Small-Molecule Inhibitors of Phosphatidylinositol 3-Kinase/Akt Signaling Inhibit Wnt/β-Catenin Pathway Cross-Talk and Suppress Medulloblastoma Growth

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Abstract

Activation of the β-catenin and receptor kinase pathways occurs often in medulloblastoma, the most common pediatric malignant brain tumor. In this study, we show that molecular cross-talk between the β-catenin and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways is crucial to sustain medulloblastoma pathophysiology. Constitutive activation of phosphoinositide-dependent protein kinase 1 (PDK1), Akt, and glycogen synthase kinase 3β (GSK-3β) was detected by immunohistochemistry in all primary medulloblastomas examined (n = 41). Small-molecule inhibitors targeting the PI3K/Akt signaling pathway affected β-catenin signaling by inhibition of GSK-3β activity, resulting in cytoplasmic retention of β-catenin and reduced expression of its target genes cyclin D1 and c-Myc. The PDK1 inhibitor OSU03012 induced mitochondrial-dependent apoptosis of medulloblastoma cells and enhanced the cytotoxic effects of chemotherapeutic drugs in a synergistic or additive manner. In vivo, OSU03012 inhibited the growth of established medulloblastoma xenograft tumors in a dose-dependent manner and augmented the antitumor effects of mammalian target of rapamycin inhibitor CCI-779. These findings demonstrate the importance of cross-talk between the PI3K/Akt and β-catenin pathways in medulloblastoma and rationalize the PI3K/Akt signaling pathway as a therapeutic target in treatment of this disease. Cancer Res; 70(1); 266-76. ©2010 AACR.

Introduction

Medulloblastoma, a primitive neuroectodermal tumor, is the most common malignant pediatric brain tumor. Medulloblastoma can originate from cerebellar granule neural precursor cells located in the external granule layer (1, 2) and typically develop in the cerebellum. Medulloblastoma cells retain many features that resemble the progenitor cells of the embryonic brain (2), and more than half of these tumors display abnormal activation of the Hedgehog or Wnt signaling pathways (3), supporting a model of embryonal tumorigenesis.

Wnt signaling plays a central role in modulating the balance between proliferation and differentiation of progenitor cells during embryonic central nervous system development (4). The canonical Wnt signaling pathway operates by stabilizing β-catenin. In the absence of Wnt/Wingless ligand activation, β-catenin is sequestered in the cytoplasm by a multiprotein complex, which encompasses the adenomatous polyposis coli protein, axin1, axin2/conductin, casein kinase 1, and glycogen synthase kinase-3β (GSK-3β). In this state, β-catenin is phosphorylated at the NH2-terminal serine and threonine residues by GSK-3β, which targets it for ubiquitination and proteolytic degradation (5). Activation of Wnt signaling by binding of Wnt ligands to a Frizzled receptor inhibits the formation of the multiprotein complex and GSK-3β–mediated phosphorylation of β-catenin, resulting in an accumulation of hypophosphorylated β-catenin in the cytosol. Stabilized hypophosphorylated β-catenin eventually translocates to the nucleus where it interacts with members of the T-cell factor/lymphoid enhancer binding factor (TCF/LEF) family of transcription factors, leading to modulated expression of a broad range of genes, such as MYC and CCND1 (6, 7).

Proteins regulating signaling through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway are frequently altered in human cancer, including medulloblastoma (8, 9). Activated Akt significantly augments Sonic-Hedgehog–induced medulloblastoma formation in mice (10). Activation of the PI3K/Akt signaling pathway is important for proliferation of human medulloblastoma cells and cancer stem cells residing in the perivascular niche following irradiation (11, 12). Moreover, medulloblastomas frequently display reduced expression of the Akt inhibitor PTEN (the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10) caused by promoter hypermethylation.
and allelic losses on chromosome region 10q23 where PTEN is localized (8, 13).

PI3Ks are key components for the activation of Akt signaling by catalyzing the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) from phosphatidylinositol-4,5-bisphosphate (PIP₂). PIP₃ translocates Akt and phosphoinositide-dependent protein kinase 1 (PKD1) from the cytoplasm to the inner cytoplasmic membrane where Akt is activated through phosphorylation by PKD1. This process is attenuated by PTEN, which removes the 3′-phosphate group of PIP₃ resulting in the regeneration of PIP₂ and decreased Akt activity (8). Activated Akt phosphorylates a plethora of downstream substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth (10).

In this study, we evaluated the importance of PI3K/Akt signaling in medulloblastomas using small-molecule inhibitors and show that molecular cross-talk between PI3K/Akt and Wnt/β-catenin signaling is significant in medulloblastoma tumorigenesis.

**Materials and Methods**

**Tumor material and patient characteristics.** Human tumor tissue samples were collected at the Karolinska University Hospital, Sweden, between 1994 and 2005. Ethical approval was obtained by the Karolinska University Hospital Research Ethics Committee. Tumor and patient characteristics are summarized in Supplementary Table S1.

