EUROPEAN JOURNAL OF CANCER 45 (2009) 1709–1720

available at www.sciencedirect.com

journal homepage: www.ejconline.com

Growth inhibitory and anti-tumour activities of OSU-03012, a novel PDK-1 inhibitor, on vestibular schwannoma and malignant schwannoma cells

Tina X. Lee[a,b], *Mark D. Packer*[a], *Jie Huang*[b,c], *Elena M. Akhmametyeva*[b,c], *Samuel K. Kulp*[d], *Ching-Shih Chen*[d], *Marco Giovannini*[e], *Abraham Jacob*[a], *D. Bradley Welling*[a], *Long-Sheng Chang*[a,b,c,*]

[a]*Department of Otolaryngology, The Ohio State University College of Medicine, Columbus, OH, USA*
[b]*Center for Childhood Cancer, The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA*
[c]*Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH, USA*
[d]*Division of Medicinal Chemistry and Pharmacognosy, The Ohio State University College of Pharmacy, Columbus, OH 43210, USA*
[e]*House Ear Institute, Los Angeles, CA, USA*

ARTICLE INFO

Article history:
Received 17 December 2008
Received in revised form 3 March 2009
Accepted 12 March 2009
Available online 7 April 2009

Keywords:
Vestibular schwannoma
Malignant schwannoma HMS-97
Neurofibromatosis type 2
The *Neurofibromatosis 2* (*NF2*) gene
Merlin
PI3K/AKT pathway
OSU-03012
Cyclooxygenase-2 inhibitor
Phosphoinositide-dependent kinase-1
Xenograft

ABSTRACT

Background: Vestibular schwannomas (VS) frequently express high levels of activated AKT. Small-molecule inhibitors of AKT signalling may have therapeutic potential in suppressing the growth of benign VS and malignant schwannomas.

Method: Primary VS and Schwann cells, human malignant schwannoma HMS-97 cells and mouse $Nf2^{-/-}$ Schwann cells and schwannoma cells were prepared to investigate the growth inhibitory and anti-tumour activities of OSU-03012, a celecoxib-derived small-molecule inhibitor of phosphoinositide-dependent kinase-1. Cell proliferation assays, apoptosis, Western blot, *in vivo* xenograft analysis using SCID mice and immunohistochemistry were performed.

Results: OSU-03012 inhibited cell proliferation more effectively in both VS and HMS-97 cells than in normal human Schwann cells. The IC_{50} of OSU-03012 at 48 h was approximately 3.1 μM for VS cells and 2.6 μM for HMS-97 cells, compared with the IC_{50} of greater than 12 μM for human Schwann cells. Similarly, mouse $Nf2^{-/-}$ schwannoma and $Nf2^{-/-}$ Schwann cells were more sensitive to growth inhibition by OSU-03012 than wild-type mouse Schwann cells and mouse schwannoma cells established from transgenic mice carrying the *NF2* promoter-driven SV40 T-antigen gene. Like VS cells, malignant schwannoma HMS-97 cells expressed high levels of activated AKT. OSU-03012 induced apoptosis in both VS and HMS-97 cells and caused a marked reduction of AKT phosphorylation at both the Ser-308 and Thr-473 sites in a dose-dependent manner. *In vivo* xenograft analysis showed that OSU-03012 was well tolerated and inhibited the growth of HMS-97 schwannoma xenografts by 55% after 9 weeks of oral treatment. The anti-tumour activity correlated with reduced AKT phosphorylation.

Conclusion: OSU-03012 is a potential chemotherapeutic agent for VS and malignant schwannomas.

* *Corresponding author*: Address: Departments of Pediatrics and Otolaryngology, The Ohio State University College of Medicine and Nationwide Children's Hospital, Columbus, OH, USA. Tel.: +1 614 355 2658; fax: +1 614 722 5895.
E-mail address: lchang@chi.osu.edu (L.-S. Chang).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.03.013

1. Introduction

Vestibular schwannomas (VS) are histologically benign nerve sheath tumours that originate on the superior or inferior vestibular branches of cranial nerve VIII.[1] Clinical symptoms of VS include sensorineural hearing loss, balance abnormalities, tinnitus, vertigo, facial weakness, hydrocephalus, blindness and even death. VS can be divided into four general categories: unilateral sporadic VS, neurofibromatosis type 2 (NF2)-associated VS, cystic schwannomas and malignant schwannomas. Among VS, unilateral sporadic tumours are by far the most common. The development of bilateral VS is the hallmark of NF2, an autosomal dominant disease caused by mutations in the *Neurofibromatosis 2* (*NF2*) tumour suppressor gene, which encodes a tumour suppressor protein named merlin, for moesin, ezrin and radixin-like protein.[2,3] Cystic schwannomas are a particularly aggressive group of unilateral schwannomas that often grow rapidly. Mutations in the *NF2* gene have also been frequently found in sporadic VS and cystic schwannomas.[1,4] Malignant VS, also called triton tumours, are rare but aggressive variants of VS. They occur either sporadically or following radiotherapy and are uniformly fatal.[5,6]

Currently, no known medical therapies are available for the treatment of VS. While improved diagnostic techniques, such as magnetic resonance imaging (MRI), detect these tumours at early stages, surgical excision or stereotactic radiation remains the only viable treatment options. Unfortunately, complications from treatment, such as hearing loss, facial weakness, dizziness, intracranial bleeding and stroke, remain major concerns.[1] Furthermore, radiation treatment may cause malignant transformation and/or growth acceleration of schwannomas.[7–9] The current management of malignant schwannomas is gross surgical resection followed by adjuvant radiotherapy. However, due to the location and extent, total excision of these invasive tumours is difficult to achieve.[10] Currently available chemotherapy regimens have little efficacy in treating triton tumours.[11,12] Together, these factors underscore the importance of developing effective chemotherapeutic agents that stop tumour growth or completely eradicate VS and malignant scwhannomas.

A better understanding of the mechanisms underlying VS tumourigenesis may provide the tools necessary to design novel targeted therapies. The loss of functional merlin protein has been implicated in VS development. Recent studies suggest that merlin plays important roles in regulating actin cytoskeleton-mediated processes, adherens junction formation and cell proliferation[13]; however, the tumour suppressor role of merlin remains elusive. We and others have identified several signalling pathways that are frequently deregulated in VS, including the PI3K/AKT pathway.[4,14–19] In addition to slightly elevated AKT mRNA and protein levels, VS tumours express substantially higher levels of phosphorylated, activated AKT than paired vestibular nerve specimens. Since the PI3K/AKT pathway serves as a convergence point for many growth stimuli, and through its downstream substrates, controls cellular processes and responses, such as cell survival, cell proliferation, insulin response, stress response and differentiation,[20] its activation likely contributes to tumourigenesis. Thus, the PI3K/AKT pathway is an attractive therapeutic target for VS, and small-molecule inhibitors of AKT signalling may have therapeutic potential in suppressing the growth of these tumours.

OSU-03012 is a recently licensed (AR-12; Arno Therapeutics Inc.) novel derivative of the cyclooxygenase-2 (COX-2) inhibitor celecoxib (Celebrex™); however, it lacks COX-2 inhibitory activity. It potently induces apoptosis in several types of cancer cells[21–25] and is currently undergoing preclinical development in the Rapid Access to Intervention Development (RAID) programme at NCI. The mechanism by which OSU-03012 induces apoptosis is mediated, at least in part, through the inhibition of phosphoinositide-dependent kinase-1 (PDK-1), an upstream kinase that phosphorylates AKT. Since VS frequently exhibit the activation of AKT, we evaluated the growth inhibitory and anti-tumour activities of OSU-03012 on cultured human and mouse Schwann cells and schwannoma cells, in addition to schwannoma xenografts in several combined immunodeficiency (SCID) mice. Our results demonstrated that OSU-03012 effectively inhibited cell proliferation and induced apoptosis in both benign and malignant human schwannoma cells, as well as mouse $Nf2^{-/-}$ Schwann cells and $Nf2^{-/-}$ schwannoma cells, with IC_{50} values in the low micromolar range. We also showed that OSU-03012 possessed potent anti-tumour activity in malignant schwannoma xenografts in SCID mice.

2. Materials and methods

2.1. Compound

OSU-03012 was synthesised as previously described[21] with purities exceeding 99% as determined by nuclear magnetic resonance spectroscopy (300 mHz).

2.2. Tissue acquisition and cell culture

The Ohio State University Institutional Review Board approved Human Subjects Protocols for the acquisition of surgically removed VS specimens from patients and normal nerves from organ donors through Lifeline of Ohio. VS were resected with informed patient consents, and their histological type was confirmed by a neuropathologist. Vestibular schwannoma and normal nerve tissues were placed in Dulbecco's modified Eagle's (DME) medium (Invitrogen) and brought to the research laboratory shortly after resection.

