Inhibition of PI3K/AKT/mTOR Pathway Enhances Temozolomide-Induced Cytotoxicity in Pituitary Adenoma Cell Lines in Vitro and Xenografted Pituitary Adenoma in Female Nude Mice

Congxin Dai,* Bo Zhang,* Xiaohai Liu, Sihai Ma, Yakun Yang, Yong Yao, Ming Feng, Xinjie Bao, Guilin Li, Janxin Wang, Kai Guo, Wenbin Ma, Bing Xing, Wei Lian, Jianqi Xiao, Feng Cai, Hongbin Zhang, and Renzhi Wang

Department of Neurosurgery (C.D., X.L., S.M., Y.Yan., Y.Yao., M.F., X.B., G.L., J.W., W.M., B.X., W.L., J.X., F.C., R.W.), Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China; Department of Neurosurgery (B.Z., K.G.), the First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011, China; and Institute of Basic Medical Sciences (H.Z.), Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China

Invasive pituitary adenomas (PAs) are often refractory to standard therapy and salvage treatment with temozolomide (TMZ). Hyperactivation of the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway contributes to chemotherapy resistance in many cancers. XL765, a novel dual-PI3K/mTOR inhibitor, has recently shown its efficacy as a monotherapy and in combination with conventional therapeutics in many cancers. The hyperactive PI3K/AKT/mTOR pathway frequently occurs in invasive PAs. In this study, we investigated whether XL765 sensitizes PA cells to TMZ in vitro and in vivo. Experiments were carried out to evaluate the effect of XL765 and TMZ alone or in combination on cell proliferation and apoptosis of PA cell lines (αT3-1, GH3, and MMQ) in vitro as well as the tumor growth and serum GH and prolactin secretions in a GH3 xenograft tumor model of female nude mice. XL765 and TMZ synergistically inhibited the growth of PA cell lines and induced apoptosis. Combination of XL765 and TMZ synergistically inhibited tumor growth, decreased serum GH and prolactin levels, and reduced the sacrifice rate of GH3 xenograft tumor models without increased systemic side effects. In addition, XL765 in combination with TMZ dramatically decreased phosphorylation of AKT and mTOR as well as the expression of Bcl-2. The increased expression of cleaved poly (ADP-ribose) polymerase and Bcl-2-associated X protein along with elevated caspase-3/7 activity were also observed in the combination group. Therefore, dual inhibitors of PI3K and mTOR may enhance alkylating agent-mediated cytotoxicity and provide a novel regimen in the treatment of invasive PAs. **(*Endocrinology* 154: 1247–1259, 2013)**

Pituitary adenomas (PAs) account for approximately 15% of intracranial tumors and are the second most common intracranial neoplasm after gliomas (1). Although most PAs are benign and noninvasive tumors that show expansive growth to surrounding tissues or remain within the sella, invasive PAs do occur and exhibit biological behavior that is in between the characteristics of benign PAs and pituitary carcinomas (2). These tumors are

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.
Copyright © 2013 by The Endocrine Society
doi: 10.1210/en.2012-1908 Received August 31, 2012. Accepted January 8, 2013.
First Published Online February 5, 2013

* C.D. and B.Z. contributed equally to this work.

Abbreviations: Bax, Bcl-2-associated X protein; CCK, cell-counting kit; CI, combination index; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IHC, immunohistochemistry; IRMA, immunoradiometric assay; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; p, phosphorylated; PA, pituitary adenoma; PARP, poly(ADP-ribose) polymerase; PI3K, phosphoinositide 3-kinase; PRL, prolactin; TdT, terminal deoxynucleotidyl transferase; TMZ, temozolomide; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

frequently massive, rapidly growing, and invasive to their surroundings, making them difficult to resect completely (3). Despite multimodal therapeutic options, including repeated surgeries, radiotherapy, and alternative medical therapies, a large number of cases are refractory to these conventional therapies and tend to recur quickly after initial treatment (4). Therefore, these invasive PAs are difficult to manage and are associated with poor prognosis and can be fatal (5).

Temozolomide (TMZ) is a second-generation, orally administered alkylating agent that can readily cross the blood-brain barrier. TMZ is used to treat malignant gliomas and metastatic melanoma by disrupting gene transcription and inducing DNA damage through attachment of a methyl group to guanine bases (6). Furthermore, TMZ has recently been shown to have significant activity as a salvage therapy for treatment of invasive PAs and pituitary carcinomas (7, 8). However, not all invasive PAs showed response to TMZ, and some patients can acquire TMZ resistance after treatment (9–11). Therefore, although the mechanisms of innate and acquired resistance remain elusive, there is an urgent need to identify new therapeutic targets and develop new agents for these refractory PAs.

The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is constitutively activated in human tumors (12). This pathway plays a pivotal role in tumor formation and progression by controlling cell growth, proliferation, apoptosis, and metastasis (13). Hyperactivation of the PI3K/AKT/mTOR pathway also has been documented in PAs, particularly the invasive type, and may be involved in PA tumorigenesis (14-16). AKT activation also contributes to resistance of chemotherapy in various cancer types, and therefore, inhibitors of the PI3K/Akt pathway have been used as single agents and in combination with chemotherapy to overcome chemotherapeutic resistance (17).

XL765, a dual inhibitor of PI3K and mTOR, can potently and selectively inhibit class I PI3K isoforms and mTOR and is currently undergoing clinical evaluation in a variety of cancer types (18). Recently it has been reported that XL765 shows antitumor activity as a monotherapy and in combination with TMZ in a range of genetically diverse glioblastoma xenografts and models of pancreatic adenocarcinoma (19, 20). These findings raise the possibility that XL765 may inhibit tumor growth as a single agent in PAs and enhance the sensitivity of TMZ as well. Therefore, in this study we sought to determine whether XL765 in combination with TMZ has a synergistic effect on cell proliferation and apoptosis in PA cell lines and a PA GH3 xenograft tumor model.

Materials and Methods

Reagents

TMZ was purchased from Sigma-Aldrich (St Louis, Missouri). XL765 was purchased from Selleckchem (Houston, Texas). The cell counting kit (CCK)-8 was obtained from Dojindo Laboratories (Kumamoto, Japan). The DeadEnd fluorometric terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) system and the Caspase-Glo 3/7 assay systems were bought from Promega (Madison, Wisconsin). Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit was acquired from Biosea Biotechnology (Beijing, China). The cell death detection kit was purchased from Roche (Indianapolis, Indiana). Antibodies against phosphorylated (p) AKT (Ser473, no. 9271), pS6 [Ser235/236, rabbit monoclonal antibody (mAb), no. 4856], pmTOR (ser2448, rabbit mAb, no. 5538), total AKT (rabbit, no. 9272), total S6 (rabbit, no. 2217), total mTOR (rabbit mAb, no. 2938), poly (ADP-ribose) polymerase (PARP; rabbit mAb, no. 9532), Ki-67 (no. 2586), horseradish peroxidase (HRP)-conjugated goat antirabbit IgG and horse antimouse IgG were obtained from Cell Signaling Technology (Beverly, Massachusetts). Anti-β-actin primary antibody, Bcl-2-associated X protein (Bax), and Bcl-2 were purchased from Santa Cruz Biotechnology, (Santa Cruz, California).

Cell lines and culture conditions

The mouse gonadotroph adenoma cell line αT3-1, which is derived from a transgenic male mouse and maintains differentiated functions unique to gonadotropes (21), was kindly provided by Dr P. L. Mellon (Department of Reproductive Medicine, University of California, San Diego, California). The GH3, which is a GH- and prolactin (PRL)-secreting cell line derived from a female Wistar-Furth rat pituitary tumor (22); and the MMQ cell line, which secretes only PRL, was isolated from the 7315a tumor in a female rat (23, 24), were obtained from the Cell Resource Center of the Chinese Academy of Medical Science (Beijing, China). The αT3-1, GH3, and MMQ cells were grown in F-12K medium containing 15% horse serum, 2.5% fetal bovine serum, and 1% penicillin-streptomycin and incubated at 37°C in a humidified incubator with 5% CO_2. For initial plating of cells in experiments, manual cell counts were performed using a hematocytometer.

Animals

Athymic 6-week-old female nude mice were used for all in vivo experiments. Animal handling and procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Peking University. GH3 cells ($\sim 2 \times 10^6$ cells) were injected into the right flank of nude mice. When tumor volumes reached 30-50 mm^3, mice were randomly assigned to groups (5-10 animals per group) and then treated with various doses of TMZ alone (5, 25, or 100 mg/kg), XL765 alone (25, 50, or 100 mg/kg), a low-dose combination of TMZ (5 mg/kg, ip) and XL765 (25 mg/kg, orally) 5 times per week, or a high-dose combination of TMZ (25 mg/kg, ip) and XL765 (50 mg/kg, orally) 5 times per week. Tumor volumes were calculated using the following formula: (cubic millimeters) = (length $\times$ width^2) $\times$ 0.5. Mouse weights were recorded every 2 days. We initiated the treatment at day 8 after implantation. After 21 days

of treatments with vehicle [dimethyl sulfoxide (DMSO)], TMZ, XL765, or TMZ/XL765 in combination, the mice were killed, and then the tumor tissues and blood samples were collected for immunohistochemistry (IHC) and immunoradiometric assay (IRMA), respectively.