**Immunohistochemistry and immunofluorescence.** Detection of phospho-PDK1Ser241 (Abcam), phospho-AktSer473, phospho-AktThr308, phospho-GSK-3βSer9, and β-catenin in primary tumor samples was performed using the corresponding specific antibodies (Cell Signaling). Sections from xenograft tumors were incubated with the primary antibodies detecting Ki-67 (NeoMarkers) or active caspase-3 (R&D Systems). As a secondary antibody, the horseradish peroxidase (HRP) SuperPicture Polymer detection kit and enhancer in TNEN buffer [50 mmol/L Tris-HCl (pH 7.8), 2 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L PMSF] or hypotonic lysis buffer [10 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 0.3% NP40, 1 mmol/L EDTA, 0.1 mmol/L PMSF], respectively. Total cell protein lysates were extracted from cells in radioimmunoprecipitation assay buffer [25 mmol/L Tris (pH 7.8), 2 mmol/L EDTA, 20% glycerol, 0.1% NP40, 1 mmol/L DTT]. Frozen tumors were disrupted with a rotor-stator homogenizer in TNEN buffer [50 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl, 0.1% NP40, 5 mmol/L EDTA] and subjected to five freeze-thaw cycles (liquid nitrogen, 37°C, before clearing by centrifugation). All protein extraction buffers were supplemented with MiniComplete protease inhibitor cocktail (Roche Diagnostic), 1 mmol/L NaF, and 1 mmol/L Na₃VO₄.

Proteins were immunoprecipitated using axin1 antibodies (Cell Signaling) coupled to agarose A beads followed by the manufacturer’s instructions (Sigma-Aldrich). Immunoblotting was performed using antibodies detecting phospho-PDK1Ser241, phospho-AktSer473, phospho-AktThr308, phospho-S6K1Thr389, phospho-4E-BP1Ser75, phospho-GSK-3βSer9, phospho-β-cateninSer33/37/Thr41, Akt, β-catenin, caspase-9, caspase-3, poly(ADP-ribose) polymerase (PARP), the BH3-interacting domain death agonist (BID), cyclin D1, c-Myc (all from Cell Signaling), and β-actin (Sigma-Aldrich). Anti-mouse IgG and anti-rabbit IgG, conjugated to horseradish peroxidase (HRP) SuperSignal (Pierce) was used for chemiluminescence detection. Detection was performed using secondary biotinylated antibody and FITC-conjugated streptavidin (Zymed Laboratories).

**Chemicals.** 2-Amino-N(4,5-(2-phenthenyl)-3-(trifluoromethyl)-1H-pyrrozol-1-yl)phenyl)aceticamide (OSU03012), LY294002 (Cell Signaling), SB-216763, doxorubicin and vinorelbine (Sigma-Aldrich), and CHIR99021 (Stemgent) were dissolved in DMSO (Sigma-Aldrich). Rapamycin (sirolimus, LC Laboratories) and CCI-779 (temsirolimus, Wyeth) were dissolved in DMSO (Sigma-Aldrich). Sodium nitroprusside dihydrate (SNP; Sigma-Aldrich) and CCI-779 (temsirolimus, Wyeth) were dissolved in 99.5% ethanol. Temozolomide and cyclophosphamide were dissolved in DMSO (Sigma-Aldrich). cisplatin (Sigma-Aldrich), and CHIR99021 (Stemgent) were dissolved in DMSO (Sigma-Aldrich). LY294002 (Cell Signaling), SB-216763, doxorubicin and vinorelbine (Sigma-Aldrich), and CHIR99021 (Stemgent) were dissolved in DMSO (Sigma-Aldrich) and diluted in PBS to the appropriate stock concentrations. Sodium nitroprusside dihydrate (SNP; Sigma-Aldrich) was dissolved in distilled water. All inhibitors were further diluted in Opti-MEM (Life Technologies) to the desired in vitro concentration. For in vivo use of CCI-779, the stock was diluted in 5% polyethylene glycol 400 (Sigma-Aldrich), 5% Tween 20 (Bio-Rad), and 0.9% sterile saline, whereas OSU03012 for oral administration was prepared as a suspension in a vehicle fluid consisting of 0.5% (w/v) methylcellulose (Sigma-Aldrich) and 0.1% (v/v) Tween 80 (Sigma-Aldrich) in sterile water.

**Cell lines and viability assays.** Origin, culturing conditions, and viability assays of human medulloblastoma and supratentorial primitive neuroectodermal (sPNET) cell lines were as described (14).

**Fluorescence-activated cell sorting analysis.** DNA content was assessed essentially as described (15). Briefly, D283 MED, D324 MED, D458 MED, UFC228-3, and MEB-MED-8A cells were treated with 3 μmol/L OSU03012 for 24 and 48 h; stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich); and subjected to cell cycle analysis using single-parameter DNA flow cytometry. The mitochondrial transmembrane potential was assessed in D283 MED, D324 MED, D458 MED, UFC228-3, and MEB-MED-8A cells after 24-h incubation with 3 μmol/L OSU03012 using tetramethylrhodamine ethyl ester (Molecular Probes) as described (16).