To prepare VS cell cultures, tumour tissues were cut into small (<1 mm) pieces and were incubated in DME medium containing 10% foetal bovine serum (FBS), 130 U/ml collagenase type I, 0.6 U/ml dispase, 100 I.U./ml penicillin and 100 μg/ml streptomycin (all from Invitrogen), for 6 h at 37 °C with moderate shaking. Dissociated tissues were further triturated by passing through a narrow Pasteur pipette. Cell suspension was collected by centrifugation, resuspended in DME medium supplemented with 10% FBS, 10 nM recombinant human NRG1-β1/HRG1-β1 EGF domain (heregulin; R&D Systems), 2 μM forskolin (Sigma–Aldrich), 100 I.U./ml penicillin and 100 μg/ml streptomycin and was plated on dishes coated with poly-L-lysine and laminin (Sigma–Aldrich).

For preparing human Schwann cell cultures, femoral nerves from organ donors were cut into small pieces and were incubated at 37 °C in DME medium supplemented with 10% foetal bovine serum, 50 U/ml penicillin and 0.05 mg/ml streptomycin for 7 d for *in vitro* Wallerian degeneration.[26] This pretreatment medium was replaced every 3 d. Pretreated nerve tissues were dissociated, and cell suspension was plated in medium containing heregulin and forskolin as described above for VS cells. Using a similar method, normal mouse Schwann cells were prepared from sciatic nerves harvested from FVB/N mice at 4–8-weeks-old.

Mouse *Nf2*$^{-/-}$ Schwann cells were prepared by two methods. Schwann cells derived from sciatic nerves of *Nf2*$^{flox2/flox2}$ mice[27] were infected with AdCMVCre recombinant adenoviruses carrying a cytomegalovirus (CMV) promoter-driven Cre gene (Gene Transfer Vector Core at University of Iowa, Iowa City, IA) to delete the *Nf2* gene. Alternatively, sciatic nerves from Nestin-CreER; *Nf2*$^{flox2/flox2}$ mice (E.M. Akhmametyeva, L.-S. Chang and colleagues, Nationwide Children's Hospital and The Ohio State University) were used to prepare Schwann cell cultures, followed by treatment with tamoxifen for 48 h.[28] To detect the deletion of the *Nf2* gene, genomic DNA was isolated from a portion of cells using the Gentra Puregene Cell Kit (Qiagen) and was analysed by the polymerase chain reaction technique accordingly.[27] We found that the *Nf2* gene was deleted in at least 90% of the Schwann cells obtained by either method.

In addition, we prepared mouse *Nf2*$^{-/-}$ schwannoma cells from the tumour developed in the P0Cre; *Nf2*$^{flox/flox}$ mice with conditional *Nf2* inactivation in Schwann cells.[27] For comparison, we established mouse *NF2*P2.4-T schwannoma cells from a schwannoma developed in a transgenic mouse carrying a 2.4-kb *NF2* promoter-driven SV40 T-antigen oncogene (E.M. Akhmametyeva, L.-S. Chang and colleagues, Nationwide Children's Hospital and The Ohio State University).[29] The human malignant schwannoma HMS-97 cells have been described previously.[30] Both mouse *NF2*P2.4-T and human HMS-97 schwannoma cells were grown in DME medium supplemented with 10% FBS.

2.3. Cell proliferation assay

VS and HMS-97 cells were grown in polylysine/laminin-coated 96-well plates (5×10^3 to 1×10^4 per well). After incubation at 37 °C for 24 h, cells were treated with various concentrations of OSU-03012 or DMSO as a vehicle control at 37 °C for another 48 h. To assess cell proliferation, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) was used according to the manufacturer's instructions. Briefly, 20 μl of CellTiter 96® AQ_{ueous} One Solution Reagent was added to each well and was incubated at 37 °C for 1–4 h. The amount of coloured formazan produced in the cells was measured at 490 nm using a Victor[3] 1420 Multilabel Counter (Perkin–Elmer). The percentage of cell proliferation was plotted against the concentrations of OSU-03012, and the IC_{50} (defined as the drug concentration at which the population of viable cells was reduced by 50%) was determined using CalcuSyn software (Biosoft). The experiments were replicated six times, and the mean IC_{50} was calculated.

2.4. Western blot analysis

VS and HMS-97 cells were treated with increasing concentrations of OSU-03012 or 0.05% DMSO as a negative control for 8–24 h. Treated cells were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich). The protein concentrations of clear lysates were determined using the Bio-Rad Protein Dye Reagent. Equal amount (20 μg/each) of protein was electrophoresed on an 8% SDS–polyacrylamide gel, followed by electroblotting to an Immobilon-P membrane (Millipore). After protein transfer, the membrane was blocked with 5% (w/v) non-fat dry milk in 10 mM Tris-buffered saline with 0.05% Tween 20 (TBST) at room temperature for 1 h and was then incubated with a primary antibody (1:1000 dilution) at 4 °C overnight. After washing with TBST three times, a horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) was added to the membrane at room temperature for 1 h, followed by incubation with the ECL Plus™ Western Blotting Reagent (GE Healthcare). The chemiluminescent activity was captured by exposure to Fiji medical X-ray films or scanning with a Typhoon 9400 phosphoimager. Primary antibodies used include: anti-AKT, anti-phospho-AKT(Ser^{473}), anti-phospho-AKT(Thr^{308}), anti-phospho-GSK-3β(Ser9) and anti-caspase 9 (all from Cell Signalling Technology). Anti-α-tubulin and anti-β-actin antibodies (Rockland Immunochemicals) were used as loading controls.

2.5. Apoptosis assays

Apoptosis was detected using the DeadEnd Fluorometric TUNEL system (Promega). VS and HMS-97 cells grown on polylysine/laminin-coated coverslips (BD Biosciences) were treated with 7.5 μM of OSU-03012 or 0.05% DMSO as the control for various durations. Treated cells were fixed in 4% paraformaldehyde for 25 min, permeabilised in 0.2% Triton X-100 in PBS for 5 min at room temperature, and then labelled with fluorescein-12-dUTP using recombinant terminal deoxynucleotidyl transferase. Labelled cells were washed three times in PBS and were covered with a drop of ProLong Gold Antifade Reagent with DAPI (Invitrogen) prior to mounting onto a glass slide. Under a Leica DM4000 fluorescence microscope, DAPI stained all nuclei in blue and fluorescein-12-dUTP incorporation resulted in localised green fluorescence within the nucleus of apoptotic cells.

Apoptosis was also confirmed by the appearance of the active large fragment of cleaved-caspase 9. HMS-97 and VS cells were exposed to different concentrations of OSU-03012 for 48 h. Soluble cell lysates were prepared and used to detect pro- and cleaved-caspase 9 by Western blot using an anti-caspase 9 antibody (Cell Signalling Technology) as described above.

2.6. Schwannoma xenografts and MRI

Schwannoma xenografts were established in severe combined immunodeficiency mice (SCID) and MRI scans were conducted as previously described.[30] Briefly, human malignant schwannoma HMS-97 cells (2.5×10^5 cells/mouse) were washed in PBS and suspended in 0.2 ml of Matrigel® (1:1 diluted in PBS; BD Biosciences). The right flank of SCID mice

was shaved and prepped using aseptic technique. An 18-gauge needle was used to inject schwannoma cells into the subcuticular plane raising a small wheal in the region overlying the sciatic nerve. The injected mice were observed for 1 week and were then divided into two groups. One group of mice was administered 200 mg/kg/day of OSU-03012 by oral gavage. The other group of mice served as controls and was gavage-fed a similar volume of the vehicle, consisting of methylcellulose (0.5%, w/v) and Tween 20 (0.1%, w/v) in sterile water. After 9 weeks of treatment, mice were examined under anaesthesia using a 4.7 T/cm magnetic resonance imaging (MRI) scanner (Bruker) as described previously.[30] T1-weighted axial and coronal images were obtained using a spin echo sequence with TR of 550–600 ms and TE of 10.5 ms. In-plane resolution was 156 μm on the axial and 195 μm on the coronal images, and the slice thickness was 0.8 mm with a 0.2-mm gap between slices. Multi-planar tumour volumes were determined by manually tracing tumour areas on axial and coronal images, followed by adding the traced areas from all slices depicting the tumour and multiplying with the distance between slices.