For evaluating the effect of TMZ, XL765 alone or TMZ/XL765 combination on the tumor burden and the relative survival of mice with GH3 xenografts, mice were observed up to 91 days after 21 days of the treatments. Mice were killed when the tumors reached a predetermined size of 3000 mm^3, when its body weight loss was greater than 20% as compared with the body weight on the first day of treatment, or its tumor became ulcerated, whichever came first, and this was considered the time to kill the animals (25).

Proliferation/viability study

The effect of TMZ, XL765, and TMZ/XL765 combination on cell viability of αT3-1, GH3, and MMQ in vitro was accessed using the CCK-8 cell viability assay. The αT3-1, GH3, and MMQ cells were centrifuged and resuspended in medium and then plated in 96-well plates at a concentration of 1×10^4 cells per 100 μL/well. The αT3-1 and GH3 cells were trypsinized before being centrifuged and resuspended because of their adherent properties. After incubation for 24 hours, TMZ alone (15.625, 31.25, 62.5, 125, or 250 μM), XL765 alone (3.125, 6.25, 12.5, 25, 50, 100, and 200 μM), or both agents (15.625, 31.25, 62.5, 125, or 250 μM TMZ + 25 or 50 μM XL765) were added into the respective wells, with DMSO serving as the solvent control. Cell viability was evaluated after 72 hours of incubation with the various agents using the CCK-8 assay. Briefly, 10 μL of CCK-8 reagent was added to each well and the cells were then incubated at 37°C for an additional 2 hours. Absorbance was then measured at a wavelength of 450-630 nm using a Victor-2 plate reader (PerkinElmer, Waltham, Massachusetts). Each CCK-8 assay was performed in triplicate. Cell viability was calculated as a percentage of the control, and the median inhibitory concentration (IC_{50}) of TMZ alone, XL765 alone, or both agents (TMZ/XL765) were calculated from growth inhibition curves fitted to the data using the OriginPro 7.5 software (Origin Labs, Northampton, Massachusetts).

Synergy analysis of combined drug effects

Drug synergy was determined by combination index (CI) methods derived from Chou-Talalay equations using the CalcuSyn software (Biosoft, Cambridge, United Kingdom) (26). Using a cell proliferation assay and computerized software data, CI values were generated between 2 drugs. A CI of 1 indicates an additive effect between 2 agents; CI greater than 1 indicates antagonism; and CI less than 1 indicates synergy.

Flow cytometry analysis of apoptosis

An apoptosis assay was performed using the Annexin V-FITC apoptosis assay kit (Biosea). Briefly, the αT3-1, GH3, and MMQ cells were seeded at a density of 1×10^5 cells in each well of a 6-well plate. These cells were treated with control solvent (DMSO), TMZ alone, XL765 alone, or TMZ/XL765 combination for 48 hours. The cells were then collected, washed twice with PBS, and resuspended with 200 μL binding buffer containing 10 μL of annexin-V-FITC. After 30 minutes of incubation in the dark at room temperature, the cells were stained with 5 μL propidium iodide (PI). Apoptotic cells were counted on a FACS flow cytometer and analyzed with CFlow Plus analysis software (Becton Dickinson, Heidelberg, Germany). Experiments were repeated at least 3 times to ensure reproducibility.

TUNEL staining in vitro

TUNEL staining was performed to detect apoptotic cells using the DeadEnd fluorometric TUNEL system (Promega). The αT3-1, GH3, and MMQ cells were seeded on glass-bottom dishes (Nest Biotechnology, Wuxi, Jiangsu, China) at a density of 2×10^4 cells/well. After treatment with control solvent (DMSO), TMZ alone, XL765 alone, or TMZ/XL765 combination for 48 hours, the cells were fixed with 4% methanol-free formaldehyde in PBS for 25 minutes. The cells were then washed 2 times with PBS and permeabilized with 0.2% Triton X-100 for 5 minutes. The cells were washed twice with PBS, equilibrated with the equilibration buffer at room temperature for 10 minutes, and then incubated at 37°C in a humidified chamber with 50 μL of terminal deoxynucleotidyl transferase (TdT) fluorescein-12-deoxyuridine 5-triphosphate for 1 hour. The reaction was terminated by the addition of 20× saline sodium citrate at room temperature for 15 minutes. The dishes were washed 3 times with PBS and then mounted using an antifading agent (Golden Bridge International, Inc, Mukilteo, Washington). The cells were then observed and photographed through a laser-scanning confocal microscope (Carl Zeiss, Heidelberg, Germany). The immunofluorescence intensity of TUNEL-positive cells in 3 randomly selected samples from each group was analyzed using Leica Confocal Software Lite (Leica Microsystems, Heidelberg, Germany).

Caspase-3/7 activity assays

To further investigate the mechanism of apoptosis induced by TMZ, XL765, or TMZ/XL765 combination, caspase-3/7 activity was measured using a kit according to the manufacturer's protocol (Promega). Cells were cultured at 5000 cells/well in 96-well, white-walled plates for 24 hours. The cells were treated with control solvent (DMSO), TMZ alone, XL765 alone, or TMZ/XL765 combination for 72 hours. The Caspase-Glo 3/7 reagent was added and incubated for 3 hours. The plates were then measured using a Veritas microplate luminometer (Turner BioSystems, Sunnyvale, California), and experiments were replicated at least 3 times.

Western blot

The effects of TMZ alone, XL765 alone, or TMZ/XL765 combination on the PI3K/mTOR pathway and the expression of Bcl-2, Bax, and PARP (intact and cleaved fragments) were analyzed by Western blot. Briefly, cells were seeded in 10-cm cell culture dishes and incubated for 24 hours. After treated with control solvent (DMSO), TMZ alone, XL765 alone, or the TMZ/XL765 combination for 72 hours, the cells were lysed in lysis buffer, and protein lysates were separated on 4%–20% Tris-glycine gels (Invitrogen, Carlsbad, California), electrophoretically transferred onto a polyvinylidene difluoride membrane (PALL Corp, East Hills, New York), and probed by standard techniques with primary antibodies against pAKT, pS6, pmTOR, total AKT, total S6, total mTOR, Bax, Bcl-2, and PARP. The HRP-conjugated goat antirabbit IgG was used as a secondary antibody for primary antibodies, whereas the horse

antimouse IgG was used as a secondary antibody for the mouse anti-β-actin primary antibody.

Immunohistochemistry

Tissues harvested from the GH3 xenograft were fixed in 4% formaldehyde overnight and then embed in paraffin for IHC. After deparaffinization and hydration, paraffin-embedded sections were pretreated for 20 minutes with 10 mmol/L sodium citrate buffer in a microwave for antigen retrieval. The sections were then stained with a primary antibody against pAKT (1:50 dilution), pS6 (1:200 dilution), and Ki-67 (1:150 dilution) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies (SP-9000; Zhongshan Gold Bridge, Beijing, China) at room temperature for 1 hour. Slides were counterstained with hematoxylin and eosin and observed and photographed with a microscope camera system.

TUNEL staining of GH3 xenografts

The TUNEL staining was performed according to the manufacturer's instructions of cell death detection kit (Roche). After 15 minutes incubation with Proteinase K (20 mg/mL) at room temperature, the sections were incubated with 2% H_2O_2 for 5 minutes to block endogenous peroxidase. The sections were then incubated with the TdT enzyme at 37°C for 2 hours. After 30 minutes incubation with antidigoxin-peroxidase solution, the sections were stained with diaminobenzidine substrate for 10 seconds to 2 minutes and counterstained with hematoxylin and eosin. A negative control was performed by omitting TdT. Slides were photographed with a microscope camera system.

