot analysis. For western blotting, samples transferred to a nitrocellulose membrane by semi-wet electrophoresis were subjected to SDS-PAGE and incubated with primary antibody rabbit anti-FAK, rabbit anti-phosphorylated FAK, rabbit anti-MMP-2, rabbit anti-MMP-9, rabbit anti-Pi3K p85, rabbit anti-phosphorylated Pi3K p85, rabbit anti-AKT, rabbit anti-phosphorylated AKT, rabbit anti-mTOR, rabbit anti-phosphorylated mTOR, rabbit anti-c-Raf, rabbit anti-phosphorylated c-Raf, rabbit anti-MEK, rabbit anti-phosphorylated MEK, rabbit anti-ERK, rabbit anti-phosphorylated ERK1/2, rabbit anti-Mcl1, rabbit anti-Bcl-2, rabbit anti-BAX, mouse anti-Bad, mouse anti-Bid, mouse anti-Bim, rabbit anti-caspase-9, rabbit anti-cleaved caspase-9, rabbit anti-caspase-3, rabbit anti-cleaved caspase-3, rabbit anti-PARP and rabbit anti-cleaved PARP antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4˚C. The samples were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology) and developed using an ECL. Membranes were tested for equal loading by probing for actin.

Statistical analysis. The results were expressed as means ± SD for repeated measures, and t-test was used for comparison of differences between groups. Differences were considered statistically significant when P≤0.05.

Results

PF403 induces cytotoxicity in SH-SY5Y neuroblastoma cells in a dose- and time-dependent manner. The MTT assay, colony forming assay and flow cytometry assay were chosen to test the cytotoxicity of PF403 in NB. Administration with different concentrations of PF403 (5x10^-12-5x10^-13 M) for 48 h, 72 and 96 h, cell viability was determined using MTT assay. The viability of the untreated cells was regarded as 100%. The results revealed that PF403 could inhibit SH-SY5Y cell proliferation significantly from 48 to 96 h, and its potency was higher than taxol (Table I). The time-dependent curve of MTT assay demonstrated the concentration of PF403 at 5x10^-11 M, its proliferation inhibitory ability in SH-SY5Y cells reached stable stage after 72 h, and PF403 had stronger antitumor proliferation ability than taxol after 48 h (Fig. 2A). The results of PF403 on proliferation were confirmed by the colony formation assay, the IC50 value was 3.81±1.29x10^-13 M. As the results show, PF403 suppressed colony formation ability of SH-SY5Y NB cells. The IC50 value was 3.81±1.29x10^-13 M. The data are presented as the mean ± standard deviation. *P<0.05, **P<0.005, ***P<0.001, compared with the control group.

| Table I. Anti-proliferative activity of PF403 on SHSY-5Y cells by MTT assay. |
|-------------------------------|-------------------------------|-------------------|
| Time (h) | PF403 (M) | Taxol (M) |
| 48 | 5.25±1.41x10^-6 | 1.57±0.42x10^-4 |
| 72 | 4.21±1.23x10^-11 | 5.16±2.24x10^-8 |
| 96 | 2.84±1.13x10^-12 | 2.61±1.43x10^-8 |

SH-SY5Y cells were treated with 5x10^-5-1x10^-13 M of PF403. Taxol was used as a positive control, and DMSO treated cells were considered as controls. After 48, 72 and 96 h of treatment, IC50 values were calculated.
The results of flow cytometry displayed that the apoptotic cells were increased dramatically in PF403 treated groups compared with control and DMSO groups, the IC₅₀ was at 10⁻¹¹ M (Fig. 2C). These results preliminarily demonstrated that PF403 had a strong anti-NB activity in vitro.

PF403 inhibits invasion of SH-SY5Y cells. To investigate the effect of PF403 on invasion in SH-SY5Y cells, we treated the SH-SY5Y NB cancer cells with various concentrations of PF403 for 24 h and observed cell invasion by Transwell assay. The results indicated that the dose required for inhibition of SH-SY5Y cell invasion was much less than the dose needed to cause apoptosis (IC₅₀ value at 10⁻³ M, at 24 h) (Table II and Fig. 3). These results suggested that PF403 was able to affect invasion ability of SH-SY5Y neuroblastoma cells at a low concentration.

CAT₃ metabolizes into PF403 in vivo. There was no significant change in the concentration of CAT₃ and PF403 incubated with artificial gastric juice for 15 min. However, the concentrations of CAT₃ were reduced, and PF403 could be detected while incubated with hepatic microsome, plasma or artificial intestinal fluid for 15 or 20 min. CAT₃ (59%) metabolized into PF403 after incubated with artificial intestinal fluid for 15 min. PF403 was detected in brain tissue after CAT₃ oral administration for 5 min, and peak time was 5 min-1 h, the average concentration of peak was 3.87-34.17 pg/ml.