2.7. Immunohistochemical analysis

Xenograft tumours grown in SCID mice with or without OSU-03012 treatment were dissected, fixed in 10% buffered formalin and embedded in paraffin. Five-micron tissue sections were obtained and processed for standard haematoxylin–eosin staining or immunostaining with antibodies against phospho-AKT.[19] Negative controls were treated with the same immunostaining procedure except without the primary antibody.

3. Results

3.1. OSU-03012 potently inhibits the growth of human VS and HMS-97 cells in vitro

Since the PI3K/AKT pathway is frequently activated in human VS, we tested whether OSU-03012 could effectively inhibit the growth of benign VS and malignant schwannoma HMS-97 cells, compared with normal human Schwann cells in culture. Exposure of primary human VS cells to OSU-03012 resulted in a dose-dependent inhibition of cell proliferation (Fig. 1A and B). To exclude the possibility of individual differences in drug sensitivity, we exposed six VS cultures from different patients with various concentrations of OSU-03012 and determined the IC_{50} values. In addition, as heregulin has been shown to synergize with intracellular cAMP to enhance Schwann cell proliferation,[31] we performed OSU-03012 treatment in the presence or absence of heregulin and forskolin. The IC_{50} values for various preparations of human VS cells tested in the absence of heregulin ranged from 2.1 to 4.0 μM (Table 1). In the presence of heregulin, only a slight increase in the IC_{50} values for various VS cell cultures was observed (IC_{50} ranging from 2.7 to 5.4 μM). Similarly, a dose-dependent inhibition of cell proliferation by OSU-03012 was observed for human malignant schwannoma HMS-97 cells with an average IC_{50} value of 2.6 μM (Fig. 1C and Table 1). For comparison, we also prepared primary normal human Schwann cells and treated them with various concentrations of OSU-03012. Normal human Schwann cells were more resistant to growth inhibition by OSU-03012 (Fig. 1D). The IC_{50} values for various preparations of normal human Schwann cells cultured in the presence of heregulin were greater than 12 μM. These results indicate that OSU-03012 potently inhibits the growth of both benign human VS and malignant schwannoma cells in culture.

3.2. Mouse $Nf2^{-/-}$ Schwann cells and $Nf2^{-/-}$ schwannoma cells are also sensitive to growth inhibition by OSU-03012

Since most, if not all, VS harbour mutations in the *NF2* gene[1–3] and since mouse $Nf2^{-/-}$ schwannoma cells also frequently exhibit up-regulation of the PI3K/Akt pathway,[16] we compared the growth inhibitory effect of OSU-03012 on various mouse $Nf2^{+/+}$ or $Nf2^{-/-}$ Schwann cells and schwannoma cells. Mouse $Nf2^{-/-}$ schwannoma cells derived from a spontaneously occurring schwannoma in the P0Cre; $Nf2^{flox/flox}$ mouse[27] showed a dose-dependent growth inhibition when exposed to various concentrations of OSU-03012 (Fig. 2A). Similarly, exposure of primary mouse $Nf2^{-/-}$ Schwann cells to OSU-03012 resulted in a dose-dependent inhibition of cell proliferation (Fig. 2B). The IC_{50} value for mouse $Nf2^{-/-}$ schwannoma cells was about 2.4 μM, while that for mouse $Nf2^{-/-}$ Schwann cells was about 2.9 μM (Table 1). In contrast, wild-type mouse Schwann cells were substantially less sensitive to growth inhibition by OSU-03012 (Fig. 2C) with an IC_{50} value of greater than 20 μM (Table 1). As an additional control, we used mouse schwannoma cells derived from a schwannoma generated in a transgenic *NF2*P2.4-T mouse carrying the SV40 T-antigen gene driven under the control of the 2.4-kb *NF2* promoter. These *NF2*P2.4-T schwannoma cells express the viral T-antigen oncoprotein but carry the wild-type *Nf2* gene. Interestingly, these *NF2*P2.4-T schwannoma cells were also relatively insensitive to growth inhibition by OSU-03012, compared with mouse $Nf2^{-/-}$ schwannoma and $Nf2^{-/-}$ Schwann cells (compare Fig. 2D to A and B). The IC_{50} value for these *NF2*P2.4-T schwannoma cells was greater than 12 μM (Table 1). Together, these results imply that the loss of a functional *Nf2* gene correlates with the higher sensitivity of mouse Schwann cells and schwannoma cells to growth inhibition by OSU-03012.

3.3. OSU-03012 inhibits AKT phosphorylation in both HMS-97 and VS cells in a dose-dependent manner

One major biochemical action of OSU-03012 is the inhibition of PDK-1, which phosphorylates and activates AKT.[21] To examine the phosphorylation status of AKT in HMS-97 and VS cells treated with various concentrations of OSU-03012, Western blots using antibodies specific to phospho-AKT at the threonine-308 [p-AKT(Thr^{308})] and serine-473 [p-AKT-(Ser^{473})] sites, and total AKT were performed. A dose-dependent inhibition of AKT phosphorylation at the threonine-308 site, which is the primary phosphorylation site of PDK1, was observed in both HMS-97 (Fig. 3A) and VS cells (Fig. 3B). The inhibition of Akt phosphorylation at the threonine-308 site in OSU-03012-treated HMS-97 cells appeared to be less than that in OSU-03012-treated VS cells; however, the reason for this observation is presently not known. Similarly, we de-

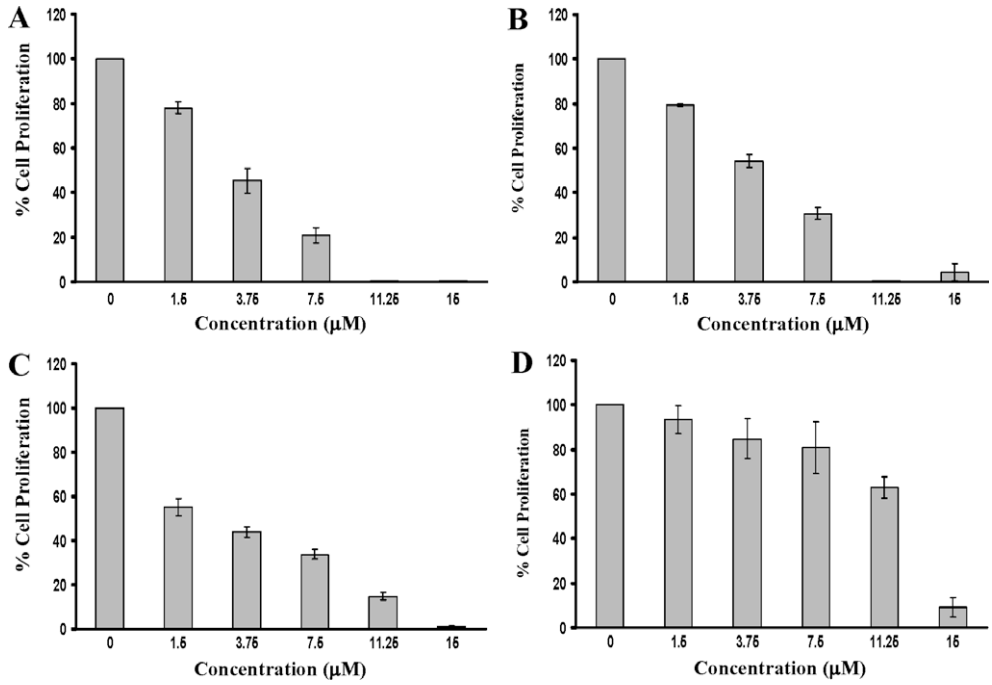

Fig. 1 – Effects of OSU-03012 on the proliferation of human VS cells grown in the absence (A) or presence (B) of heregulin, human malignant schwannoma HMS-97 cells (C) and normal human Schwann cells (D). Primary human VS and Schwann cells were prepared as described in Section 2, and were plated out at a density of 5×10^3 cells/well in a poly-D-lysine/laminin-coated 96-well plate in growth medium with or without heregulin at 37 °C for 24 h. HMS-97 cells were seeded at the same density in DME medium supplemented with 10% FBS overnight. Various concentrations of OSU-03012 were then added to the culture. DMSO (0.05%) in which the stock solution of OSU-03012 was prepared was used as the vehicle control. After treatment for 48 h, cell proliferation was measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) and was recorded using a Victor^3 1420 Multilabel Counter (Perkin–Elmer). The percentage of cell proliferation was plotted against the concentrations of OSU-03012. The experiments were performed in six replicates. Columns show the mean of the replicates and error bars denote the standard deviation. Six different preparations of primary VS cells (Table 1) were studied and the representative result was shown.