**Protein fractionation, immunoprecipitation, and immunoblotting.** Fractionated nuclear and cytosolic protein lysates were obtained using nuclear extraction buffer [20 mmol/L Tris-HCl (pH 7.5), 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 25% glycerol, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF)] or hypotonic lysis buffer [10 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 0.3% NP40, 1 mmol/L EDTA, 0.1 mmol/L DTT, 0.1 mmol/L PMSF], respectively. Total cell protein lysates were extracted from cells in radioimmunoprecipitation assay buffer [25 mmol/L Tris (pH 7.8), 2 mmol/L EDTA, 20% glycerol, 0.1% NP40, 1 mmol/L DTT]. Frozen tumors were disrupted with a rotor-stator homogenizer in TNEN buffer [50 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl, 0.1% NP40, 5 mmol/L EDTA] and subjected to five freeze-thaw cycles (liquid nitrogen, 37°C, before clearing by centrifugation). All protein extraction buffers were supplemented with MiniComplete protease inhibitor cocktail (Roche Diagnostic), 1 mmol/L NaF, and 1 mmol/L Na₃VO₄.

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**Transfection, small interfering RNA knockdown, and luciferase reporter gene assays.** Cells were transfected with...
were approved by the regional ethics committee for animal chemotherapeutic drugs (SA Biosciences) and/or the full-length cDNA of β-catenin (pSPORT3-β-cat; Open Biosystems) using Lipofectamine 2000 (Invitrogen) for 48 h. Cells were then treated for 6 h with 3 μmol/L OSU3012, and reporter gene activity was assessed using a Dual-Luciferase Reporter Assay System (Promega Biotech AB). GSK-3β knockdown was achieved using the SignalSilence GSK-3α/β kit (Cell Signaling) according to the manufacturer’s instructions.

**Xenografts and in vivo administration of OSU3012 and CCI-779.** Four- to 6-wk-old female NMRI nu/nu mice were s.c. injected with 7 × 10⁶ human D283 MED medulloblastoma cells. Mice were randomly assigned into six treatment groups (eight mice in each treatment group) and the drugs were given in daily doses as follows: (a) 7 mg/kg OSU3012 or (b) 70 mg/kg OSU3012 orally via a gastric feeding tube, (c) 20 mg/kg CCI-779 i.p., (d) 7 mg/kg OSU3012 and 20 mg/kg CCI-779 i.p., (e) 70 mg/kg OSU3012 and 20 mg/kg CCI-779 i.p., or (f) no treatment. Each mouse was treated for 8 d and treatment was started on the appearance of palpable tumors (mean, 0.13 mL). All animal experiments were approved by the regional committee for animal research (N234-05) in accordance with national regulations (SFS 1988:534, SFS 1988:539, and SFS 1988:541).

**Statistical analysis.** Calculation of EC₅₀ values and testing for synergistic or additive effects of combination therapy were performed as previously described (15, 17). Mann-Whitney U test and the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn’s multiple comparison test were used for analysis of statistical differences between two and several independent populations, respectively. All statistical tests were two-sided.

**Results**

**Key molecules in the PI3K/Akt signaling pathway are constitutively activated in primary medulloblastomas.** By immunohistochemical analysis of primary human medulloblastoma and sPNET tumor samples (Supplementary Table S1), specific phosphorylation of PDK1Ser241, AktThr308, and AktSer473 was detected in the cytoplasm of all primary tumor samples (Fig. 1). Interestingly, GSK-3β was also phosphorylated Ser9 in all primary tumor samples analyzed (Fig. 1), whereas nuclear accumulation of β-catenin was detected in 27% of the cases. Thirty-one percent of the samples had mixed cytoplasmic/nuclear β-catenin staining (Fig. 1; Supplementary Table S1).

**Inhibition of PDK1 using the small-molecule inhibitor OSU3012 potently suppresses medulloblastoma cell proliferation and clonogenic capacity.** OSU3012, a third-generation celecoxib derivative, has been shown to affect PI3K/Akt signal transduction through inhibition of PDK1 phosphorylation (18). As shown in Fig. 2A, treatment of medulloblastoma cells with 3 μmol/L OSU3012 resulted in reduced phosphorylation of PDK1Ser241, AktThr308, and AktSer473. All medulloblastoma cell lines showed a concentration-dependent decrease in cell viability (Fig. 2B) and significant dose-dependent inhibition of colony formation (P < 0.001; Fig. 2C) after 48-hour exposure of OSU3012. Concentrations associated with 50% decrease in cell viability (biological EC₅₀) ranged from 1.13 to 7.23 μmol/L (mean, 2.47 μmol/L; Supplementary Table S2).

**OSU3012 induces S-phase arrest followed by apoptosis in medulloblastoma cells.** Cell cycle analysis revealed a pronounced S-phase arrest that was evident after both 24-hour and 48-hour incubation with 3 μmol/L OSU3012 in D283 MED and D324 MED cells (see also Supplementary Fig. S1A–E). We also observed an accumulation of cells with a hypodiploid DNA content (sub-G₁). This was most pronounced in MEB-MED-8A, D458 MED, and PFSK-1 cells incubated with 3 μmol/L OSU3012 (Supplementary Fig. S1C–E).