**Table II. PF403 affected the invasion capacity in SH-SY5Y cells.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration (M)</th>
<th>Cell count ± SD</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>8981±1710</td>
<td>-</td>
</tr>
<tr>
<td>PF403</td>
<td>5x10⁻⁹</td>
<td>1313±355</td>
<td>83.36</td>
</tr>
<tr>
<td></td>
<td>5x10⁻¹⁰</td>
<td>4192±1087</td>
<td>53.34</td>
</tr>
<tr>
<td></td>
<td>5x10⁻¹¹</td>
<td>6648±1836</td>
<td>26.06</td>
</tr>
</tbody>
</table>

SH-SY5Y cells were treated with concentrations of PF403 for 24 h. Results were reported as mean migrated cells/5 field ± SD of three independent experiments. The inhibition rate was calculated according to the formula: Inhibition rate = (Cell count (Control)-Cell count (PF403))/Cell count (Control). *P<0.001; **P<0.005.
in the experimental group, whereas tumors occupied almost the whole brain of mice in the control group (Fig. 5B). Tumor was observed in 9 successive slices for control group, and 8 slices for TMZ (120 mg/kg) group, but only 6 and 4 slices for CAT3 low group (6 mg/kg) and CAT3 high group (12 mg/kg). Histopathological examination of control mice showed that experimental groups developed smaller tumors in the brain (Fig. 5C). H&E staining proved the tumor as neuroblastoma, tumor cells packed closely, cell morphology was basically the same, mostly the small round cells were distributed like islands. Shape and boundary of the tumors were consistent with MRI results. The tumor cells had less cytoplasm and their nucleus was clear. Capillaries surrounding tumor cells were arranged in a rosette structure (Fig. 5D). Tumor cells invaded into the surrounding normal tissue, but in CAT3 treated groups this phenomenon was not observed (Fig 5E). The volume of tumor through MRI and H&E stain sections were calculated, and showed consistency of the two methods (Table III). These results proved that the pro-drug of PF403 had potent anti-neuroblastoma activity in situ.
PF403 induces the change of multiple cell signaling pathways involved in tumor cell invasion and cell apoptosis. We explored which factors were involved in the events where PF403 inhibited invasion and induced apoptosis of neuroblastoma cells. In the process of tumor cell invasion, focal adhesion kinase (FAK) and matrix metalloproteinases (MMPs) play an important role (10). The levels of MMP-2, MMP-9, phosphorylated and total FAK in PF403 (5x10^{-11} M, 5x10^{-12} M and 5x10^{-13} M) treated SH-SY5Y cells were detected by western blot analysis at different times. The results showed that the expression of p-FAK and MMP-9 was, respectively, significantly decreased after treatment with PF403 for 4 and 72 h (Figs. 6A and 7A). However, the total of FAK did not alter (Figs. 6A and 7A). The expression of FAK is related to the Ras-mitogen-activated protein kinases (MAPKs) and PI3K/AKT cell signaling pathway (11,12). To assess whether the PF403 inhibited phosphorylation of FAK was associated with these cell signaling pathways, SH-SY5Y cells were treated with various concentrations of PF403 (5x10^{-11} M, 5x10^{-12} M and 5x10^{-13} M) for 4 and 72 h. The results of protein expression of Raf/MEK/ERK and PI3K/AKT/mTOR pathway demonstrated that PF403 downregulated the proteins expression...
Table III. Antitumor activity of CAT3 on neuroblastoma in situ.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight</th>
<th>H&amp;E staining</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>End</td>
<td>Lmax (mm)</td>
</tr>
<tr>
<td>Control</td>
<td>16.2±0.9</td>
<td>17.3±2.9</td>
<td>5.4±1.3</td>
</tr>
<tr>
<td>TMZ</td>
<td>16.7±1.1</td>
<td>17.5±2.1</td>
<td>4.3±1.2</td>
</tr>
<tr>
<td>CAT3 low</td>
<td>16.5±1.1</td>
<td>17.7±2.3</td>
<td>4.0±1.5</td>
</tr>
<tr>
<td>CAT3 high</td>
<td>16.3±1.2</td>
<td>17.9±1.9</td>
<td>2.8±1.0</td>
</tr>
</tbody>
</table>

There was no significant body weight loss, or death, after oral administration of CAT3 and TMZ for 8 days successive. Lmax is the maximum length in the maximum cross-sectional tumor with H&E staining. W is the maximum wide perpendicular to the Lmax. In the MR imaging, Lmax and W were measured as the H&E staining, and T (thickness) was calculated by number of splices multiplying the thickness of the splice. H&E stained tumor volume formula was \( V_H = L_{max} \times W^2 \), and MRI tumor volume calculation was \( V_M = L_{max} \times W \times T \). **P<0.001.