Table 1 – IC_{50} values of OSU-03012 for various human and mouse Schwann cells and schwannoma cells. Primary VS cells (VS 1–6) were prepared from six different patients. Normal human Schwann cells were also prepared as described in Section 2. VS cells, normal Schwann cells and human malignant schwannoma HMS-97 cells were treated with various concentrations of OSU-03012 in the absence (–) or presence (+) of heregulin (Fig. 1). Likewise, mouse $Nf2^{+/+}$ (wild-type) and $Nf2^{-/-}$ Schwann cells, $Nf2^{-/-}$ schwannoma cells, and NF2P2.4-T schwannoma cells were also prepared and treated with various concentrations of OSU-03012 (Fig. 2). Cell proliferation was analysed as described in Section 2. The IC_{50} value was defined as the concentration at which cell proliferation decreased by 50% and was calculated using CalcuSyn software (Biosoft). The experiments were repeated at least three times, and the IC_{50} value was shown as the means + standard deviation. N.D., not determined.

Species	Type of cells	– Heregulin (μM)	+ Heregulin (μM)
Human	VS 1	2.1 ± 0.6	2.7 ± 0.9
	VS 2	3.0 ± 0.1	5.4 ± 0.2
	VS 3	3.6 ± 0.5	4.1 ± 0.8
	VS 4	3.5 ± 0.3	3.5 ± 0.2
	VS 5	4.0 ± 0.1	4.5 ± 0.5
	VS 6	2.8 ± 0.1	3.1 ± 0.3
	HMS-97	2.6 ± 0.1	N.D.
	Normal Schwann cells	N.D.	>12
Mouse	$Nf2^{-/-}$ schwannoma	N.D.	2.4 ± 0.3
	$Nf2^{-/-}$ Schwann cells	N.D.	2.9 ± 0.2
	$Nf2^{+/+}$ Schwann cells	N.D.	>20
	NF2P2.4-T schwannoma	N.D.	>12

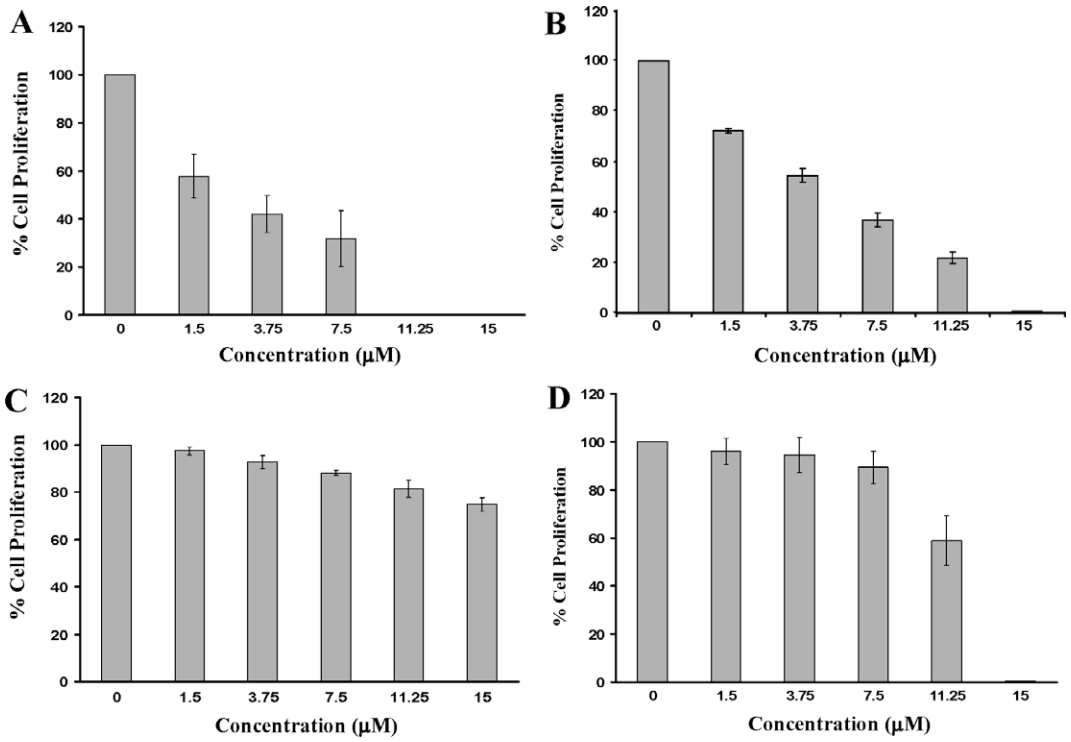

Fig. 2 – Effects of OSU-03012 on the proliferation of (A) *Nf2*$^{-/-}$ mouse schwannoma cells from the P0Cre; *Nf2*$^{flox/flox}$ mouse, (B) *Nf2*$^{-/-}$ mouse Schwann cells, (C) *Nf2*$^{+/+}$ mouse Schwann cells and (D) T antigen-transformed mouse NF2P2.4-T schwannoma cells. Various mouse schwannoma and Schwann cells were plated out in a poly-D-lysine/laminin-coated 96-well plate (5×10^3 cells/well) in a complete growth medium at 37 °C for 24 h and then treated with various concentrations of OSU-03012 or DMSO control for another 48 h before cell proliferation assay was performed. The percentage of cell proliferation was plotted against the concentrations of OSU-03012. Each experiment was performed in triplicates and was repeated at least two times. Columns show the mean of the replicates and error bars denote the standard deviation.

tected a dose-dependent inhibition of AKT phosphorylation at the serine-473 site when cells were treated with increasing concentrations of OSU-03012. Likewise, the phosphorylation of glycogen synthase kinase-3β [p-GSK-3β(Ser9)], a downstream substrate of AKT, was also inhibited by OSU-03012 in a dose-dependent manner. In contrast, the expression level of total AKT remained constant in both the malignant and benign schwannoma cells treated with up to 7.5 μM of OSU-03012. Furthermore, we showed that under similar treatment conditions, OSU-03012 had little or no effect on Akt phosphorylation in both NF2P2.4-T schwannoma cells (Fig. 3C) and normal mouse Schwann cells (Fig. 3D), both of which were relatively insensitive to growth inhibition by OSU-03012 (Fig. 2). These results indicate that the inhibition of AKT phosphorylation at both the threonine-308 and serine-473 sites by OSU-03012 correlates with its anti-proliferative effect in both human malignant and benign schwannoma cells.

3.4. *OSU-03012 induces apoptosis in both HMS-97 and VS cells*

Since AKT is critical to cell proliferation and cell survival, we next examined whether OSU-03012 induced programmed cell death in both HMS-97 and VS cells by two methods, TUNEL and cleavage of caspase 9. For the TUNEL assay, we treated HMS-97 and VS cells with 7.5 μM of OSU-03012 for various durations before end-labelling to detect apoptosis-induced DNA fragmentation. TUNEL-positive HMS-97 cells were found as early as 6 h after treatment (data not shown), and were readily observed after 14 h (Fig. 4A). Nearly all HMS-97 cells were TUNEL-labelled 24 h after OSU-03012 treatment, while DMSO-treated control cells showed no labelling. Similarly, TUNEL-positive VS cells were detected 18 h after OSU-03012 treatment, and nearly all OSU-03012-treated cells were labelled after 24 h (Fig. 4B). To confirm apoptotic induction in OSU-03012-treated cells, Western blot analysis to detect cleaved-caspase 9 was performed. Treatment with increasing concentrations of OSU-03012 resulted in a dose-dependent decrease of caspase 9 with a concomitant increase of the 35-kDa active fragment of cleaved-caspase 9, evidence of apoptotic induction, in both HMS-97 and VS cells (Fig. 5). In contrast, OSU-03012 treatment had little or no effect on caspase 9 cleavage in normal mouse Schwann cells under similar conditions (data not shown). Together, these results indicate that OSU-03012 potently induced apoptosis in both benign and malignant schwannoma cells.