Depolarization of the mitochondrial membrane potential was detected in all medulloblastoma cell lines on treatment with OSU3012 (Supplementary Fig. S2A–E). Western blotting confirmed activation of caspase-9, caspase-3, and PARP, whereas no activation of BID was observed in any of the five medulloblastoma cell lines investigated (Fig. 2D).

**OSU3012 significantly augments the cytotoxic effect of chemotherapeutic drugs in vitro.** To further investigate OSU3012 as a potential drug in the treatment of medulloblastoma, we studied the effects of OSU3012 in combination with mammalian target of rapamycin (mTOR) inhibitors or conventional chemotherapeutic drugs. D324 MED and D283 MED cells were treated with increasing concentrations of OSU3012, mTOR inhibitors, and chemotherapeutic drugs. The activities of the individual cytostatic drugs were determined in initial experiments (data not shown). Fixed concentration ratios of the drugs were used with serial dilutions for combination and single-drug treatments. The results are summarized in Table 1, which shows the combination index (CI) at IC₅₀. OSU3012 induced a synergistic or additive effect in D324 MED and D283 MED cells when used in combination with CCI-779 (IC₅₀, 0.57–0.80; 0.78–0.94), rapamycin (IC₅₀, 0.57–0.83; 0.88–0.92), cyclophosphamide (IC₅₀, 0.45–0.62; 0.56–0.99), or doxorubicin (IC₅₀, 0.97–1.12; 0.75–1.00). In contrast, OSU3012 in combination with vincristine had an additive effect in D283 MED cells (IC₅₀, 0.98–1.20), whereas an antagonistic effect was observed in D324 MED cells (IC₅₀, 1.01–1.35). OSU3012 in combination with temozolomide had an antagonistic effect in both cell lines at the doses used (IC₅₀, 1.18–1.38; 1.07–1.30).

Furthermore, a combination of OSU3012 and the mTOR inhibitor CCI-779 significantly augmented inhibition of the clonogenic capacity of D324 MED, PFSK-1, and UW228-3 cells compared with single-drug treatment (P < 0.001; all cell lines; Supplementary Fig. S3).

**Effects of OSU3012 on Akt downstream proteins in medulloblastoma cells.** The cell viability assays showed a synergistic induction of cell cytotoxicity when medulloblastoma cells were treated with a combination of OSU3012 and the mTOR inhibitors rapamycin or CCI-779 (Table 1). However, increased levels of AktSer473 phosphorylation were observed in both D324 MED and D283 MED cells treated with CCI-779. This activation of Akt was inhibited with concomitant treatment of the cells with OSU3012 (Fig. 3A). Both OSU3012 and CCI-779...
reduced the phosphorylation of the mTOR downstream targets S6K1 and 4E-BP1 (Fig. 3B).

**OSU03012 has profound effects on the growth of established medulloblastoma xenografts.** Athymic mice carrying established D283 MED xenografts were treated with either 7 or 70 mg/kg of OSU03012 by daily oral gavages. Tumor growth was significantly inhibited after treatment for 2 days with 70 mg/kg/d OSU03012 (P < 0.005) or 4 days with 7 mg/kg/d OSU03012 (P < 0.05) compared with untreated controls (Fig. 4A–C). At the end of treatment, tumor volumes were reduced by 66% or 40% in mice receiving 70 or 7 mg/kg/d, respectively, compared with controls. Significant elevated activated caspase-3 (P < 0.0001) expression and reduced expression of the proliferation marker Ki-67 (P < 0.0001) were detected in tumors from mice treated with OSU03012 (Fig. 4D).

**OSU03012 augments the effect of the mTOR inhibitor CCI-779 on medulloblastoma growth in vivo.** Because both OSU03012 and CCI-779 inhibit components in the PI3K/Akt/mTOR signaling pathway and had synergistic cytotoxic effects on medulloblastoma cells in vitro (Table 1), we examined the in vivo activity of these drugs in combination. CCI-779 significantly inhibited the growth of established medulloblastoma tumors from day 2 of treatment (P < 0.05), and the tumor volume was reduced by 65% compared with controls at the end of treatment (Supplementary Fig. S4A–C). However, CCI-779 in combination with 7 or 70 mg/kg/d of OSU03012 reduced the tumor growth by 73% or 75%, respectively, compared with controls (Supplementary Fig. S4A and B).