**Figure 7.** (A) PF403 affected invasion protein expression on SH-SY5Y cells at 72 h. (B) PF403 affected Raf/MEK/ERK signaling on SH-SY5Y cells at 72 h. (C) PF403 affected PI3K/AKT/mTOR signaling on SH-SY5Y cells at 72 h. (D) PF403 affected apoptosis protein expression on SH-SY5Y cells at 72 h. SH-SY5Y cells were cultured and treated with 5x10^{-11} M, 5x10^{-12} M and 5x10^{-13} M of PF403 for 72 h, and the cell lysates immunoblotted with antibodies. Blots were stripped and immunoblotted with anti-β-actin antibody. The results clearly indicate p-FAK, MMP-2, MMP-9, proteins of Raf/ERK, PI3K/AKT, apoptosis pathway was downregulated in PF403 treated cells in 72 h. **P<0.05, **P<0.005 and ***P<0.001. of Raf/MEK/ERK and PI3K/AKT/mTOR pathways at the concentration of 5x10^{-11} M in 4 h (Fig. 6B and C), and PF403 downregulated proteins expression of Raf/MEK/ERK and PI3K/AKT/mTOR pathway at 5x10^{-13} M for 72 h in a concen-
tration dependent manner (Fig. 7B and C). PF403 accelerated apoptosis by upregulated expression of pro-apoptotic proteins (Bad, Bid, Bim and Bax), downregulated anti-apoptosis proteins (Mcl-1 and Bcl-2), and activated caspase-dependent mitochondrial apoptosis pathways (cleave-caspase-9, cleave-caspase-3 and c-PARP) in cells administrated with PF403 for 72 h (Fig. 7D).

**Discussion**

The clinical characteristics of NB are heterogeneity, metastasis and high malignancy, resulting in shorter survival in patients (2,13). Few chemotherapeutic or molecularly targeted antineoplastic agents have shown efficacy in brain tumors, therefore, development of novel chemotherapy agents are urgent. Our earlier study had found that PF403 exerted potent antitumor activity in various tumors in vitro, such as, melanoma, hepatoma, ovarian cancer, glioma and neuroblastoma (9). As this compound could penetrate the blood-brain barrier (8), we supposed that it could suppress growth of brain tumors, and neuroblastoma was chosen in this study. The results of MTT, colony formation and flow cytometry illustrated that PF403-induced SH-SY5Y cell death was dose- and time-dependent. Significant increase in cell deaths was observed at the lowest concentration of PF403 treated groups than controls. PF403 induced SH-SY5Y cell apoptosis with EC50 value of 10^{-11}-10^{-12} M at 72 h by MTT assay. This is much lower than its precursor compound CAT (at 72 h IC50 value was 10^{-7}-10^{-8} M) (7), and the traditional cytotoxic drug taxol (at 72 h IC50 value at 10^{-6}-10^{-7} M). Colony forming assay showed that PF403 inhibited SH-SY5Y NB cell proliferation in a long-term, and it had a good dose-effect relationship. Flow cytometry assay showed that SH-SY5Y cells cultured with PF403 for 72 h induced intense apoptosis.

**Invasion of growth** is the most characteristic biological phenotype of NB, which is also the major reason causing poor prognosis. The present study found that PF403 could inhibit the invasion ability of NB cells in a dose-dependent manner, which is essential for NB treatment.

As PF403 displayed a strong anti-neuroblastoma and anti-invasion activity in vitro, subsequently its antitumor activity in vivo was studied. The barrier function of the BBB is critical for regulating transport to the brain, but also represents a significant hurdle in developing therapies for CNS diseases including brain cancer, and most convention of chemotherapeutic agents cannot penetrate BBB (14-16). Besides, the microenvironment in brain is different from other tissues in human body. Thus, an in situ NB model was used to evaluate the proliferation inhibitory activity of PF403. Our results exhibited that, 14 days after SH-SY5Y cell implantation, the tumor could be located by MRI in mouse brain, and CAT3 significantly inhibited NB cell proliferation. H&E staining confirmed its potent anti-proliferation and anti-invasion ability in situ.