3.5. *OSU-03012 suppressed the growth of malignant schwannoma xenografts in vivo*

To evaluate the anti-tumour potential of OSU-03012, SCID mice bearing HMS-97 malignant schwannoma xenografts

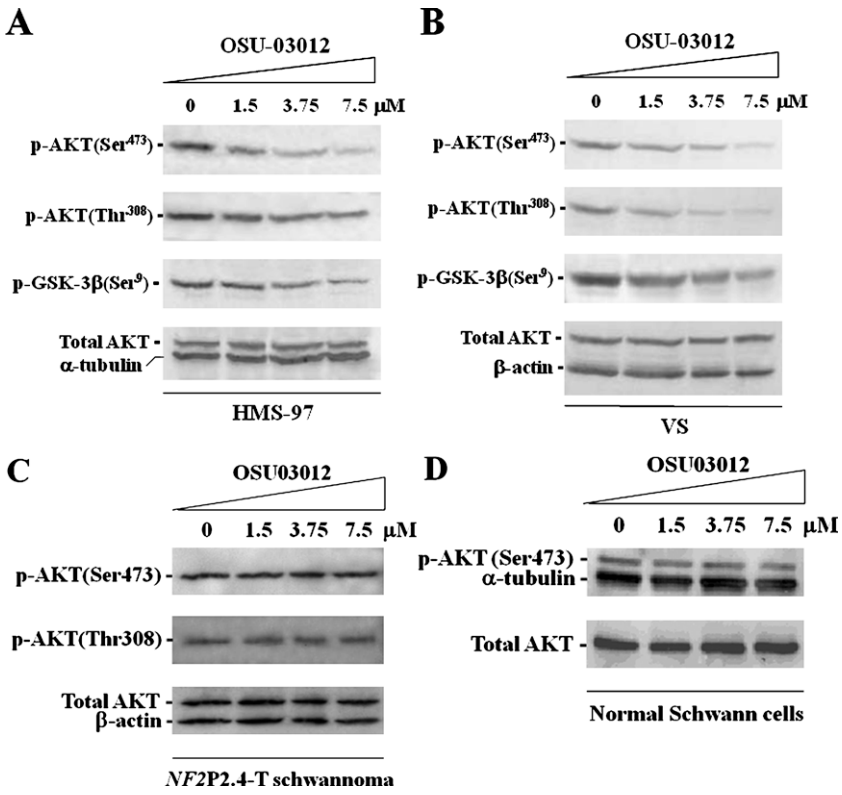

Fig. 3 – Effects of OSU-03012 on AKT phosphorylation in human malignant schwannoma HMS-97 cells (A), primary human VS cells (B), mouse *NF2*P2.4-T schwannoma cells (C) and normal mouse Schwann cells (D). Various concentrations of OSU-03012 were added to the cells for 12 h. Cell lysates were analysed for p-AKT(Ser^{473}), p-AKT(Thr^{308}), p-GSK-3β(Ser9) and total AKT by Western blots. In addition, the expression of α-tubulin or β-actin was detected and served as a loading control.

were treated orally with OSU-03012 at 200 mg/kg/day or with vehicle for 9 weeks. Then, the tumour volume of schwannoma xenograft in each mouse was quantified using high-field small-animal MRI.[30] Treatment of mice with OSU-03012 inhibited HMS-97 tumour growth by 55%, relative to the vehicle-treated control (Fig. 6). This *in vivo* efficacy after oral administration confirmed the oral bioavailability of OSU-03012, which was previously determined to be approximately 40% in rats using the same vehicle.[32] SCID mice appeared to tolerate OSU-03012 at 200 mg/kg/day for 9 weeks. We did not observe any signs of toxicity or significant changes in body weight of the treated animals, compared with the vehicle-treated group. Importantly, histopathological examination revealed that the malignant schwannoma xenograft tumour from the OSU-03012-treated mouse harboured islands of necrosis, while those from the vehicle-treated control exhibited minimal necrosis (Fig. 7A). To correlate this *in vivo* tumour-suppressive response to an intratumoural biomarker of drug activity, HMS-97 xenograft tumours from untreated and treated mice were harvested, and tumour sections were immunostained with the anti-p-AKT(Ser^{473}) antibody. As shown in Fig. 7B, the phosphorylated AKT protein was readily detected in both the nucleus and cytoplasm of HMS-97 tumour cells treated with the vehicle control. In contrast, the level of phospho-AKT staining was substantially reduced in OSU-03012-treated tumour cells (Fig. 7B). Collectively, these results demonstrate that OSU-03012 possessed potent anti-tumour activity in malignant schwannoma xenografts in SCID mice.

4. Discussion

Vestibular schwannomas (VS) are intracranial tumours arising from Schwann cells of cranial nerve VIII. VS are frequently seen in patients with NF2 and some of them have multiple schwannomas, which may be difficult to remove.[1] Currently, no medical therapies are available for the treatment of all types of benign VS. Malignant schwannomas are rare but highly aggressive with a 5-year survival rate of only 10–20%.[6] These tumours do not respond to currently available chemotherapy, and total excision is difficult to achieve due to their location and extent at presentation.[10–12] Therefore, the development of chemotherapies to stop tumour growth or completely eradicate VS and malignant schwannomas is of urgent clinical need. Since both VS[17,19] and malignant schwannomas (Figs. 3 and 7) frequently express high levels of activated AKT, drugs targeting the AKT pathway, such as OSU-03012, are likely to have therapeutic potential in suppressing the growth of these tumours. In the present study, we assessed the *in vitro* and *in vivo* efficacy of OSU-03012 in human benign VS and malignant schwannoma HMS-97 cells. We showed that OSU-03012 possesses potent growth inhibitory and anti-tumour activities on both benign and malignant human schwannoma cells, as well as mouse *Nf2*$^{-/-}$ Schwann cells and schwannoma cells.

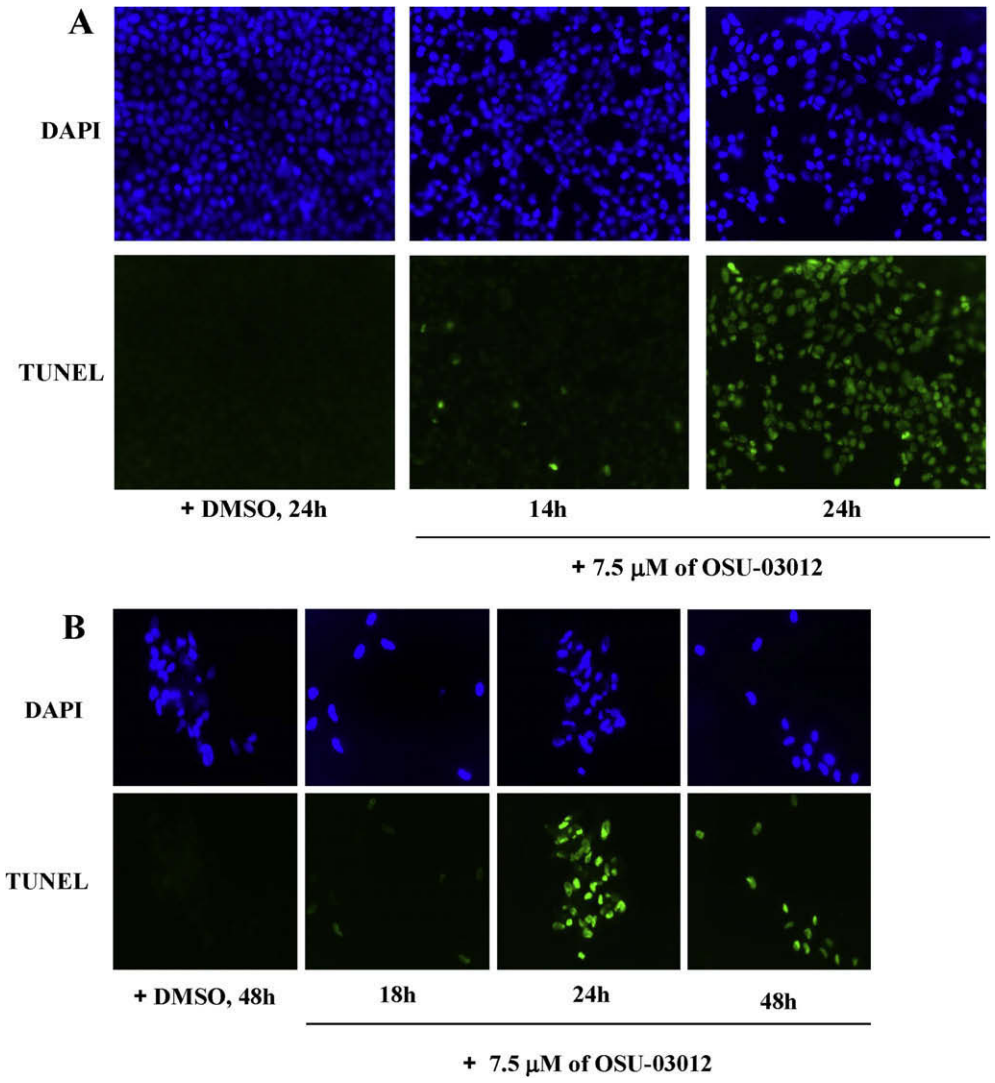

Fig. 4 – OSU-03012 treatment induced apoptosis in both human malignant schwannoma HMS-97 cells (A) and primary human VS cells (B). OSU-03012 (7.5 μM) was added to the cells for the indicated time periods. As controls, HMS-97 cells were treated with DMSO (0.05%) for 24 h and VS cells were treated for 48 h. Treated cells were fixed and labelled by TUNEL staining (shown in green). DAPI was used to counter stain the nuclei in blue.

