Glycogen synthase kinase 3β inhibitors induce apoptosis in ovarian cancer cells and inhibit in-vivo tumor growth

Tyvette S. Hilliard, Irina N. Gaisina, Amanda G. Muehlbauer, Arsen M. Gaisin, Franck Gallier and Joanna E. Burdette

Ovarian cancer is the most lethal gynecological malignancy among US women. Paclitaxel/carboplatin is the current drug therapy used to treat ovarian cancer, but most women develop drug resistance and recurrence of the disease, necessitating alternative strategies for treatment. A possible molecular target for cancer therapy is glycogen synthase kinase 3β (GSK3β), a downstream kinase in the Wnt signaling pathway that is overexpressed in serous ovarian cancer. Novel maleimide-based GSK3β inhibitors (GSK3βi) were synthesized, selected, and tested *in vitro* using SKOV3 and OVCA432 serous ovarian cancer cell lines. From a panel of 10 inhibitors, GSK3βi 9ING41 was found to be the most effective *in vitro*. 9ING41 induced apoptosis as indicated by 4′,6-diamidino-2-phenylindole-positive nuclear condensation, poly (ADP-ribose) polymerase cleavage, and terminal deoxynucleotidyl transferase dUTP nick end labeling staining. The mechanism for apoptosis was through caspase-3 cleavage. GSK3βi upregulated phosphorylation of the inhibitory serine residue of GSK3β in OVCA432 and SKOV3 cell lines and also inhibited phosphorylation of the downstream target glycogen synthase. An in-vivo xenograft study using SKOV3 cells demonstrated that tumor progression was hindered by 9ING41 *in vivo*. The maximum tolerated dose for 9ING41 was greater than 500 mg/kg in rats. Pharmacokinetic analysis showed 9ING41 to have a bioavailability of 4.5% and to be well distributed in tissues. Therefore, GSK3β inhibitors alone or in combination with existing drugs may hinder the growth of serous ovarian cancers. *Anti-Cancer Drugs* 22:978–985 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy (M/C 870), University of Illinois at Chicago, Chicago, Illinois, USA

Correspondence to Dr Joanna E. Burdette, PhD, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy (M/C 870), University of Illinois at Chicago, Chicago, IL 60607, USA
Tel: +1 312 996 6153; fax: +1 312 996 7107;
e-mail: joannab@uic.edu

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Introduction

Ovarian cancer kills approximately 14 000 women annually in the United States [1]. Standard drug therapy consists of treatment with paclitaxel/carboplatin [2], but most women die from recurrence in 5 years because of drug resistance. Therefore, combination therapy or alternative strategies for resistant disease are of critical importance. Inhibitors of the Wnt signaling pathway, including its downstream kinase glycogen synthase kinase 3β (GSK3β), offer possible options for drug therapy in resistant ovarian cancers [3].

Wnt signaling controls embryogenesis, cell differentiation, proliferation, and migration [4,5]. Wnt ligands are secreted molecules that bind to frizzled receptors. Wnt signals through both canonical and noncanonical pathways, but all pathways initially begin with one of the 19 ligands binding to one of the 10 receptors in the human genome [6]. In the canonical pathway, a frizzled receptor phosphorylates GSK3β. GSK3β is a dual-regulated kinase in the Wnt pathway, originally found to regulate glycogen metabolism but now it is known to affect a variety of cellular events by phosphorylation of different substrates [7]. Kinase activity is dependent on phosphorylation of GSK3β at tyrosine 216. In contrast, inhibition of kinase activity is dependent on phosphorylation of GSK3β at serine 9 [3]. Activation of GSK3β phosphorylates β-catenin and glycogen synthase. Phosphorylation of β-catenin targets it for proteolytic degradation. If β-catenin is not phosphorylated and degraded, it enters the nucleus, where it is available for transcription of the T-cell factor (TCF)/lymphoid enhancer-binding factor 1 pathway. Transcriptional targets downstream of this pathway include c-myc, E-cadherin, and cyclin D1 [8]. Inhibition of GSK3β kinase activity could block phosphorylation of β-catenin, a signaling event that investigators have suggested would promote cancer. However, in cancers of the peritoneal cavity including ovarian surface epithelium, colon, and pancreatic cells, GSK3β inhibitors (GSK3βi) induce apoptosis or reduce cell viability [3,9–12].