**PDK1 inhibition by OSU03012 downregulates cyclin D1 and c-Myc protein expression in medulloblastoma via activation of GSK-3β and inhibition of β-catenin function.** Incubation of medulloblastoma cells with OSU03012 or the PI3K inhibitor LY294002 inhibited phosphorylation of GSK-3βSer9 (Fig. 5A; Supplementary Fig. S7). Subcellular protein fractionation and immunofluorescence cytology showed that medulloblastoma cell lines contained significant levels of β-catenin in the nucleus that was reduced in a dose-dependent manner on treatment with increasing concentrations of OSU03012 (Fig. 5B). OSU03012 induced β-catenin phosphorylation and reduced the protein levels of c-Myc and cyclin D1 (Fig. 5A). Similarly, treatment of medulloblastoma cells with the PI3K inhibitor LY294002 also resulted in reduced expression of cyclin D1 and c-Myc, whereas concomitant treatment of medulloblastoma cells with OSU03012 and the mTOR inhibitor CCI-779 resulted in an almost complete suppression of cyclin D1 and c-Myc expression (Supplementary Figs. S6 and S7). OSU03012 significantly inhibited β-catenin–TCF/LEF transactivation in D324 MED and D283 MED cells (P < 0.001; Fig. 5C). Forced overexpression of β-catenin by cotransfection with a β-catenin expression plasmid (pSPORTβ-cat) was also inhibited by OSU03012 (Fig. 5C).
To analyze the involvement of GSK-3β in the potential cross-talk between PI3K/Akt signaling and nuclear translocation of β-catenin in medulloblastoma, we treated D283 MED, D324 MED, and MEB-MED-8A (data not shown) cells with GSK-3β inhibitors or activators in combination with OSU03012. As shown in Fig. 5D, the GSK-3β inhibitors CHIR99021 and SB-216763 significantly rescued medulloblastoma cells from the toxic effects of OSU03012 (P < 0.001.

Figure 2. Inhibition of PDK1 signal transduction impairs the growth of medulloblastoma cells and induces apoptosis. A, OSU03012 reduces phosphorylation of PDK1Ser241, AktThr308, and AktSer473 in medulloblastoma cells. D324 MED, PFSK-1, D283 MED, and MEB-MED-8A were incubated with 3 μmol/L OSU03012 for 0.5, 6, or 24 h, and protein extracts were subjected to Western blot analysis to detect phosphorylated PDK1Ser241, AktThr308, AktSer473, and total Akt. β-Actin was used as a control for equal loading of samples. B, OSU03012 inhibits the growth of medulloblastoma cells. Medulloblastoma cells were treated with increasing concentrations of OSU03012 for 48 h, and cell survival was measured using an MTT assay. C, OSU03012 impairs the clonogenic capacity of medulloblastoma cells. D324 MED, PFSK-1, and UW228-3 cells were treated with 10, 100, or 500 nmol/L of OSU03012 for 48 h. Cells were then incubated in drug-free medium for 10 d and colonies (>75 cells) with 50% plate efficiency were counted. D, medulloblastoma cells were treated with 3 μmol/L OSU03012 for 6 and 24 h, and protein extracts were subjected to Western blotting using antibodies detecting full-length and cleaved caspase-9, caspase-3, PARP, and BID. β-Actin was used to ensure equal protein loading.
Discussion

Recent studies indicate that numerous components of the PI3K/Akt signaling pathway are frequent targets for amplification, translocations, and mutations in cancer, resulting in activation of the pathway (8). In medulloblastoma, activation of PI3K/Akt signaling seems to be a frequent event because the majority of primary medulloblastomas exhibit phosphorylation of AktSer473 independent of histologic staging (9, 19, 20). In this study, we show that PDK1, a protein regulating the phosphorylation, and thereby Akt are constitutively activated in primary medulloblastomas (Fig. 1). Moreover, GSK-3β, a protein that is constitutively active in resting cells (21), was found to be functionally inactivated by phosphorylation in all of the primary medulloblastomas analyzed (Fig. 1). Similarly, phosphorylation of GSK-3βSer9 was detected by Western blot in three of six primary medulloblastomas. However, no increased phosphorylation of PDK1 and only weak activation of Akt were detected in these samples (22).

Medulloblastoma cells exhibit increased expression of several growth factor receptors that may transmit their signals through the PI3K/Akt signaling pathway, such as the insulin-like growth factor-I (IGF-I) receptor (23), platelet-derived growth factor B (24, 25), tyrosine receptor kinase B (26), the chemokine receptor (CXCR4; ref. 27), the epidermal growth factor (EGF) receptor (28), and c-KIT (29). The importance of PI3K/Akt signaling in cancer cells has resulted in an intense search for agents that specifically inhibit key components of this pathway. Several of these compounds show promising preclinical antitumor effects, and a few of them are already in clinical trials (30). In contrast to PI3Ks or Akt for which several subunits or isoforms exist, only one single isoform of PDK1 has been reported in humans. PDK1 has high specificity for phosphorylation and activation of all three Akt isoforms (PKα1/Akt1, PKβ1/Akt2, and PKγ/Akt3). PDK1 hypomorphic mice display no deleterious phenotype, and the formation of PTEN-deficient tumors is abrogated when PDK1 hypomorphic mice are crossed with PTEN heterozygote mice. Taken together, this makes PDK1 an attractive candidate as an anticancer drug target (30, 31). Celecoxib, a drug designed to specifically inhibit the activity of cyclooxygenase-2, has shown promising effects as an anticancer agent. One of the most prominent off-target effects of celecoxib is the specific inhibition of PDK1 activity possibly by competing with ATP for binding to PDK1 (32). OSU03012 was isolated by structure-based optimization of celecoxib for compounds lacking cyclooxygenase-2 activity but retaining PDK1-inhibiting activity (18). We have recently shown that celecoxib inhibits medulloblastoma growth both in vitro and in vivo (14).