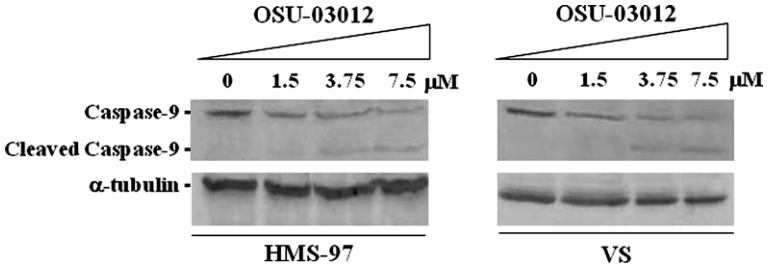

Fig. 5 – OSU-03012 treatment induced cleavage of caspase 9. Human malignant schwannoma HMS-97 cells and primary human VS cells were treated with increasing concentrations of OSU-03012 for 12 h. Soluble cell lysates were prepared and analysed by Western blot using an anti-caspase-9 antibody to detect pro-caspase-9 (47 kDa) and the cleaved-caspase 9 fragment (37 kDa). The expression of α-tubulin was also detected and used as a loading control.

Consistent with the previous studies using other human cancer cells,[21–24] OSU-03012 potently induces growth inhibition and apoptosis in both benign and malignant schwannoma cells in association with PDK-1 inhibition and subsequent inactivation of Akt and downstream signalling molecules. Intriguingly, we found a dose-dependent

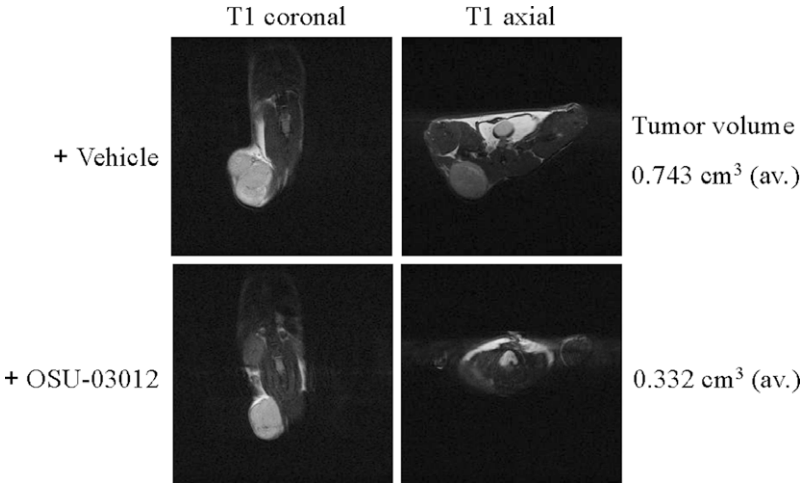

Fig. 6 – Volumetric measurement of human malignant schwannoma xenografts in SCID mice with or without OSU-03012 treatment by MRI. HMS-97 cells (2.5×10^5 cells/mouse) were implanted in the right flank of SCID mice as previously described.[30] The mice were observed for 1 week to allow the growth of xenografts. Subsequently, one group of mice was administered 200 mg/kg/day of OSU-03012 by oral gavage. The other group of mice was gavage-fed a similar volume of the vehicle as the control. After 9 weeks of treatment, mice were examined under anaesthesia using a 4.7 T/cm MRI scanner (Bruker). Both T1-weighted axial and coronal images were obtained and multiplanar tumour volumes were determined as described in Section 2. Images from representative mice are shown. Note that the average tumour volume of schwannoma xenografts was greatly reduced in the OSU-03012-treated group.

reduction of AKT phosphorylation at both the threonine-308 and serine-473 sites in HMS and VS cells upon treatment with increasing concentrations of OSU-03012. Although the threonine-308 site is considered the primary phosphorylation site by PDK1, three models have been proposed for AKT phosphorylation at the serine-473 site, including phosphorylation by PDK1, by a putative kinase named PDK2, or by autophosphorylation.[33] If the serine-473 site is phosphorylated by a kinase other than PDK1,[34] our results argue that OSU-03012 may also inhibit the other AKT kinase, PDK2. In addition, OSU-03012 may induce apoptosis in cancer cells by affecting pathways other than PDK-1/Akt signalling, including the disruption of mitochondrial membrane potential and activation of caspase 9,[23,35] induction of endoplasmic reticulum stress responses,[36] inhibition of p21-activated kinase-1 (PAK1) activity,[24] inhibition of Janus-activated kinase 2/signalling transducer activator of transcription 3 and mitogen-activated protein kinase (MAPK) pathways, and down-regulation of cyclins A and B and the inhibitor of apoptosis protein members, X-linked inhibitor of apoptosis and survivin.[25] Consistently, we also found that OSU-03012 treatment led to the activation of caspase 9 in both VS and HMS-97 malignant schwannoma cells.

VS frequently harbour mutations in the *NF2* tumour suppressor gene.[1–3] Evidence suggests that merlin, the *NF2* gene product, may exert its growth suppressive activity by inhibiting the PI3K/AKT pathway.[18,37] PI3K is a membrane-associated lipid kinase that converts phosphatidylinositol diphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3), which recruits AKT from the cytoplasm to the cell membrane where it is phosphorylated and activated by PDK-1.[20] Merlin could bind to the PI3K enhancer long isoform (PIKE-L), a brain-specific GTPase that binds to PI3K and stimulates its lipid kinase activity,[37] and could inhibit PI3K activity by disrupting the binding of PIKE-L to PI3K. In addition, recent evidence suggests that AKT could also bind to and phosphorylate merlin on residues threonine-230 and serine-315, which abolish merlin's intra-molecular interactions and binding to PIKE-L and other binding partners.[18] Although the reciprocal experiment for the effect of merlin binding on AKT activity has not been demonstrated, it is conceivable that merlin inactivation, as frequently seen in VS, could result in the activation of the PI3K/AKT pathway. Consistent with this notion, mouse *Nf2*$^{-/-}$ Schwann cells or schwannoma cells derived from mice with conditional *Nf2* knockout in Schwann cells show the activation of Akt.[16] Analogous to human VS and malignant schwannoma cells, both mouse *Nf2*$^{-/-}$ Schwann cells and *Nf2*$^{-/-}$ schwannoma cells display higher sensitivity to the inhibition of AKT by OSU-03012 than normal mouse Schwann cells and *NF2*P2.4-T mouse schwannoma cells. These results indicate that biallelic loss of a functional *NF2* gene and the activation of the AKT pathway in *Nf2*$^{-/-}$ schwannoma and Schwann cells correlate with chemotherapeutic sensitivity to OSU-03012.

It should be mentioned that the SV40 T-antigen oncoprotein is capable of transforming a variety of cell types. T antigen transforms cells by binding and inactivating the p53 and retinoblastoma protein family of tumour suppressors.[38] The fact that the *NF2*P2.4-T mouse schwannoma cells are relatively resistant to growth inhibition by OSU-03012 compared with *Nf2*$^{-/-}$ mouse schwannoma or Schwann cells implies that T antigen induces tumour formation by targeting pathways other than the PI3K/AKT pathway. In addition, these results further implicate the specificity of OSU-03012 action to the AKT pathway. The *NF2*P2.4-T mouse schwannoma cells should be useful as negative controls for future testing of pathway-specific inhibitors.