Serum GH and PRL secretion assay

After 3-week treatments with vehicle (DMSO), TMZ (5 mg/kg), XL765 (25 mg/kg), or TMZ/XL765 combination, mice were anesthetized with an ip injection of chloral hydrate (300 mg/kg) and blood was drawn from the retroorbital sinus. The athymic female nude mice without implantation with GH3 cells were used as a negative control group. Blood rat-PRL and rat-GH were measured using a 2-site IRMA (27). Briefly, blood was centrifuged at 3000 × *g* for 10 minutes at 4°C, and plasma was collected for the hormone assay. Serum rat-GH and rat-PRL

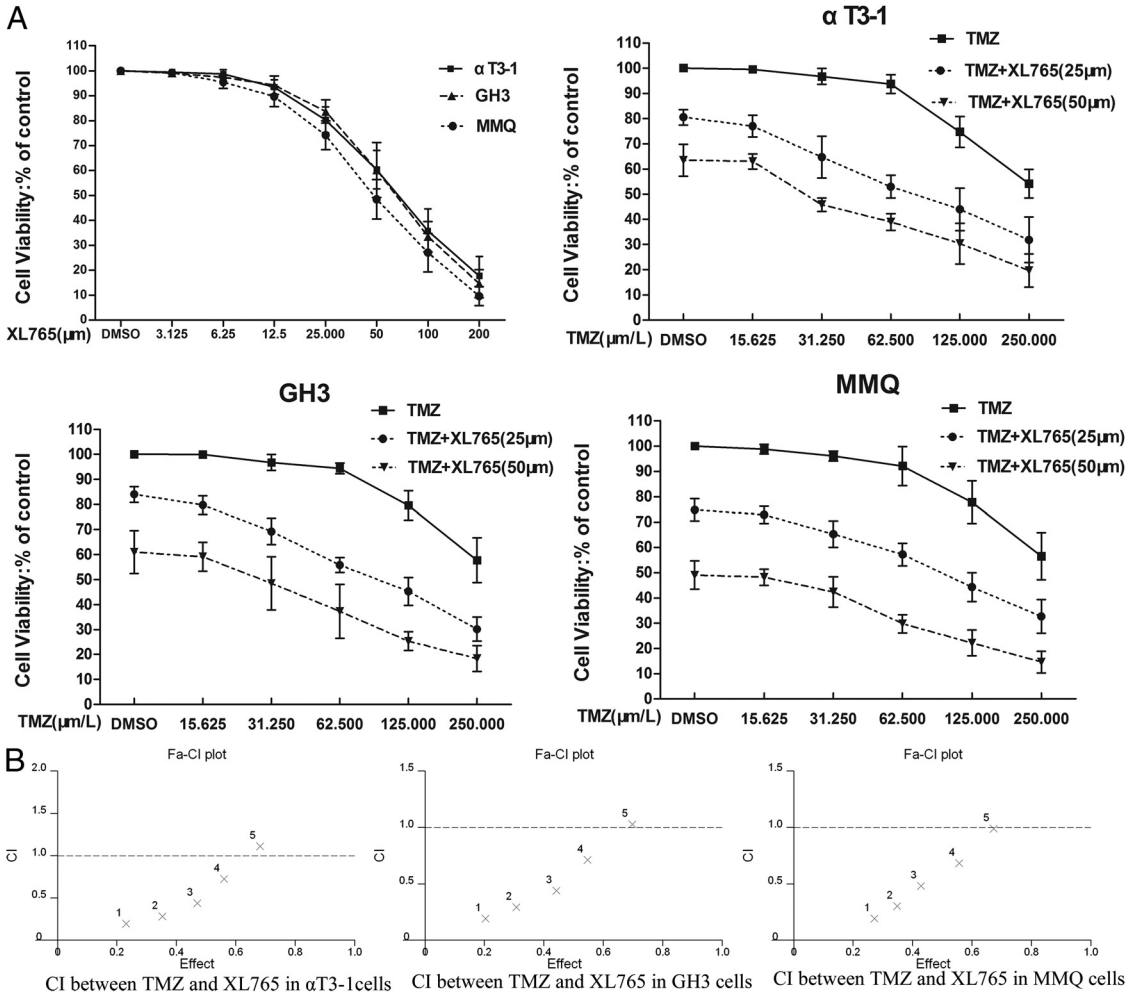

Figure 1. The TMZ/XL765 combination inhibits synergistically the proliferation of αT3-1, GH3, and MMQ cells. A, The cells were treated with DMSO or with various concentrations of XL765 (3.125–200 μM), various concentrations of TMZ (15.625–250 μM), or combinations for 72 hours, and the cell viability was assessed by the CCK-8 assay. Data are expressed as percentage of vehicle-treated control (mean ± SD). B, The CI for TMZ and XL765 in αT3-1, GH3, and MMQ cells. The synergy between TMZ and XL765 was analyzed based on data from panel A using CalcuSyn software (Biosoft). The ×1 represents a CI of 15.625 μM TMZ and 25 μM XL765; ×2 represents a CI of 31.25 μM TMZ and 25 μM XL765; ×3 represents a CI of 62.5 μM TMZ and 25 μM XL765; ×4 represents a CI of 125 μM TMZ and 25 μM XL765; and ×5 represents a CI of 250 μM TMZ and 25 μM XL765.

were measured by IRMA. Results are expressed as the mean ± SD from 3 independent experiments.

Statistical analysis

The normal distribution and homogeneity of variance of the data sets were analyzed by the Kolmogorov-Smirnov test and Levene's test, respectively. Comparisons of the means for cell proliferation, annexin V staining, and Caspase-Glo assay for αT3-1, GH3, and MMQ cells treated with DMSO, TMZ alone, XL765 alone, or combined TMZ/XL765 at the indicated time points were analyzed using 1-way ANOVA. The body weight and tumor volumes of GH3 xenograft tumor-bearing mice were analyzed by a Student's *t* test between 2 groups and 1-way ANOVA for comparisons among multiple groups. A $P < .05$ was considered to be statistically significant.

Results

TMZ and XL765 synergistically inhibit PA cell lines in vitro

To investigate the ability of XL765 to modify the response of PA cells to TMZ treatment, we evaluated the effects of various concentrations of TMZ alone, XL765 alone, and both TMZ and XL765 on the viability of PA cell lines αT3-1, GH3, and MMQ. Cell viability was determined using the CCK8 assay. The IC_{50} values for TMZ after 72 hours treatment in αT3-1, GH3, and MMQ cells were 259.88, 288.64, and 268.45 μM, respectively, which were consistent with our previous study (28) and the study reported by Sheehan et al (29). The IC_{50} values for XL765 after 72 hours treatment in αT3-1, GH3, and MMQ cells were 83.52, 72.74, and 61.45 μM, respectively. To further assess the ability of XL765 to sensitize PA cell lines to TMZ, cells were incubated with 25 or 50 μM XL765 in combination with various concentrations of TMZ. As shown in Figure 1A, the addition of 25 or 50 μM XL765 resulted in a significant decrease in the IC_{50} of TMZ in all cell lines tested (Table 1).

To further assess the synergistic effect of TMZ and XL765 on PA cell lines, the proliferation data were analyzed using the method established by Chou and Talalay with CalcuSyn software (Biosoft). Drug synergy between TMZ and XL765 was determined using CI methods. As shown in Figure 1B and Table 2, the CI values were less than 1 for most of the effect ranges of the drugs (fractional effect 0.2-0.6) at low concentrations of TMZ (range 15.625-125 μM), demonstrating that the combination of low doses of XL765 and TMZ synergistically inhibits proliferation of αT3-1, GH3 and MMQ cells. However, when 250 μM of TMZ was used in combination with 25 μM XL765, the CI values were greater than 1 for αT3-1 and GH3 cells, indicating that the 2 drugs were antagonistic at high concentrations. However, the CI value for 250 μM of TMZ and 25 μM of XL765 in MMQ was 0.991, which was approximately 1, indicating that the 2 drugs were addictive at a high dose. Taken together, these data showed that the combination treatment of low doses of TMZ and XL765 exerted a synergistic antiproliferation effect on the PA cell lines αT3-1, GH3, and MMQ in vitro.

XL765 enhances TMZ-induced apoptosis of PA cell lines in vitro

To investigate the effect of XL765, TMZ, or TMZ/XL765 combination on apoptosis of PA cell lines, GH3 cells were treated with the drugs for 48 hours and then assessed by flow cytometry. As shown in Figure 2A, TMZ (125 μM) or XL765 (25 μM) alone induced apoptosis of GH3 cells by 8.5% and 9.1%, respectively, compared with 2% in the control group. Strikingly, the combination of XL765 and TMZ further induced apoptosis up to 25.7% in GH3 cells. As expected, the increase in the apoptotic rate was reflected as a concomitant increase in the activity of caspase-3/7, which are key effectors of apoptotic pathways. As shown in Figure 2B, the combination of

Table 1. The IC_{50} for TMZ, XL765 alone, or TMZ/XL765 Combination in Pituitary Adenomas Cell Lines

Cell Lines	IC_{50} Values				
	XL765	TMZ	TMZ/XL765 (25 μM)	TMZ/XL765 (50 μM)	*P*
αT3-1	83.52	259.88	71.79	35.43	.000
GH3	72.74	288.64	78.73	30.60	.000
MMQ	61.45	268.45	72.75	24.34	.000

The 1-way ANOVA was used to analyze the IC_{50} values for TMZ alone, TMZ/XL765 (25 μM), and TMZ/XL765 (50 μM) in pituitary adenoma cell lines.