We therefore investigated the effects of OSU03012 on medulloblastoma cells both at the molecular level and as a potential future drug in the treatment of this disease. We observed that OSU03012 potently modulated the effect of key molecules in the PI3K/Akt signaling pathway (Fig. 2A). Cytotoxic effects of OSU03012 on a panel of medulloblastoma cell lines revealed that OSU03012 was significantly more potent compared with

**Table 1. Effect of OSU03012 in combination with chemotherapeutic drugs and mTOR inhibitors in medulloblastoma cells**

<table>
<thead>
<tr>
<th>Combination</th>
<th>OSU03012 + CCI-779</th>
<th>OSU03012 + rapamycin</th>
<th>OSU03012 + vincristine</th>
<th>OSU03012 + doxorubicin</th>
<th>OSU03012 + temozolomide</th>
<th>OSU03012 + cyclophosphamide</th>
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<tr>
<td><strong>D324 MED</strong></td>
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<tr>
<td>Mean CI at IC₅₀ (95% CI)</td>
<td>0.69 (0.57–0.80)</td>
<td>0.70 (0.57–0.83)</td>
<td>1.21 (1.01–1.35)</td>
<td>1.05 (0.97–1.12)</td>
<td>1.28 (1.18–1.38)</td>
<td>0.53 (0.45–0.62)</td>
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<td>Effect</td>
<td>Synergistic</td>
<td>Synergistic</td>
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<td><strong>D283 MED</strong></td>
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<tr>
<td>Mean CI at IC₅₀ (95% CI)</td>
<td>0.86 (0.78–0.94)</td>
<td>0.90 (0.88–0.92)</td>
<td>1.09 (0.98–1.20)</td>
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<td>1.15 (1.07–1.30)</td>
<td>0.78 (0.56–0.99)</td>
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<td>Effect</td>
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<td>Additive</td>
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CHIR99021; P < 0.001, SB-216763), whereas the GSK-3β activator SNP significantly increased the toxic effects of OSU03012 (P < 0.01). CHIR99021 and SB-216763 increased GSK-3βSer9 phosphorylation and c-Myc protein expression in OSU03012-treated medulloblastoma cells. SNP treatment resulted in decreased GSK-3βSer9 phosphorylation and reduced c-Myc expression (Supplementary Fig. S5). Moreover, OSU03012 treatment of medulloblastoma cells resulted in increased binding of GSK-3β to axin1 compared with nontreated cells (Supplementary Fig. S5B). Similar small interfering RNA (siRNA) knockdown of GSK-3β inhibited the cytotoxic effect of OSU03012 and increased c-Myc expression in D324 MED and D283 MED cells (Fig. 5D; Supplementary Fig. S5C).

Finally, decreased phosphorylation of PDK1Ser241 and GSK-3βSer9 and decreased levels of β-catenin, cyclin D1, and c-Myc proteins were observed in D283 MED xenografts treated with OSU03012 (Supplementary Fig. S8).

Medulloblastoma cells exhibit increased expression of several growth factor receptors that may transmit their signals through the PI3K/Akt signaling pathway, such as the insulin-like growth factor-I (IGF-I) receptor (23), platelet-derived growth factor B (24, 25), tyrosine receptor kinase B (26), the chemokine receptor (CXCR4; ref. 27), the epidermal growth factor (EGF) receptor (28), and c-KIT (29). The importance of PI3K/Akt signaling in cancer cells has resulted in an intense search for agents that specifically inhibit key components of this pathway. Several of these compounds show promising preclinical antitumor effects, and a few of them are already in clinical trials (30). In contrast to PI3Ks or Akt for which several subunits or isoforms exist, only one single isoform of PDK1 has been reported in humans. PDK1 has high specificity for phosphorylation and activation of all three Akt isoforms (PKα1/Akt1, PKβ1/Akt2, and PKγ/Akt3). PDK1 hypomorphic mice display no deleterious phenotype, and the formation of PTEN-deficient tumors is abrogated when PDK1 hypomorphic mice are crossed with PTEN heterozygote mice. Taken together, this makes PDK1 an attractive candidate as an anticancer drug target (30, 31). Celecoxib, a drug designed to specifically inhibit the activity of cyclooxygenase-2, has shown promising effects as an anticancer agent. One of the most prominent off-target effects of celecoxib is the specific inhibition of PDK1 activity possibly by competing with ATP for binding to PDK1 (32). OSU03012 was isolated by structure-based optimization of celecoxib for compounds lacking cyclooxygenase-2 activity but retaining PDK1-inhibiting activity (18). We have recently shown that celecoxib inhibits medulloblastoma growth both in vitro and in vivo (14).