Like many other key regulatory proteins, merlin is regulated by phosphorylation,[39] possibly mediated through Rac1

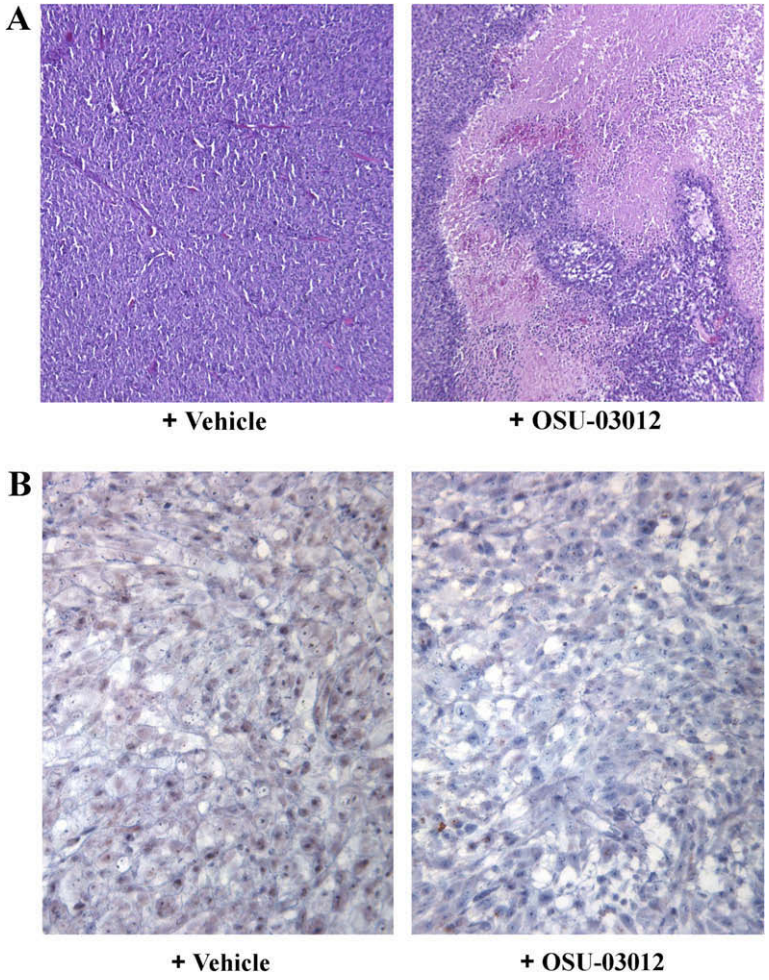

Fig. 7 – Immuno-histopathological examination of HMS-97 xenograft tumours from SCID mice with or without OSU-03012 treatment. (A) Haematoxylin–eosin staining of tissue sections of HMS-97 tumours from mice treated with OSU-03012 or vehicle control for 9 weeks by oral gavages. (B) Immunostaining for p-AKT(Ser^{473}) expression in HMS-97 tumour tissue sections from mice treated with OSU-03012 or vehicle control for 9 weeks.

and other Rho GTPase family members.[40] Among the Rac/Cdc42 effectors, PAK1 and PAK2 can phosphorylate merlin, affecting merlin's localisation and function.[37,39,41–43] Conversely, merlin inhibits PAK1, and the PAK1-specific inhibitors could reduce the growth of *NF2*-deficient mesothelioma cells.[44] Interestingly, Porchia et al.[24] recently demonstrated that OSU-03012 could also inhibit PAK activity in thyroid carcinoma cells. Since PAK expression is frequently up-regulated in *NF2*-deficient cells,[4,16,42] experiments are in progress to examine whether OSU-03012 could inhibit PAK activation in schwannoma cells.

Two lines of evidence support the notion that OSU-03012 induces apoptosis in schwannoma cells. First, OSU-03012 treatment induced chromosome fragmentation in the nucleus of both VS and HMS-97 schwannoma cells as visualised by TUNEL staining. This effect is accompanied by the decreased level of activated AKT. Second, OSU-03012 treatment results in a dose-dependent cleavage of caspase 9, leading to the appearance of the 35-kDa active fragment of cleaved-caspase 9. Cardone et al.[45] previously showed that murine Akt could phosphorylate caspase on serine-196 and inhibit its protease activity. A phosphorylation-inactive caspase 9 mutant, when expressed in the cell, resulted in Akt-resistant induction of apoptosis. Thus, it is possible that the inhibition of AKT phosphorylation and activation by OSU-03012 may allow cleavage and generation of an active caspase 9, ultimately leading to apoptosis. In this manner, OSU-03012 induces the caspase-dependent pathway of apoptosis in both benign VS and malignant HMS-97 schwannoma cells. It is noteworthy that OSU-03012 may also induce apoptosis via caspase-independent mechanisms in other tumour types.[22,25,36]

Chemotherapeutic agents that could reduce the size of VS while sparing associated nerves are attractive options for treating these tumours. OSU-03012 is a derivative of celecoxib, but lacks the COX-2 inhibitory activity of the parent drug.[21] Such a unique feature may limit the known side-effects of COX-2 inhibitors and make OSU-03012 an attractive choice for the treatment of benign tumours such as VS. Our *in vivo* data showing the effect of OSU-03012 on the growth of malignant schwannoma xenografts in SCID mice further corroborate the *in vitro* findings. Also, the results indicate the oral bioavailability and tolerability of OSU-03012 at the

dose used in the mice, consistent with the previous reports using murine xenograft models for liver, breast and lung cancers.[46–48] Intriguingly, we observed that, in addition to reducing tumour size, OSU-03012 treatment induced necrosis within the HMS-97 malignant schwannoma xenograft grown in SCID mice. Although the mechanism underlying the induction of this necrosis is presently unknown, it is tempting to speculate that OSU-03012 may induce cell death by down-regulating genes that encode autocrine growth factors, cytokines and/or angiogenic factors. Some of these genes could be downstream targets of the PI3K/AKT pathway. Consistent with this notion, we observed that the *in vivo* tumour-suppressive response to OSU-03012 activity correlates with the decrease of activated AKT in the xenograft tumour. Presently, we are conducting a similar experiment to test the *in vivo* efficacy of OSU-03012 using VS xenografts.

Several additional characteristics of OSU-03012 make it an attractive candidate for further drug development. Due to their intracranial location, treatment of VS may require drugs that penetrate the blood–brain barrier. Our preliminary analysis reveals that OSU-03012 effectively crosses the blood–brain barrier after intravenous (i.v.) dosing. OSU-03012 has also been reported to interact synergistically with other chemotherapeutic agents, such as the protein tyrosine kinase inhibitor imatinib mesylate[49] and EGFR inhibitors,[48] to suppress tumour growth. In addition, OSU-03012 could sensitise tumour cells to radiation.[36] Pretreatment with OSU-03012 may lower stereotactic radiation doses to levels that minimise toxicity, preserve cranial nerve function and decrease long-term risk for malignant transformation. Together, these results suggest that OSU-03012 has potential to become a novel treatment option for VS and malignant schwannomas.

Conflict of interest statement

None declared.

Acknowledgments

We sincerely thank Georgeta Mihai for volumetric MRI analysis, Lifeline of Ohio for nerve specimens, Chang laboratory members for discussion and suggestions throughout the study, and Sarah S. Burns for critical reading of the manuscript. This work was supported by grants from the National Institute of Deafness and Communicative Disorders (D.B.W./L.S.C.), the Department of Defense Neurofibromatosis Research Programme (L.S.C.), Children's Tumour Foundation (A.J.), The Triological Society (A.J./L.S.C./D.B.W.) and the National Cancer Institute-supported Ohio State University Comprehensive Cancer Center.