Table 2. Combination Index for Combination TMZ and XL765 in Pituitary Adenomas Cell Lines

Cell Lines	Drug combination	CI	Interpretation
αT3-1	TMZ (15.625), XL765 (25)	0.195	Synergy
	TMZ (31.25), XL765 (25)	0.284	Synergy
	TMZ (62.5), XL765 (25)	0.44	Synergy
	TMZ (125), XL765 (25)	0.729	Synergy
	TMZ (250), XL765 (25)	1.111	Antagonism
GH3	TMZ (15.625), XL765 (25)	0.195	Synergy
	TMZ (31.25), XL765 (25)	0.294	Synergy
	TMZ (62.5), XL765 (25)	0.441	Synergy
	TMZ (125), XL765 (25)	0.713	Synergy
	TMZ (250), XL765 (25)	1.031	Antagonism
MMQ	TMZ (15.625), XL765 (25)	0.192	Synergy
	TMZ (31.25), XL765 (25)	0.303	Synergy
	TMZ (62.5), XL765 (25)	0.484	Synergy
	TMZ (125), XL765 (25)	0.686	Synergy
	TMZ (250), XL765 (25)	0.991	Synergy

XL765 and TMZ increased the activity of caspase-3/7 dramatically in compared with either TMZ or XL765 alone. Importantly, a similar synergistic effect on apoptosis induction between TMZ and XL765 was also observed in the αT3-1 and MMQ cells (Supplemental Figures 1 and 2, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

To further classify the proapoptotic effect of the drug combination, the morphological changes of GH3 cells were evaluated by TUNEL staining. After treatment with TMZ, XL765, or the combination, GH3 cells exhibited distinct morphological features of apoptosis, including early coalescence of nuclear chromatin and margination and nuclear shrinkage (Figure 2C). The immunofluorescence intensity of TUNEL-positive cells in the TMZ/XL765 combination-treated group was much higher than either TMZ or XL765 alone (Figure 2D).

Combination of TMZ and XL765 suppresses GH3-derived tumor growth

To assess whether the TMZ and XL765 combination could exert a synergistic antitumor effect on the PA cell line in vivo, we first treated GH3 xenograft tumor-bearing nude mice with various doses of TMZ (5, 25, or 100 mg/kg) or XL765 (25, 50, or 100 mg/kg). As shown in Figure 3A, either TMZ or XL765 had dose-dependent antitumor

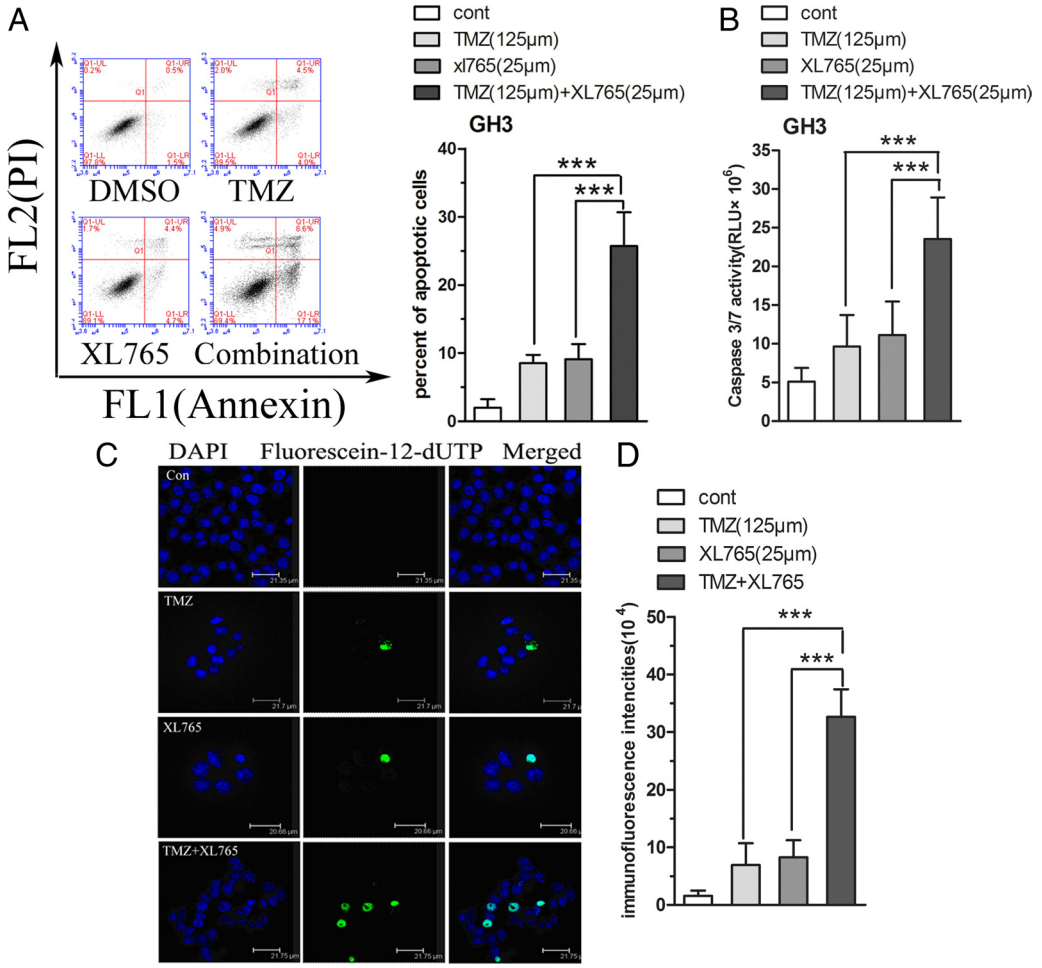

Figure 2. XL765, TMZ, or the TMZ/XL765 combination induces cell apoptosis of GH3. A, After 48 hours treatment with DMSO, TMZ, XL765, or the TMZ/XL765 combination, the apoptotic cells of GH3 was determined using a FACS flow cytometer (Becton Dickinson). Data are expressed as the mean ± SD from 3 independent experiments and analyzed using 1-way ANOVA (TMZ or XL765 vs vehicle: $P < .001$, TMZ/XL765 combination vs TMZ or XL765 alone: $^{***}P < .001$). cont, control. B, The activity of cellular caspase-3/7 was evaluated after treatment with DMSO, TMZ, XL765, or TMZ and XL765 in combination for 48 hours with Caspase-Glo reagent (Promega) and a Veritas Microplate Luminometer. The results, recorded as relative light units (RLUs), are expressed as the mean ± SD from 3 independent experiments (TMZ/XL765 combination vs TMZ or XL765 alone: $^{***}P < .001$). C, The GH3 cells were incubated with the DMSO, TMZ, XL765, or TMZ/XL765 combination for 48 hours, and then TUNEL staining was performed to detect the apoptotic cells. The nuclei of TUNEL-positive cells were stained green. The chromatin condensation and margination were present in apoptotic cells. DAPI, 4′,6′-diamino-2-phenylindole; dUTP, deoxyuridine 5-triphosphate D, The immunofluorescence intensity of TUNEL-positive cells in 3 randomly selected samples from each group was analyzed using Leica Confocal Software Lite. The result of immunofluorescence intensity is expressed as the mean ± SD (TMZ/XL765 combination vs TMZ or XL765 alone: $^{***}P < .001$).