The induction of apoptosis by GSK3βi has been reported for ovarian cancer cells, and the goal of this research was to characterize novel maleimide inhibitors for increased efficacy [13]. Lithium chloride (LiCl) is a common GSK3βi, which could perturb the pathway in a nonselective manner and with relatively weak potency. Previously, LiCl induced $pGSK3\beta^{ser9}$ expression and increased apoptosis in immortalized ovarian surface epithelium

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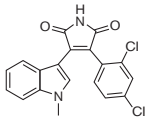
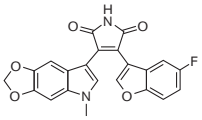
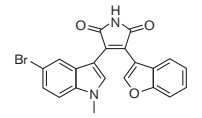
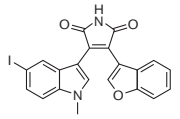
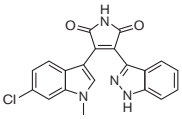
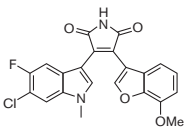
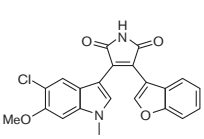
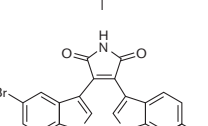
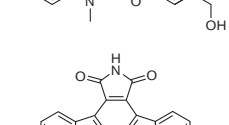
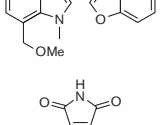
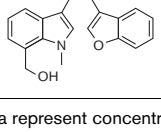
cells [14]. Tumor growth was inhibited by LiCl, which suggests that potent and more selective GSK3β inhibition could be effective in treating ovarian cancer [3,14,15]. A series of novel GSK3βi were profiled against two cancer cell lines and evaluated *in vitro* and *in vivo* for efficacy in slowing cell and tumor growth [13].

Table 1 Inhibitory concentration required for 50% cell death of glycogen synthase kinase 3 β inhibitors in ovarian cancer cells

Compound	Name code	IC_{50} (μmol/l) OVCA432	IC_{50} (μmol/l) SKOV3
	SB216763	>50	>50
	9-ING-41	11.2	10.5
	5-ING-135	33.6	74.5
	9-ING-49	20.0	26.7
	2-ING-173	13.0	36.7
	9-ING-87	10.7	42.7
	10-ING-52	26.6	>50
	FG2-007B	14.2	>50
	FG1-022A	58.3	>50
	FG1-029	>50	46.6
			

Data represent concentration required to kill 50% of the cells as measured using four concentrations of inhibitors and fit using sigmoidal dose–response curve with a variable slope.

Materials and methods

Cell culture and materials

OVCA432 ovarian cancer cells (RC Bast, MD Anderson, Houston,Texas,USA) were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acid, 1% sodium pyruvate, and 1% penicillin–streptomycin (Invitrogen, Carlsbad, California, USA). SKOV3 cells from American Type Culture Collection were grown in McCoy's 5A (Sigma Aldrich, St Louis, Missouri, USA), 10% fetal bovine serum, and 1% penicillin–streptomycin. Both cell lines were incubated at 37°C, 5% CO_2. Both SKOV3 and OVCA432 cell lines are sensitive to cisplatin treatment [16]. All GSK3β inhibitors were synthesized by Dr Kozikowski's group at the University of Illinois at Chicago as described previously [13,17,18]. SB216763 and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich, and LiCl was obtained from Fisher Science (Hanover Park, Illinois, USA).

Proliferation assays

Cells were seeded into 96-well plates at 5×10^3 cells per 100 μl in minimum essential medium media. The next day, fresh media with DMSO or test compounds in Table 1 at various concentrations were added to plates, and the cells were allowed to grow for 4 days. Proliferation was measured with CellTiter 96 Aqueous One Solution (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Spectrophotometric analysis was completed using a Biotek EL312e microplate reader (Fisher Biotek, Pittsburgh, Pennsylvania, USA). All conditions were tested in six replicates in triplicate experiments. The half-maximal inhibitory concentration (IC_{50}) value was determined as the concentration that caused 50% reduction in survival of cells.