REFERENCES

1. Welling DB, Packer MD, Akhmametyeva EM, Chang LS. The biology and genetics of vestibular schwannomas. In: Bambakidis N, Megerian C, Spetzler R, editors. *Surgery of the cerebellopontine angle*. B.C. Dekker Inc.; 2008.
2. Rouleau GA, Merel P, Lutchman M, et al. Alteration in a new gene encoding a putative membrane-organising protein causes neurofibromatosis type 2. *Nature* 1993;**363**:515–21.
3. Trofatter JA, MacCollin MM, Rutter JL, et al. A novel Moesin-, Exrin-, Radixin-like gene is a candidate for the neurofibromatosis 2 tumor-suppressor. *Cell* 1993;**72**:791–800.
4. Welling DB, Lasak JM, Akhmametyeva E, Ghaheri B, Chang LS. CDNA microarray analysis of vestibular schwannomas. *Otol Neurotol* 2002;**23**:736–48.
5. Shin M, Ueki K, Kurita H, Kirino T. Malignant transformation of a vestibular schwannoma after gamma knife radiosurgery. *Lancet* 2002;**360**:309–10.
6. Miettinen M. *Diagnostic soft tissue pathology*. Washington (DC): Churchill Livingstone; 2003. p. 367–8.
7. Takenobu A, Okamoto H, Hirao J, et al. Malignant nerve sheath tumour of the jugular foramen followed by meningeal dissemination in a patient with neurofibromatosis. *Brain Tumor Pathol* 1993;**10**:125–30.
8. Mut M, Cataltepe O, Sovlemezoglu F, Akalan N, Ozgen T. Radiation-induced malignant triton tumor associated with severe spinal cord compression. Case report and review of the literature. *J Neurosurg* 2004;**100**(3):298–302.
9. Balasubramaniam A, Shannon P, Hodaie M, Laperriere N, Michaels H, Guha A. Glioblastoma multiforme after stereotactic radiotherapy for acoustic neuroma: case report and review of the literature. *Neuro-Oncol* 2007;**9**:447–53.
10. Chibbaro S, Herman P, Povlika M, George B. Malignant trigeminal schwannoma extending into the anterior skull base. *Acta Neurochir (Wien)* 2008;**150**:599–604.
11. Sordillo PP, Helson L, Hajdu SI, et al. Malignant schwannoma – clinical characteristics, survival, and response to therapy. *Cancer* 1981;**47**:2503–9.
12. Victoria L, McCulloch TM, Callaghan EJ, Bauman NM. Malignant triton tumor of the head and neck: a case report and review of the literature. *Head Neck* 1999;**21**:663–70.
13. McClatchey AI, Giovannini M. Membrane organization and tumorigenesis – the NF2 tumor suppressor, Merlin. *Genes Dev* 2005;**19**:2265–77.
14. Lasak JM, Welling DB, Akhmametyeva EM, Salloum M, Chang LS. Retinoblastoma-cyclin-dependent kinase pathway deregulation in vestibular schwannomas. *Laryngoscope* 2002;**112**:1555–61.
15. Hanemann CO, Bartelt-Kirbach B, Diebold R, Kampchen K, Langmesser S, Utermark T. Differential gene expression between human schwannoma and control Schwann cells. *Neuropathol Appl Neurobiol* 2006;**32**:605–14.
16. Manent J, Kaiser S, Lallemand D, et al. NF2 molecular dissection across species and models: evidence for activation of the PI3K/Akt pathway, a mechanism previously correlated with Schwann cell differentiation, survival and tumorigenesis. In: The Children's Tumor Foundation international neurofibromatosis consortium; 2006 [abstract P27].
17. Lee T, Jacob A, Packer M, Chen CS, Welling DB, Chang LS. OSU03012 and (S)-HDAC-42, two novel inhibitors of the PI3K/AKT pathway, are potential therapeutic agents for vestibular schwannomas. In: *The Children's Tumor Foundation-sponsored NF conference*; 2007 [abstract P36].
18. Tang X, Jang SW, Wang X, et al. Akt phosphorylation regulates the tumour-suppressor merlin through ubiquitination and degradation. *Nat Cell Biol* 2007;**9**:1199–207.
19. Jacob A, Lee TX, Neff BA, Miller S, Welling B, Chang LS. Phosphatidylinositol 3-kinase/AKT pathway activation in human vestibular schwannoma. *Otol Neurotol* 2008;**29**:58–68.
20. LoPiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat* 2008;**11**:32–50.

21. Zhu J, Huang JW, Tseng PH, et al. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. *Cancer Res* 2004;**64**:4309–18.
22. Johnson AJ, Smith LL, Zhu J, et al. A novel celecoxib derivative, OSU03012, induces cytotoxicity in primary CLL cells and transformed B-cell lymphoma cell line via a caspase- and Bcl-2-independent mechanism. *Blood* 2005;**105**:2504–9.
23. Tong Z, Wu X, Chen CS, Kehrer JP. Cytotoxicity of a non-cyclooxygenase-2 inhibitory derivative of celecoxib in non-small-cell lung cancer A549 cells. *Lung cancer* 2006;**52**:117–24.
24. Porchia LM, Guerra M, Wang YC, et al. OSU-03012, A celecoxib derivative, directly targets p21 activated kinase. *Mol Pharmacol* 2007;**72**:1124–31.
25. Zhang S, Suvannasankha A, Crean CD, et al. OSU-03012, a novel celecoxib derivative, is cytotoxic to myeloma cells and acts through multiple mechanisms. *Clin Cancer Res* 2007;**13**:4750–8.
26. Morrissey TK, Kleitman N, Bunge RP. Isolation and functional characterization of Schwann cells derived from adult peripheral nerve. *J Neurosci* 1991;**11**:2433–42.
27. Giovannini M, Robanus-Maandag E, van der Valk M, et al. Conditional biallelic *Nf2* mutation in the mouse promotes manifestations of human neurofibromatosis type 2. *Genes Dev* 2000;**14**:1617–30.
28. Kerkhoff E, Rapp UR. Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. *Mol Cell Biol* 1997;**17**:2576–86.
29. Chang LS, Akhmametyeva EM, Wu Y, Zhu L, Welling DB. Multiple transcription initiation sites, alternative splicing, and differential polyadenylation contribute to the complexity of human neurofibromatosis 2 transcripts. *Genomics* 2002;**79**:63–76.
30. Chang LS, Jacob A, Lorenz M, et al. Growth of benign and malignant schwannoma xenografts in severe combined immunodeficiency mice. *Laryngoscope* 2006;**116**:2018–26.
31. Monje PV, Bartlett Bunge M, Wood PM. Cyclic AMP synergistically enhances neuregulin-dependent ERK and Akt activation and cell cycle progression in Schwann cells. *Glia* 2006;**53**:649–59.
32. Chan KK, Chen P, Xie X, Kulp SK, Chen CS, Covey JM. Poster 602 – pharmacokinetics of Akt inhibitor NSC728209 in the rat by LC/MS/MS method. *Eur J Cancer* 2006(Suppl. 4):182.
33. Dong LQ, Liu F. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. *Am J Physiol Endocrinol Metab* 2005;**289**:E187–196.
34. Nakamura K, Sakaue H, Nishizawa A, et al. PDK1 regulates cell proliferation and cell cycle progression through control of cyclin D_1 and $p27^{kip1}$ expression. *J Biol Chem* 2008;**283**:17702–11.
35. Ding H, Han C, Zhu J, Chen CS, D'Ambrosio SM. Celecoxib derivatives induce apoptosis via the disruption of mitochondrial membrane potential and activation of caspase 9. *Int J Cancer* 2005;**113**:803–10.
36. Yacoub A, Park MA, Hanna D, et al. OSU-03012 promotes caspase-independent but PERK-, cathepsin B-, BID-, and AIF-dependent killing of transformed cells. *Mol Pharmacol* 2006;**70**:589–603.
37. Rong R, Tang X, Gutmann DH, Ye K. Neurofibromatosis 2 (NF2) tumor suppressor merlin inhibits phosphatidylinositol 3-kinase through binding to PIKE-L. *Proc Natl Acad Sci USA* 2005;**101**:18200–5.
38. Ahuja D, Saenz-Robles MT, Pipas JM. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. *Oncogene* 2005;**24**:7729–45.
39. Shaw RJ, Paez JG, Curto M, et al. The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling. *Dev Cell* 2001;**1**:63–72.
40. Morrison H, Sperka T, Manent J, Giovannini M, Ponta H, Herrlich P. Merlin/neurofibromatosis type 2 suppresses growth by inhibiting the activation of Ras and Rac. *Cancer Res* 2007;**67**:520–7.
41. Kissil JL, Johnson KC, Eckman MS, Jacks T. Merlin phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization. *J Biol Chem* 2002;**277**:10394–9.
42. Xiao GH, Beeser A, Chernoff J, Testa JR. P21-activated kinase links Rac/Cdc42 signaling to merlin. *J Biol Chem* 2002;**277**:883–6.
43. Surace EI, Haipek CA, Gutmann DH. Effect of merlin phosphorylation on neurofibromatosis 2 (NF2) gene function. *Oncogene* 2004;**23**:580–7.
44. Hirokawa Y, Tikoo A, Huynh J, et al. A clue to the therapy of neurofibromatosis type 2: NF2/merlin is a PAK1 inhibitor. *Cancer J* 2004;**10**:20–6.
45. Cardone MH, Roy N, Stennicke HR, et al. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998;**282**:1318–21.
46. Gao M, Yeh PY, Lu YS, et al. OSU-03012, a novel celecoxib derivative, induces reactive oxygen species-related autophagy in hepatocellular carcinoma. *Cancer Res* 2008;**68**:9348–57.
47. Weng SC, Kashida Y, Kulp SK, et al. Sensitizing estrogen receptor-negative breast cancer cells to tamoxifen with OSU-03012, a novel celecoxib-derived phosphoinositide-dependent protein kinase-1/Akt signaling inhibitor. *Mol Cancer Ther* 2008;**7**:800–8.
48. Wang YC, Kulp SK, Wang D, et al. Targeting endoplasmic reticulum stress and Akt with OSU-03012 and gefitinib or erlotinib to overcome resistance to epidermal growth factor receptor inhibitors. *Cancer Res* 2008;**68**:2820–30.
49. Tseng PH, Lin HP, Zhu J, et al. Synergistic interactions between imatinib mesylate and the novel phosphoinositide-dependent kinase-1 inhibitor OSU-03012 in overcoming imatinib mesylate resistance. *Blood* 2005;**105**:4021–7.