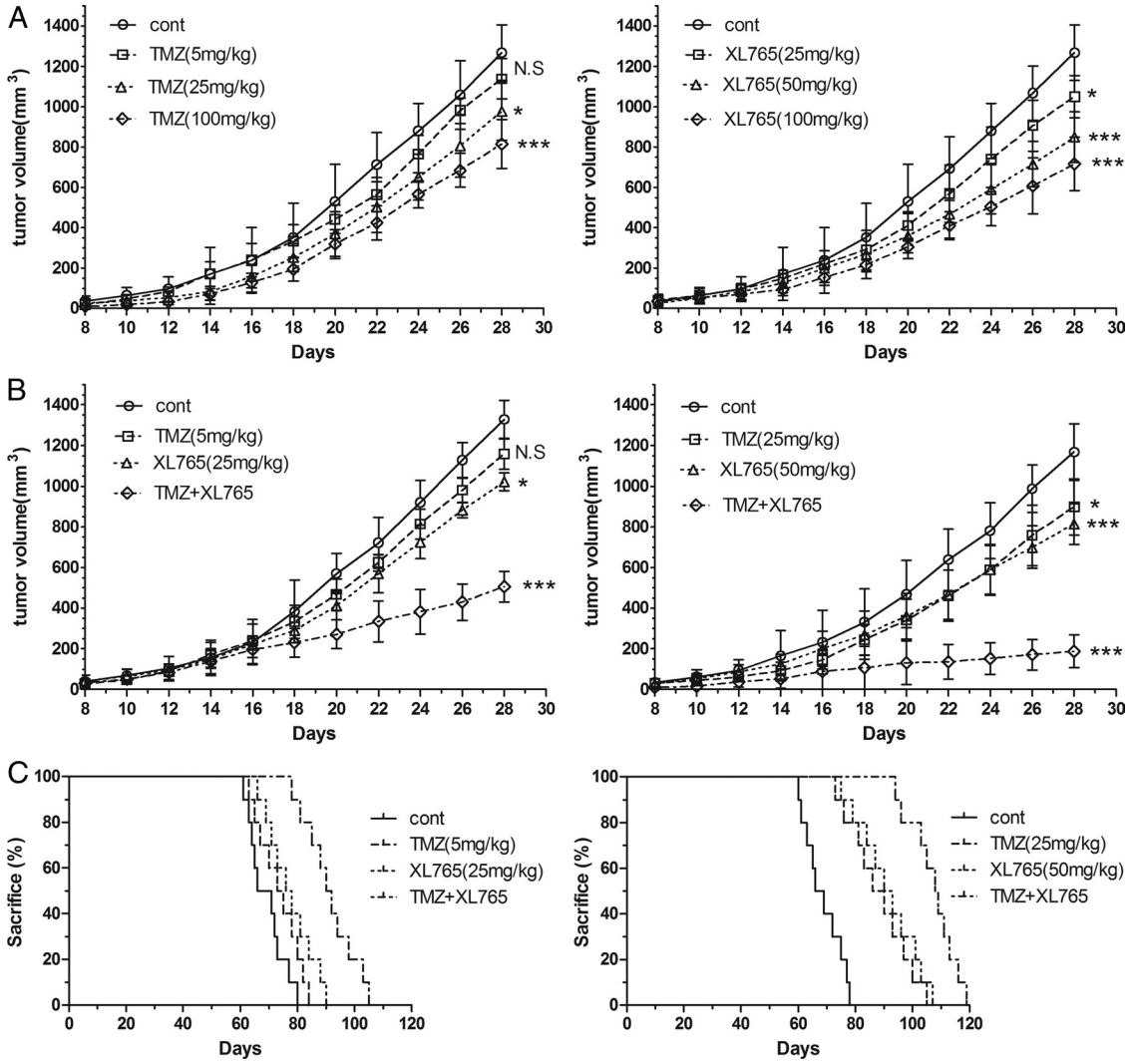

Figure 3. The TMZ/XL765 combination treatment inhibits the tumor growth and delays the killing of mice with GH3 xenograft tumors. A, GH3 cells were injected sc into nude mice (n = 5). When tumor volumes reached 30-50 mm^3, the animals received different doses of TMZ (5, 25, or 100 mg/kg) through an ip injection 5 times a week and XL765 (25, 50, or 100 mg/kg) through oral gavage 5 times a week. Tumor volumes and mice weights were recorded every 2 days. Results are shown as means ± SD. Statistical analysis of tumor volume was performed by 1-way ANOVA followed by a Bonferroni test for multiple comparisons. $*P < .05$, $**P < .01$, and $***P < .001$ compared with the respective controls. cont, control. B, Mice with GH3 xenograft tumors were given DMSO, 5 mg/kg TMZ, 25 mg/kg XL765, or 25 mg/kg TMZ and 50 mg/kg XL765 in combination. The treatment period was 21 days. Tumor volumes and body weights were measured every 2 days until the mice were euthanized at the end of treatment. C, Kaplan-Meier curves were used to illustrate the rate of the killing of the GH3 xenograft-bearing mice due to tumor burden. The *P* values were determined using the log-rank test. Each group consisted of 10 mice. Data are shown as means ± SD. $*P < .05$, $**P < .01$, and $***P < .001$ compared with the respective controls.

activity, compared with the DMSO-treated control group. However, the body weights of the mice decreased in a dose-dependent manner as well (Supplemental Figures 3 and 4), indicating a high dose of TMZ or XL765 resulted in significant systemic cytotoxicity.

To investigate synergy, we next treated GH3 xenograft tumor-bearing nude mice with nontoxic doses of TMZ (5 mg/kg), XL765 (25 mg/kg), or the TMZ/XL765 combination. As shown in Figure 3B, we found that a low dose of TMZ (5 mg/kg) or XL765 (25 mg/kg) alone had no or marginal antitumor activity. In contrast, the combination of TMZ and XL765 in these dosages showed significant inhibition of tumor growth. Furthermore, the combination treatment produced synergistic antitumor activity without any significant body weight loss, indicating the combination drugs at these doses had no significant sys-

Table 3. Effect of Low Dose of Combination on Killing of GH3 Xenograft-Bearing Mice

Comparison	Median Survival	*P* Values
TMZ (5 mg/kg)/control	74 vs 68.5 d	.111
XL765 (25 mg/kg)/control	77 vs 68.5 d	.016
Combination/control	91 vs 68.5 d	<.001
Combination/TMZ (5 mg/kg)	91 vs 74 d	<.001
Combination/XL765 (25 mg/kg)	91 vs 77 d	.002

Table 4. Effect of High Dose of Combination on Killing of GH3 Xenograft-Bearing Mice

Comparison	Median Survival	*P* Values
TMZ (25 mg/kg)/control	88 vs 67.5 d	<.001
XL765 (50 mg/kg)/control	91.5 vs 67.5 d	<.001
Combination/control	108.5 vs 67.5 d	<.001
Combination/TMZ (25 mg/kg)	108.5 vs 88 d	<.001
Combination/XL765 (50 mg/kg)	108.5 vs 91.5 d	<.001

temic side effects (Supplemental Figure 5). We next treated the GH3 xenograft nude mice with a higher dose of TMZ (25 mg/kg) or XL765 (50 mg/kg) alone, which produced modest systemic cytotoxicity, or the same dose of the drugs in combination. We found that the high-dose combination also had greater antitumor effect than each single agent alone, and tumor growth was almost completely arrested (Figure 3B). Importantly, the high-dose combination did not produce greater systemic cytotoxicity than either single agent alone because no difference in body weight was observed between the treatment groups (Supplemental Figure 6).

To evaluate the long-term antitumor effect of the TMZ/XL765 combination, we further assessed the effects of TMZ, XL765, or the combination treatment on the rate of the killing of GH3 xenograft nude mice, which indirectly reflect the relative survival of tumor-bearing mice due to their tumor burden. Our results showed that treatment with a low dose of TMZ (5 mg/kg) alone did not reduce the tumor burden on the mice compared with the control groups. However, the combination treatment with TMZ and XL765 significantly decreased the rate of the killing of the mice compared with either agent alone at both low and high doses, respectively (Figure 3C and Tables 3 and 4). Taken together, these results demonstrate that the combination of TMZ and XL765 can inhibit tumor growth and prolong the survival of mice bearing a GH3 xenograft

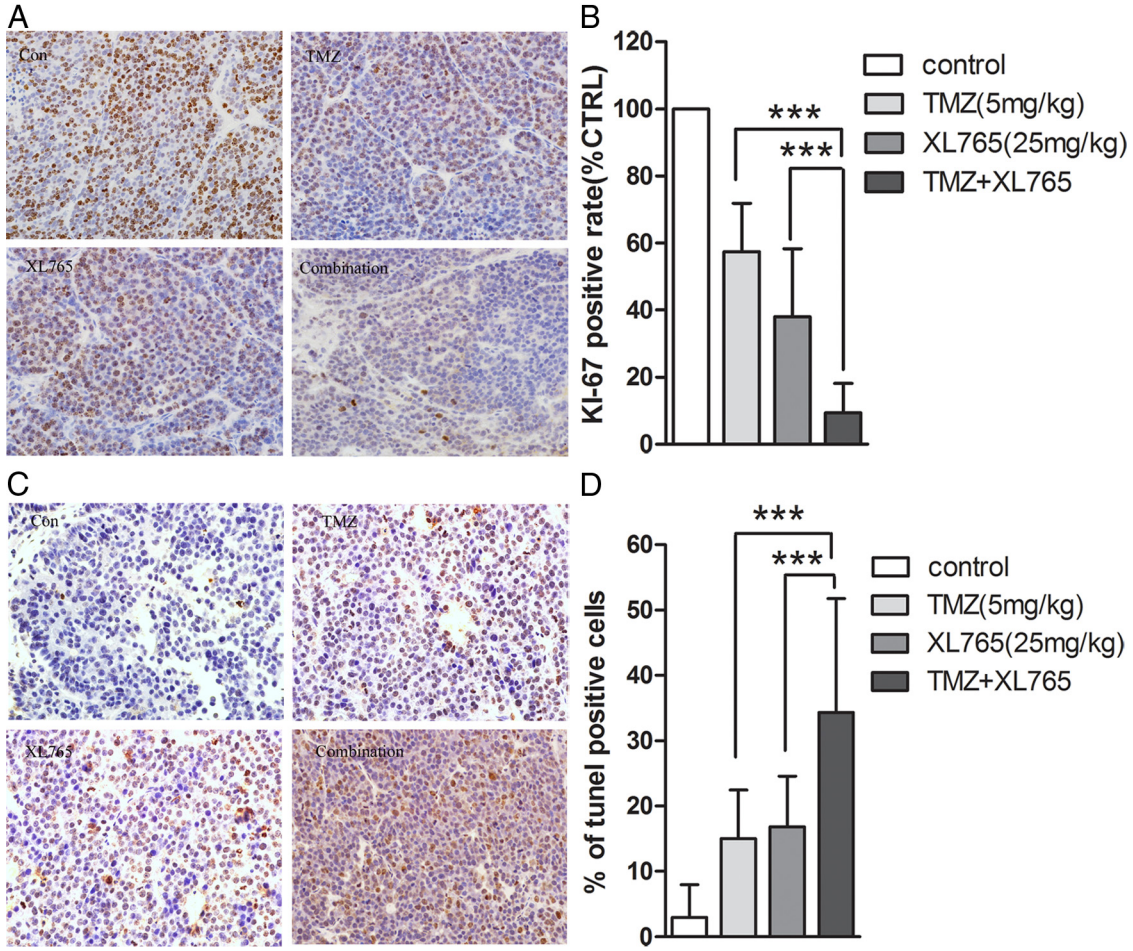

Figure 4. TMZ, XL765, or the TMZ/XL765 combination treatment inhibits the cell proliferation and induces the cell apoptosis of GH3 cells in vivo. A and B, Ki-67 expression in the section of GH3 tumors treated with DMSO, TMZ, XL765, or the TMZ/XL765 combination was determined by IHC. Positive cells appear brown, whereas negative cells remain blue (n = 10 tumors per group). Magnification, ×40 as indicated. CTRL, control. C and D, The apoptotic cells of tumor tissues from various treatments were detected by TUNEL staining. TUNEL-positive cells appear brown, whereas negative cells remain blue (n = 10 tumors per group). Magnification, ×40 as indicated. (TMZ/XL765 combination vs TMZ or XL765 alone; ***$P < 0.001$).

tumor, suggesting that the combination of TMZ with XL765 has a significant therapeutic benefit in vivo.