FAK is a mediator of cell-extracellular matrix signaling events and its expression plays an important role in the process of NB invasion (17). Compiling evidence implies that FAK regulates focal adhesion signaling by phosphorylating various substrates, as well as the MMPs-mediated matrix degradation, which consecutively regulates downstream signaling cascades (18). FAK signaling played a critical role in the production of MMPs such as MMP-2 and MMP-9, and subsequently activated tumor invasion (10). Sun et al (19) demonstrated that inhibition of the expression and phosphorylation of FAK, activities of MMP-2 and MMP-9 could be downregulated. FAK is also essential for the Ras/ERK signaling pathway (20). The residues surrounding Tyr577 (FAK) can also constitute a sequence that binds to the Ras/ERK signaling pathway (21). FAK and MAPK signaling involved in MMP-2 secretion has been shown in QG90 lung cancer cell (22). Moreover, it was found that the MMP-9 gene promoter is partially regulated through activation of the Ras/ERK pathway (23). Also, PI3K is one of the critical downstream signal molecules of the FAK pathway (24). Activated FAK binds the SH2 of PI3K, thereby transporting the catalytic subunit of PI3K to the membrane, where it catalyzes the phosphorylation of inositol lipids in lung cancer cell migration (25). Several studies have indicated that FAK/PI3K/Akt is involved in the regulation of MMP-2 and MMP-9 activities on different cell types (12,26). In the present study, PF403 inhibited the activation of FAK, reduced phosphorylation of FAK, and regulated the expression of MMP-2 and MMP-9. In addition, it showed that PF403 inhibited PI3K/AKT/mTOR and Raf/MEK/ERK pathways in SH-SY5Y cells. Thus, it seems that PF403 inhibited SH-SY5Y NB cell migration in concert with downregulated protein expression of PI3K/AKT and Raf/ERK signaling pathways, and associated with p-FAK, MMP-2 and MMP-9.

PI3K/AKT/mTOR and Raf/MEK/ERK pathways influence protein expression of the Bcl-2 family, they play a central role in apoptosis regulating mitochondrial outer membrane permeabilisation (MOMP) and releasing apoptosis-inducing proteins such as cytochrome c, and SMAC sequestered within the mitochondria (27). The Bcl-2 family is divided into three groups based on functionality and presence of conserved Bcl-2 homology (BH1-4) domains: multi-domain anti-apoptotic proteins (Bcl-2 and Mcl-1), multi-domain pro-apoptotic proteins (BAX) and BH3-only proteins (Bad, Bid and Bim). Interactions between these groups of proteins dictate whether the cells die or not. Multi-domain anti-apoptotic proteins prevent MOMP by interacting with and sequestering the multi-domain pro-apoptotic proteins (28,29). BAX allows for oligomerization at the mitochondrial outer membrane and subsequent MOMP through pore formation (30). The BH3-only proteins, competitively bind and neutralize anti-apoptotic proteins, allowing BAX oligomerization and promoting cell death, whereas Bid and Bim can also interact with and activate BAX, facilitating membrane insertion and MOMP (27,31). In this study, upregulation of BAX and downregulation of Bcl-2 was observed in a PF403 concentration-dependent manner. PF403 could upregulate expression of Bim, Bid and Bad, and downregulate expression of Mcl1. The regulation of expression of the Bcl-2 family proteins was a response to the accelerated SH-SY5Y cell death.

Apoptotic pathways in PF403-mediated cell death in SH-SY5Y neuroblastoma cells were examined. The activity of PI3K/AKT and Ras/ERK pathways was downregulated in PF403 treated cells, which were important to proliferation and apoptosis of NB. Our results also strongly demonstrated caspase-9, downstream of Bcl-2 family, was activated in PF403 treated SH-SY5Y cells, suggesting activation of the intrinsic
apoptotic pathway. Downstream target of caspase-9, caspase-3, was also activated in PF403 treated SH-SY5Y cells. Activation of caspase-3 has been reported to have targets in nuclear compartment for the initiation of DNA damage suggesting its nuclear localization (32). PARP is a nuclear target of cleaved caspase-3 and proteolysis cleavage of PARP is considered to be a hallmark feature of apoptosis. Our results demonstrated the presence of only full-length PARP protein in untreated cells but a strong PARP fragment was produced in all PF403 treated cells, strongly suggesting involvement of apoptosis. Taken together, our results clearly suggest that the intrinsic apoptotic pathway was contributed to PF403-mediated SH-SY5Y cell death.

In conclusion, the results presented in the present study demonstrated the effect and novel mechanisms associated with cytotoxic property of PF403 on NB cells. Our studies show that PF403, a novel antitumor agent, could induce apoptosis and inhibit invasion and metastasis of SH-SY5Y NB cells in vitro; CAT1, the pro-drug of PF403, had anti-neuroblastoma activity in situ; PF403 inhibited invasion of NB cells through downregulating protein expression of p-FAK, following MMP-2 and Ral/ERK and PI3K/AKT pathways; PF403-mediated neuroblastoma cell death partly through downregulating Ral/MEK/ERK and PI3K/AKT/mTOR pathways, and then activating apoptotic pathway through regulation of proteins expression of Bcl-2 family activating the intrinsic apoptotic pathway. Thus, the primary biochemical mechanism of action of PF403 was as speculated. In summary, our results strongly suggests that PF403 has potential as a new anticancer drug for the treatment of neuroblastoma.

References