We therefore investigated the effects of OSU03012 on medulloblastoma cells both at the molecular level and as a potential future drug in the treatment of this disease. We observed that OSU03012 potently modulated the effect of key molecules in the PI3K/Akt signaling pathway (Fig. 2A). Cytotoxic effects of OSU03012 on a panel of medulloblastoma cell lines revealed that OSU03012 was significantly more potent compared with
the nonsteroidal anti-inflammatory drugs celecoxib or diclofenac in inducing cytotoxicity of medulloblastoma cells (Fig. 2B and C; Supplementary Table S2; ref. 14). The growth-inhibitory effect of OSU03012 was associated with induction of mitochondrial-dependent apoptosis of medulloblastoma cells (Fig. 2D; Supplementary Fig. S2). Cell cycle analysis showed an S-phase arrest in D283 MED and D324 MED cells, whereas no evident S-phase arrest was observed for MEB-MED-8A, D458 MED, or PFSK-1 cells treated with OSU03012. The observed S-phase arrest may be due to OSU03012-mediated down-regulation of G1-S phase–specific cyclin D1 expression (Fig. 5A). Others have reported a combination of caspase-dependent and caspase-independent mechanisms for OSU03012-mediated induction of tumor cell death (33–35). Recently, OSU03012 was reported to induce autophagy through accumulation of reactive oxygen species (ROS) in hepatocellular carcinoma (36). This is particularly interesting because Akt, in contrast to its ability to inhibit apoptosis induced by chemotherapeutic agents, is not able to inhibit ROS-mediated apoptosis (37).

Our results show that OSU03012 augmented the cytotoxic effects of conventional chemotherapeutic drugs in a synergistic or additive manner (Table 1). Similarly, inhibition of the catalytic PI3K p110α isoform enhances the effect of doxorubicin in medulloblastoma cells (38). Because the mTOR inhibitor CCI-779 has been shown to have prominent effects on preclinical medulloblastoma models and is currently undergoing clinical testing in medulloblastoma patients (39, 40), we focused on this agent in more detail. A potential dilemma when designing anticancer therapies using agents that inhibit mTOR is that mTOR inhibitors have been shown to activate Akt through a feedback loop (15). However, our data show that a combination of OSU03012 and CCI-779 inhibits Akt and acts synergistically to induce medulloblastoma cell cytotoxicity in vitro (Table 1). Therefore, treatment with agents that simultaneously target key components in the PI3K/Akt/mTOR pathway may prove to be more efficacious.

Nuclear transactivation and activity of β-catenin has been directly related to signaling through the Wnt pathway, and this coordinates a diverse array of developmental processes, including the proliferation and fate of neural progenitor cells (5). Wnt signaling has emerged as an important step in human oncogenesis, and ~15% of sporadic medulloblastomas contain mutations in proteins of the Wnt pathway (41–43). In contrast, ~25% of children with medulloblastoma have nucleopositive β-catenin (44). Clearly, there must be other mechanisms involved in regulating β-catenin activation. Indeed, it has been shown that β-catenin–mediated transactivation of TCF/LEF can be activated by growth factors such as EGF, hepatocyte growth factor, IGF-I, IGF-II, and insulin (45–48). We found that treatment of medulloblastoma cells with OSU03012 diminished the amount of nuclear β-catenin and induced β-catenin phosphorylation of NH2-terminal serine and threonine residues followed by decreased TCF/LEF transactivation. Treatment also reduced the expression of cyclin D1 and c-Myc (Fig. 5), two proteins that are important in medulloblastoma tumorigenesis (49). Inhibition of nuclear β-catenin translocation is likely to be due to the observed OSU03012-mediated inhibition of Akt activity, leading to activation of GSK-3β function, because activated Akt has been shown to effectively suppress the role of GSK-3β in regulating the degradation of β-catenin (25, 50). This is supported by the findings that the GSK-3β activator SNP significantly increased the cytotoxic effects of OSU03012, whereas the GSK-3β inhibitors CHIR99021 and SB-216763 or siRNA knockdown of GSK-3β inhibited OSU03012-mediated medulloblastoma cell death (Fig. 5D). Attenuation of GSK-3β phosphorylation, growth, and survival has also been shown in medulloblastoma cells treated with the IGF-I receptor inhibitor NVP-AEW541 (23). These results suggest that molecular cross-talk between the PI3K/Akt and the Wnt signaling pathways

Figure 3. PDK1 inhibition abolishes CCI-779–mediated increase of Akt phosphorylation and inactivates the function of S6K1 and 4E-BP1 in medulloblastoma cells. A and B, D324 MED and D283 MED cells were incubated with 3 μmol/L OSU03012, 6 μmol/L CCI-779, or a combination of 3 μmol/L OSU03012 and 6 μmol/L CCI-779. Protein extracts were isolated after 6- and 24-h incubation and subjected to Western blotting using antibodies to detect phosphorylated AktSer473, total Akt, phosphorylated S6K1Thr389, and phosphorylated 4E-BP1Ser65. β-Actin was used to ensure equal protein loading.
exists in medulloblastoma and that GSK-3β is the key enzyme bridging these pathways. Furthermore, we also show that OSU03012 inhibits the phosphorylation of S6K1 and 4E-BP1 (Fig. 3), two proteins located downstream in the PI3K/Akt/mTOR signaling pathway. Both proteins have important functions in regulating protein translation and thereby cell growth and proliferation (8).