Combination of TMZ and XL765 inhibits cell proliferation and induces apoptosis of GH3 cells in vivo

To determine the effect of TMZ, XL765, or the TMZ/XL765 combination on cell proliferation and apoptosis of GH3 cells in vivo, we performed Ki-67 immunohistochemistry to assess the growth potential of PAs as well as TUNEL staining of paraffin-embedded sections from tumor tissues collected from the GH3 xenograft nude mice treated with DMSO, TMZ, XL765, or the TMZ/XL765 combination. TMZ (5 mg/kg) or XL765 (25 mg/kg) alone moderately decreased the number of Ki-67-positive cells (Figure 4, A and B) and increased the number of TUNEL-positive cells (Figure 4, C and D) compared with the DMSO-treated tumors. However, the TMZ/XL765 combination dramatically increased the number of TUNEL-positive cells and decreased the number of Ki-67-positive cells compared with either agent alone.

Combination of TMZ and XL765 inhibits secretion of GH and PRL in GH3 xenograft tumor-bearing nude mice

The GH3 cell line used in this study was derived from a pituitary tumor in a 7-month-old female Wistar-Furth rat and secreted rat-GH and rat-PRL in tissue culture as well as in xenografted nude mice (30, 31). To detect the functional inhibition of TMZ, XL765, or the TMZ/XL765 combination on rat-GH and rat-PRL levels in GH3 xenograft tumor-bearing mice, serum rat-GH and rat-PRL levels were measured by IRMA. As shown in Figure 5, A and B, TMZ (5 mg/kg) and XL765 (25 mg/kg) alone only marginally decreased serum rat-GH and rat-PRL levels. However, the TMZ/XL765 combination decreased both serum rat-GH and rat-PRL dramatically compared with either agent alone.

Combination of TMZ and XL765 inhibits AKT/mTOR signaling and induces apoptosis

To determine the mechanisms involved in the synergy between TMZ and XL765, we extracted total protein from GH3 cells after treatment with DMSO, TMZ, XL765, or the combination and performed Western blot analysis. As shown in Figure 6A, XL765 markedly down-regulated the phosphorylation of AKT, mTOR, and S6, whereas TMZ alone did not. Interestingly, the TMZ/XL765 combination dramatically decreased phosphorylation of AKT, mTOR and S6 compared with TMZ or XL765 alone. Previous studies have shown that AKT controls the expression of some apoptosis-related proteins, such as Bax, Bcl-2, and PARP (32, 33). In accordance with these studies, our results showed that TMZ alone could partially regulate the expression of these proteins. However, when XL765 was added, the TMZ-mediated Bcl-2 inhibitory effect and Bax or PARP stimulatory effect were significantly enhanced (Figure 6B).

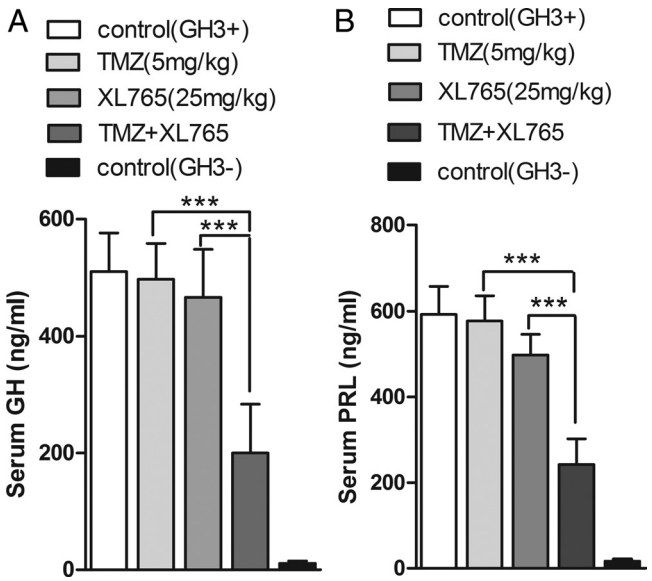

Figure 5. The TMZ/XL765 combination decreases serum GH and PRL of mice bearing GH3 xenografts. A and B, After treatment with DMSO, TMZ, XL765, or the XL765/TMZ combination, the blood of the nude mice xenograft with GH3 tumor was collected and serum rat-GH and rat-PRL were measured by IRMA (TMZ/XL765 combination vs TMZ or XL765 alone: $***P < .001$).

Our in vivo data were consistent with results from the in vitro study. As shown in Supplemental Figure 7 and Figure 6, C and D, the expression of pAKT and pS6 in GH3 xenograft tumors was markedly down-regulated by treatment with XL765 (25 mg/kg) but not by TMZ (5 mg/kg). However, the TMZ/XL765 combination substantially down-regulated pAKT and pS6 expression compared with TMZ or XL765 alone. Taken together, our in vivo and in vitro data indicate that the synergistic effect between TMZ and XL765 on the inhibition of tumor growth occurs by inhibiting the activation of PI3K/mTOR pathways and regulating apoptosis-related proteins.

Discussion

Invasive PAs are difficult to be resected completely due to massive invasion of surrounding anatomical structures and rapid growth, and these tumors are often refractory to standard therapy and salvage treatment with TMZ (3). The present study shows that the administration of XL765 in combination with TMZ inhibits the proliferation and

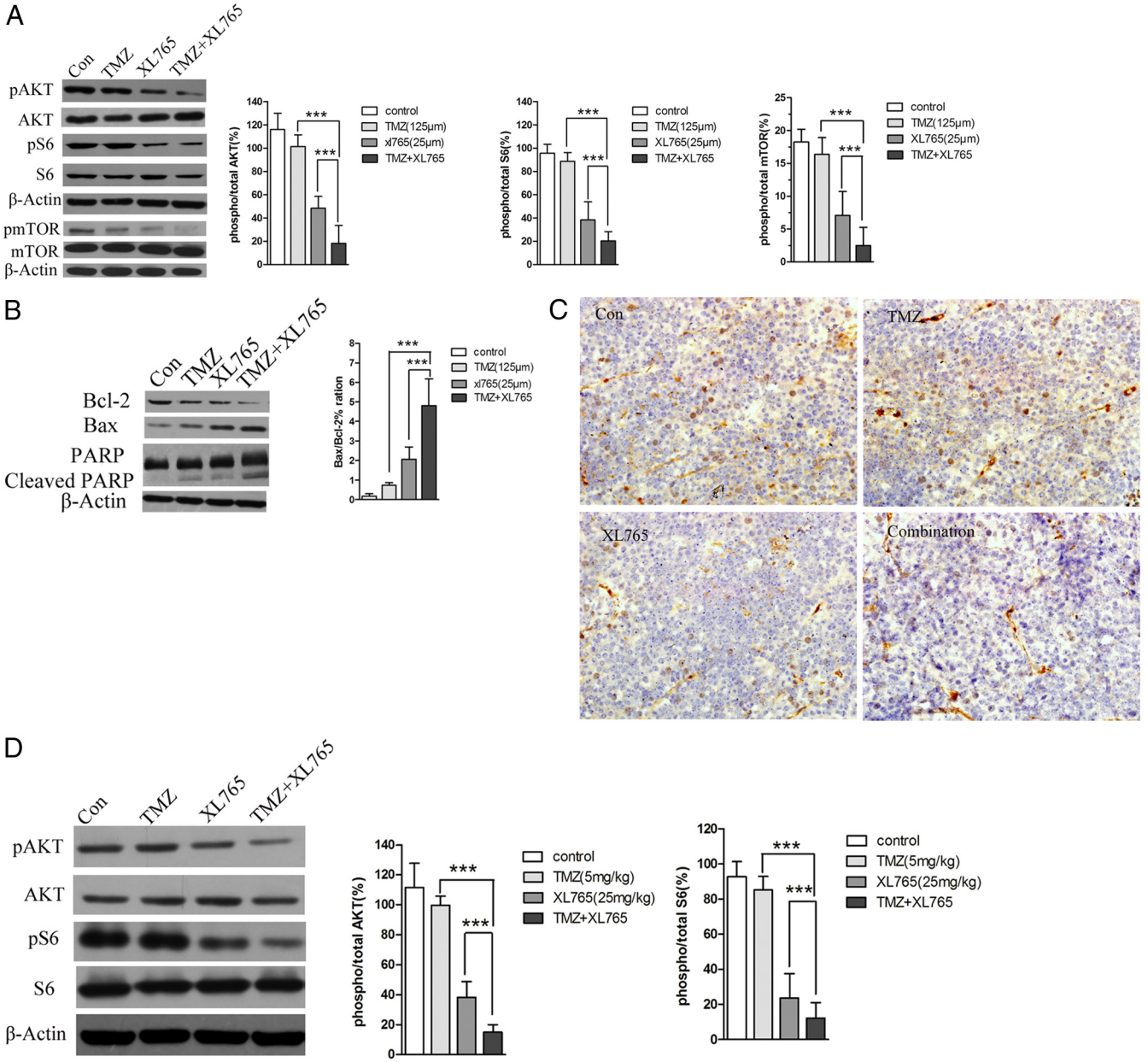

Figure 6. Combination of TMZ and XL765 inhibits AKT/mTOR signaling and induces apoptosis in vitro and in vivo. A, After treatment with DMSO, TMZ, XL765, or the TMZ/XL765 combination, the phosphorylation of AKT, mTOR, and S6 in GH3 cells were analyzed by Western blot. Con, control. B, The expression of Bax, Bcl-2, and PARP in GH3 after the same treatment was examined by Western blot in vitro. C, After treatment with DMSO, TMZ, XL765, or the TMZ/XL765 combination, tumor tissues were collected, and the expression of pS6 was determined by IHC. D, The expression of pS6 and pAKT of tumor tissues after the same treatment was detected by Western blot. Bars are mean ± SD from three independent experiments (TMZ/XL765 combination vs TMZ or XL765 alone: $***P < 0.001$).

promotes the apoptosis of PA cells through inhibition of AKT/mTOR pathway in vitro and in vivo.

Constitutive activation of AKT has been observed in many tumors and plays a pivotal role in the pathogenesis and progression of several human cancers, including PAs (12, 16, 34). Inhibition of the PI3K/AKT signaling pathway can lead to tumor growth inhibition in some types of cancers, further underscoring the important role of this pathway in mediating tumor growth and progression (35, 36). However, a previous study also suggested that AKT inhibition alone does not exert long-term cytotoxic effects in human tumors due to disruption of feedback mechanisms (37). Furthermore, sole inhibition of mTOR activity through the use of mTOR inhibitors can also reactivate PI3K/AKT signaling and in turn stimulate the activity of MAPK pathways in human cancers (38, 39). In the clinic, an mTOR inhibitor has been used in 1 case of pituitary carcinoma, but it was ineffective for controlling the secretion of hormone and tumor growth, and recent data suggest that the feedback of rapamycin on AKT phosphory-

lation may be associated with the mechanisms of mTOR inhibitor resistance in PAs (40, 41). Considering these adverse effects, several dual PI3K/mTOR inhibitors have been developed, which have been shown to exert an effective therapeutic modality in treating many types of tumor, such as pancreatic adenocarcinoma, glioma, renal cell carcinoma, and follicular lymphoma (19, 20, 43, 44). Here we demonstrated that XL765, a dual inhibitor of the PI3K/mTOR pathway, inhibited the proliferation of PA cells and simultaneously promoted the apoptosis of these tumor cells. Furthermore, XL765 enhanced TMZ-induced cytotoxicity in PA cells, showing that this tumor type may be a new therapeutic target for XL765 treatment and further suggesting a critical role of the PI3K/mTOR pathway in the formation of PAs.

Tumor cell resistance to chemotherapeutic drugs, especially that mediated by the PI3K/mTOR signaling pathway, remains a major problem that is responsible for the failure of many cancer treatments (45). It has been reported that inhibition of the PI3K-AKT-mTOR pathway potently increases the sensitivity of glioblastoma, melanoma, and renal cell carcinoma to chemotherapy (19, 43, 47). Of note, we found that inhibition of PI3K/mTOR signaling by XL765 sensitized PA cell lines to TMZ. Our in vitro study shows that the combination of TMZ and XL765 has a stronger inhibitory effect on the growth of PA cell lines than either agent alone. Moreover, the combination of XL765 and TMZ at low concentrations had an even stronger inhibitory effect on the proliferation of PA cells than TMZ alone at a high concentration (250 μM). The combination of XL765 and TMZ induced a 25.7% apoptotic rate in GH3 cells, which was higher than 8.5% and 9.1% produced by TMZ and XL765 alone, respectively. The increase in caspase-3/7 activity together with the apoptotic rate of cells after treatment with the combination of drugs, suggesting XL765/TMZ combination markedly enhances the efficacy of TMZ by inducing caspase-3/7 activity and promoting apoptosis in GH3 cells. The combination of XL765 and TMZ inhibited cell proliferation and promoted the apoptosis of other PA cell lines derived from mouse or rat, including αT3-1 and MMQ, further confirming the broad applicability of this combination for treating PAs.

Our in vivo study also confirmed the results of the in vitro study. The pituitary gland is located outside the blood-brain barrier. Even though the sc xenografted tumor used in our study is not an authentic pituitary tumor, the pharmacokinetic requirements for TMZ and XL765 to reach a sc tumor vs a tumor in the pituitary gland should not differ dramatically. In the absence of an animal model with pituitary tumor in normal anatomical location, this imperfect tumor model does provide us a window of opportunity to evaluate the efficacy of therapeutic compounds in a preclinical study. We found that a low dose of TMZ (5 mg/kg) alone could not reduce tumor load in mice harboring GH3 xenografts. Although high doses of TMZ did exert antitumor activity in these mice, it was accompanied with a body weight loss, indicating markedly systemic toxicity. TMZ alone did not inhibit activation of PI3K/mTOR pathways, regardless of the dose. Strikingly, when XL765 was administered with TMZ, the date of the killing of the mice was significantly postponed compared with mice treated with TMZ or XL765 alone, suggesting that the 2 drugs synergized to prolong the survival of the nude mice xenograft with GH3. The combination of XL765 and TMZ also exerted a more efficient antitumor effect and inhibited AKT (a downstream protein in the PI3K pathway) and mTOR/S6 activation in the GH3 tumors compared with each agent alone, further confirming that this combination may be an effective strategy for the treatment of PAs in vivo.

In the present study, the combination of XL765 and TMZ at low doses did not cause increased systemic toxicity. A previous study found that 50 mg/kg XL765 induced systemic toxicity and subsequently reduced the body weight of treated animals despite being able to effectively inhibit the tumor growth of a glioblastoma xenograft (19). Importantly, the low dose of TMZ (5 mg/kg) used in our study was significantly lower than the clinically relevant dose of TMZ (200 mg/m^2). The IC_{50} values for TMZ in combination with 25 μM XL765 in this study were relatively low compared with clinically achievable serum levels during chemotherapy (100 μM) (42). Taken together, our in vitro and in vivo studies suggest that the combination of XL765 and TMZ produces more than an additive effect and may be synergistic effect in PA treatment.

We are not sure at present how TMZ and XL765 reduce blood GH and PRL levels. Previous studies have shown that inhibition of PI3K could down-regulates the PRL secretion (46). We hypothesize that these therapeutic agents decreased the secretion of these hormones by shrinking tumor mass and suppressing the PI3K pathway.

In summary, we report here that XL765 inhibits tumor growth and enhances the efficacy of TMZ by inhibiting the PI3K/mTOR signaling pathway and regulating apoptosis-related proteins. XL765 in combination with TMZ may thus be useful in the treatment of PAs.

Acknowledgments

We thank Dr P. L. Mellon for providing the αT3-1 cell line. Authorship contributions include the following: R.W. and C.D.

conceived and designed the experiments; C.D., B.Z., X.L., S.M., Y.Ya., Y.Yao., M.F., X.B., G.L., J.W., K.G., W.M., B.X., W.L., J.X., and F.C. performed the experiments; and C.D., H.Z., and R.W. analyzed the data and wrote the paper.

Address all correspondence and requests for reprints to: Renzhi Wang, Department of Neurosurgery, Peking Union Medical College Hospital, Beijing 100730, China. E-mail: wangrz@126.com.