**Figure 4.** OSU03012 impairs medulloblastoma growth in vivo. NMRI nu/nu mice engrafted with 7 × 10⁶ D283 MED cells s.c. were randomized to receive either 7 mg/kg/d (n = 8) or 70 mg/kg/d (n = 10) through gastric feeding for 8 d or no treatment (n = 10). A, comparison of tumor volume indices from mice treated with 7 mg/kg/d OSU03012 and untreated controls. Significant treatment effect was obtained after 4 d of treatment with OSU03012 (P < 0.05). B, comparison of tumor volumes from mice receiving 70 mg/kg/d OSU03012 and untreated controls. Significant treatment effect was achieved after 2 d of OSU03012 treatment (P < 0.005). C, individual tumor growth in mice receiving the different treatments. D, immunohistochemical staining of untreated xenograft tumors (top row) or xenografts from mice treated with 70 mg/kg/d OSU03012 (middle row). Proliferation was detected by Ki-67 (left; ×400 magnification) and apoptosis by cleaved caspase-3 (right; ×400 magnification). Quantification (bottom row) of proliferation and apoptosis of xenografts from mice treated with 2 mg OSU03012. Bars, SD.
Because limited information about the in vivo antitumorogenic effects of OSU03012 is available, we investigated the effects of OSU03012 on established human medulloblastoma xenografts in nude mice. We found that OSU03012 effectively inhibited the growth of medulloblastoma in vivo in a dose-dependent manner (Fig. 4A–C). This was accompanied by reduced proliferation and increased caspase-3 activity in OSU03012-treated tumors
(Fig. 4D). OSU03012 also augmented the antitumor effect of the mTOR inhibitor CC1-779 (Supplementary Fig. S4A–C).

Taken together, our data show that molecular cross-talk between two of the major molecular mechanisms controlling embryonic development, the receptor tyrosine kinase PI3K/Akt signal transduction pathway and the canonical Wnt/β-catenin pathway, is important in medulloblastoma. We also show that small-molecule inhibitors targeting key components in the PI3K/Akt signaling pathway may represent an approach for the treatment of medulloblastoma and that the effect of OSU03012 should be further investigated in a clinical setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction: Small-Molecule Inhibitors of Phosphatidylinositol 3-Kinase/Akt Signaling Inhibit Wnt/β-Catenin Pathway Cross-Talk and Suppress Medulloblastoma Growth

In this article (Cancer Res 2010;70:266–76), which was published in the January 1, 2010 issue of Cancer Research (1), there are a number of errors. There are errors in the third and fourth sentences of the abstract; the sentences should read as follows:

"Constitutive activation of phosphoinositide-dependent protein kinase 1 (PDK1), Akt, and phosphorylation of glycogen synthase kinase 3β (GSK-3β) was detected by immunohistochemistry in all primary medulloblastomas examined (n = 41). Small-molecule inhibitors targeting the PI3K/Akt signaling pathway affected β-catenin signaling by activation of GSK-3β, resulting in cytoplasmic retention of β-catenin and reduced expression of its target genes cyclin D1 and c-Myc."

In the final sentence of the fourth paragraph of the Results section, “median” should appear instead of “mean”. In the paragraph "PDK1 inhibition by OSU03012 downregulates cyclin D1 and c-Myc protein expression in medulloblastoma via activation of GSK-3β and inhibition of β-catenin function” on page 269, the Supplementary Figure referenced should be S6, not S7.

The first two sentences of the Fig. 4 legend should read as follows:

"OSU03012 impairs medulloblastoma growth in vivo. NMRI nu/nu mice engrafted with 7 × 10^6 D283 MED cells s.c. were randomized to receive either 7 mg/kg/d (n = 8) or 70 mg/kg/d (n = 8) through gastric feeding for 8 d or no treatment (n = 8). A, comparison of tumor volume indexes from mice treated with 7 mg/kg/d OSU03012 and untreated controls."

The legend for Fig. 4D should read as follows:

"D, immunohistochemical staining of untreated xenograft tumors (left column) or xenografts from mice treated with 70 mg/kg/d OSU03012 (middle column). Proliferation was detected by Ki-67 (top row; ×400 magnification) and apoptosis by cleaved caspase-3 (bottom row; ×400 magnification). Quantification (right column) of proliferation and apoptosis of xenografts from mice treated with 2 mg OSU03012. Bars, SD."

Finally, there are errors in Figs. 3 and 5D; the corrected figures appear below.

---

**Figure 3.**

(A) Western blot analysis of D324 and D283 cells treated with OSU03012 or CCI-779 for 24 h. (B) Densitometry analysis of p-Akt, Akt, p-S6K1, p-4E-BP1, and β-actin.

Figure 5D.

<table>
<thead>
<tr>
<th>Condition</th>
<th>D324</th>
<th>D283</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 μM OSU</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 μM CHIR99021</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 μM SB-216763</td>
<td>-</td>
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</table>

* D324 cells were treated with 5 μM OSU/03012

Reference

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Small-Molecule Inhibitors of Phosphatidylinositol 3-Kinase/Akt Signaling Inhibit Wnt/ $\beta$-Catenin Pathway Cross-Talk and Suppress Medulloblastoma Growth

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