This work was supported by the National Natural Science Foundation of China (Grant 81072084). The funding institution had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Asa SL, Ezzat S.** The pathogenesis of pituitary tumours. *Nat Rev Cancer*. 2002;2:836–849.
2. **Scheithauer BW, Kovacs KT, Laws EJ, Randall RV.** Pathology of invasive pituitary tumors with special reference to functional classification. *J Neurosurg*. 1986;65:733–744.
3. **Colao A, Grasso LF, Pivonello R, Lombardi G.** Therapy of aggressive pituitary tumors. *Expert Opin Pharmacother*. 2011;12:1561–1570.
4. **Hornyak M, Couldwell WT.** Multimodality treatment for invasive pituitary adenomas. *Postgrad Med*. 2009;121:168–176.
5. **Buchfelder M.** Management of aggressive pituitary adenomas: current treatment strategies. *Pituitary*. 2009;12:256–260.
6. **Bei R, Marzocchella L, Turriziani M.** The use of temozolomide for the treatment of malignant tumors: clinical evidence and molecular mechanisms of action. *Recent Pat Anticancer Drug Discov*. 2010;5:172–187.
7. **Raverot G, Sturm N, de Fraipont F, et al.** Temozolomide treatment in aggressive pituitary tumors and pituitary carcinomas: a French multicenter experience. *J Clin Endocrinol Metab*. 2010;95:4592–4599.
8. **Syro LV, Ortiz LD, Scheithauer BW, et al.** Treatment of pituitary neoplasms with temozolomide: a review. *Cancer*. 2011;117:454–462.
9. **Murakami M, Mizutani A, Asano S, et al.** A mechanism of acquiring temozolomide resistance during transformation of atypical prolactinoma into prolactin-producing pituitary carcinoma: case report. *Neurosurgery*. 2011;68:E1761–E1767.
10. **Morin E, Berthelet F, Weisnagel J, Bidlingmaier M, Serri O.** Failure of temozolomide and conventional doses of pegvisomant to attain biochemical control in a severe case of acromegaly. *Pituitary*. 2012;15:97–100.
11. **Losa M, Mazza E, Terreni MR, et al.** Salvage therapy with temozolomide in patients with aggressive or metastatic pituitary adenomas: experience in six cases. *Eur J Endocrinol*. 2010;163:843–851.
12. **Shaw RJ, Cantley LC.** Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*. 2006;441:424–430.
13. **Liang J, Slingerland JM.** Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle*. 2003;2:339–345.
14. **Dworakowska D, Wlodek E, Leontiou CA, et al.** Activation of RAF/MEK/ERK and PI3K/AKT/mTOR pathways in pituitary adenomas and their effects on downstream effectors. *Endocr Relat Cancer*. 2009;16:1329–1338.
15. **Cakir M, Grossman AB.** Targeting MAPK (Ras/ERK) and PI3K/Akt pathways in pituitary tumorigenesis. *Expert Opin Ther Targets*. 2009;13:1121–1134.
16. **Lin Y, Jiang X, Shen Y, et al.** Frequent mutations and amplifications of the PIK3CA gene in pituitary tumors. *Endocr Relat Cancer*. 2009;16:301–310.
17. **West KA, Castillo SS, Dennis PA.** Activation of the PI3K/Akt pathway and chemotherapeutic resistance. *Drug Resist Update*. 2002;5:234–248.
18. **Molckovsky A, Siu LL.** First-in-class, first-in-human phase I results of targeted agents: highlights of the 2008 American Society of Clinical Oncology Meeting. *J Hematol Oncol*. 2008;1:20.
19. **Prasad G, Sottero T, Yang X, et al.** Inhibition of PI3K/mTOR pathways in glioblastoma and implications for combination therapy with temozolomide. *Neuro-oncology*. 2011;13:384–392.
20. **Mirzoeva OK, Hann B, Hom YK, et al.** Autophagy suppression promotes apoptotic cell death in response to inhibition of the PI3K-mTOR pathway in pancreatic adenocarcinoma. *J Mol Med (Berl)*. 2011;89:877–889.
21. **Windle JJ, Weiner RI, Mellon PL.** Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Mol Endocrinol*. 1990;4:597–603.
22. **Ostlund RJ, Leung JT, Hajek SV, Winokur T, Melman M.** Acute stimulated hormone release from cultured GH3 pituitary cells. *Endocrinology*. 1978;103:1245–1252.
23. **Judd AM, Login IS, Kovacs K, et al.** Characterization of the MMQ cell, a prolactin-secreting clonal cell line that is responsive to dopamine. *Endocrinology*. 1988;123:2341–2350.
24. **Bates RW, Garrison MM, Morris HP.** Comparison of two different transplantable mammotropic pituitary tumors. Hormone content and effect on host. *Proc Soc Exp Biol Med*. 1966;123:67–70.
25. **Liu Q, Sun JD, Wang J, et al.** TH-302, a hypoxia-activated prodrug with broad in vivo preclinical combination therapy efficacy: optimization of dosing regimens and schedules. *Cancer Chemother Pharmacol*. 2012;69:1487–1498.
26. **Chou TC.** Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res*. 2010;70:440–446.
27. **Goji K.** Pulsatile characteristics of spontaneous growth hormone (GH) concentration profiles in boys evaluated by an ultrasensitive immunoradiometric assay: evidence for ultradian periodicity of GH secretion. *J Clin Endocrinol Metab*. 1993;76:667–670.
28. **Ma S, Liu X, Yao Y, et al.** Effect of temozolomide on cell viability in gonadotroph adenoma cell lines. *Oncol Rep*. 2011;26:543–550.
29. **Sheehan J, Rainey J, Nguyen J, Grimsdale R, Han S.** Temozolomide-induced inhibition of pituitary adenoma cells. *J Neurosurg*. 2011;114:354–358.
30. **Yasamura Y, Tashjian AJ, Sato GH.** Establishment of four functional, clonal strains of animal cells in culture. *Science*. 1966;154:1186–1189.
31. **Heaney AP, Fernando M, Melmed S.** PPAR-γ receptor ligands: novel therapy for pituitary adenomas. *J Clin Invest*. 2003;111:1381–1388.
32. **Malla R, Gopinath S, Alapati K, et al.** Downregulation of uPAR and cathepsin B induces apoptosis via regulation of Bcl-2 and Bax and inhibition of the PI3K/Akt pathway in gliomas. *PLoS One*. 2010;5:e13731.
33. **Li SL, Huang CH, Lin CC, et al.** Antitumor effect of BPR-DC-2, a novel synthetic cyclic cyanoguanidine derivative, involving the inhibition of MDR-1 expression and down-regulation of p-AKT and PARP-1 in lung cancer. *Invest New Drugs*. 2011;29:195–206.
34. **Musat M, Korbonits M, Kola B, et al.** Enhanced protein kinase B/Akt signalling in pituitary tumours. *Endocr Relat Cancer*. 2005;12:423–433.
35. **Hoeflich KP, Merchant M, Orr C, et al.** Intermittent administration of MEK inhibitor GDC-0973 plus PI3K inhibitor GDC-0941 triggers robust apoptosis and tumor growth inhibition. *Cancer Res*. 2012;72:210–219.
36. **Roccaro AM, Sacco A, Husu EN, et al.** Dual targeting of the PI3K/Akt/mTOR pathway as an antitumor strategy in Waldenstrom macroglobulinemia. *Blood*. 2010;115:559–569.

37. **Chandarlapaty S, Sawai A, Scaltriti M, et al.** AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. *Cancer Cell*. 2011;19:58–71.
38. **Gomez-Pinillos A, Ferrari AC.** mTOR signaling pathway and mTOR inhibitors in cancer therapy. *Hematol Oncol Clin North Am*. 2012;26:483–505.
39. **Park S, Zhao D, Hatanpaa KJ, et al.** RIP1 activates PI3K-Akt via a dual mechanism involving NF-κB-mediated inhibition of the mTOR-S6K-IRS1 negative feedback loop and down-regulation of PTEN. *Cancer Res*. 2009;69:4107–4111.
40. **Jouanneau E, Wierinckx A, Ducray F, et al.** New targeted therapies in pituitary carcinoma resistant to temozolomide. *Pituitary*. 2012; 15:37–43.
41. **Cerovac V, Monteserin-Garcia J, Rubinfeld H, et al.** The somatostatin analogue octreotide confers sensitivity to rapamycin treatment on pituitary tumor cells. *Cancer Res*. 2010;70:666–674.
42. **Hammond LA, Eckardt JR, Kuhn JG, et al.** A randomized phase I and pharmacological trial of sequences of 1,3-bis(2-chloroethyl)-1-nitrosourea and temozolomide in patients with advanced solid neoplasms. *Clin Cancer Res*. 2004;10:1645–1656.
43. **Roulin D, Waselle L, Dormond-Meuwly A, Dufour M, Demartines N, Dormond O.** Targeting renal cell carcinoma with NVP-BEZ235, a dual PI3K/mTOR inhibitor, in combination with sorafenib. *Mol Cancer*. 2011;10:90.
44. **Bhende PM, Park SI, Lim MS, Dittmer DP, Damania B.** The dual PI3K/mTOR inhibitor, NVP-BEZ235, is efficacious against follicular lymphoma. *Leukemia*. 2010;24:1781–1784.
45. **Fan QW, Weiss WA.** Targeting the RTK-PI3K-mTOR axis in malignant glioma: overcoming resistance. *Curr Top Microbiol Immunol*. 2010;347:279–296.
46. **Hayakawa J, Ohmichi M, Tasaka K, et al.** Regulation of the PRL promoter by Akt through cAMP response element binding protein. *Endocrinology*. 2002;143:13–22.
47. **Sinnberg T, Lasithiotakis K, Niessner H, et al.** Inhibition of PI3K-AKT-mTOR signaling sensitizes melanoma cells to cisplatin and temozolomide. *J Invest Dermatol*. 2009;129:1500–1515.

THE ENDOCRINE SOCIETY®

Take advantage of The Endocrine Society's online **ABIM approved Maintenance of Certification (MOC) self-assessment resources.**

www.endoselfassessment.org