4′,6-Diamidino-2-phenylindole and terminal deoxynucleotidyl transferase dUTP nick end labeling staining of ovarian cancer cells

Cells were plated at 5×10^4 cells per well in chamber culture slides from BD Biosciences (Bedford, Massachusetts, USA). The cells were allowed to attach overnight at 37°C and then treated with 0.1% DMSO, 50 μmol/l LiCl, 5 μmol/l 9ING41, or 25 μmol/l SB216763. One day later, the cells were fixed with 4% paraformaldehyde and washed with PBS. After fixation, slides were coverslipped with Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California, USA). For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, the DeadEnd Colorimetric TUNEL system (Promega) was used according to the manufacturer's protocol. All cells were imaged using a ×20 objective (Nikon DS-Ri1,

Huntley, Illinois, USA). Apoptotic cells were counted using ImageJ (National Institutes of Health). Cell death was determined by the appearance of condensed nuclear DNA and nuclear membrane fragmentation visible on the DAPI stains or positive brown TUNEL staining in three fields from triplicate experiments.

Western blot

Cell lysates were collected after 24 h treatment with 0.1% DMSO, 50 μmol/l LiCl, 5 μmol/l 9ING41, or 25 μmol/l SB216763. Cells were lysed in 150 μl lysis buffer [25 mmol/l Tris HCl pH 7.6, 150 mol/l NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Pierce, Rockford, Illinois, USA)], supplemented with Complete Mini Protease Inhibitor Cocktail tablets (Roche, Indianapolis, Indiana, USA) and Phosphatase Inhibitor Cocktail II (Sigma Aldrich). Equal protein concentrations were confirmed through a bicinchoninic acid assay (Pierce). Samples were run under reducing conditions in a Tris-glycine buffer using 10% acrylamide Bis–Tris gels. Gels were transferred to a polyvinylidene fluoride membrane using the iBlot dry transferring system (Invitrogen). Membranes were blocked for 1 h in 5% nonfat milk in Tris-buffered saline with 0.1% Tween, except for cleaved caspase 3, which was blocked in 5% bovine serum albumin in PBS with 0.5% Tween, and cyclin D1, which was blocked in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween. Phospho-GSK3β, phospho-glycogen synthase, cyclin D1, cleaved caspase 3, and cleaved poly-ADP ribose polymerase (PARP) antibodies were purchased from Cell Signaling Technologies (Beverly, Massachusetts, USA). Antiactin was purchased from Sigma Aldrich. The secondary antibody was a goat anti-rabbit horseradish peroxidase (Cell Signaling Technologies). Proteins were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, Illinois, USA) on an Alpha Innotech gel documentation system. Blots were performed in triplicate, and densitometry analysis was performed using ImageJ.

Xenograft

Female nude (nu/nu) mice aged 6–7 weeks were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA). Mice were placed in three separate categories ($n = 4$): DMSO/0.85% saline solvent (9 : 7), LiCl (340 mg/kg), and 9ING41 (40 mg/kg) [15]. Mice were injected with 4×10^6 SKOV3 cells in the rear hind limb subcutaneously. Once the tumors were palpable, drugs were injected intraperitoneally (i.p.) in 100 μl volume every other day for 21 days [3,15]. Tumor volumes were measured using digital calipers and quantified using the formula $(\pi/6) \times (\text{larger diameter}) \times (\text{smaller diameter})^2$. Body weights were measured every other day. At the end of the study, tumors were excised, weighed, and fixed in paraformaldehyde.

Range-finding toxicity studies

Sprague–Dawley rats were used for toxicity studies conducted at the Stanford Research Institute (SRI) International in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. 9ING41 was administered orally at 100 mg/kg or at 500 mg/kg to three male and three female Sprague–Dawley rats. On day 3, blood was collected for evaluation of the hematology and clinical chemistry parameters. Animals were killed on day 8, and macroscopic examinations were performed.

Bioavailability and pharmacokinetic studies

Male Sprague–Dawley rats were used for clearance studies conducted at SRI. Blood samples (approximately 300 μl) were collected ($n = 3$) in tubes containing EDTA as the anticoagulant. Samples were kept on ice and processed within 30 min of collection. Plasma was prepared by centrifuging blood samples at 2600 rpm at 2–8°C for 15 min and stored at −80°C. The extraction method included the addition of 100 μl acetonitrile containing 100 ng/ml hexylnicotinate (internal standard) to 50 μl of plasma. Samples were vortexed for 15 min, centrifuged for 10 min at 18 000*g*, and 100 μl of the supernatant was removed to a 150 μl glass insert in a 2 ml high-performance liquid chromatography vial for subsequent liquid chromatography tandem mass spectrometry analysis. Pharmacokinetic analysis was performed using noncompartmental methods and WinNonlin Professional (Version 5.2, Pharsight Corp, Mountain View, California, USA). The human-Eag-related gene (hERG) potassium channel assay was performed by Pharmaron Inc. (*http://www.pharmaron.com*).

Statistical analysis

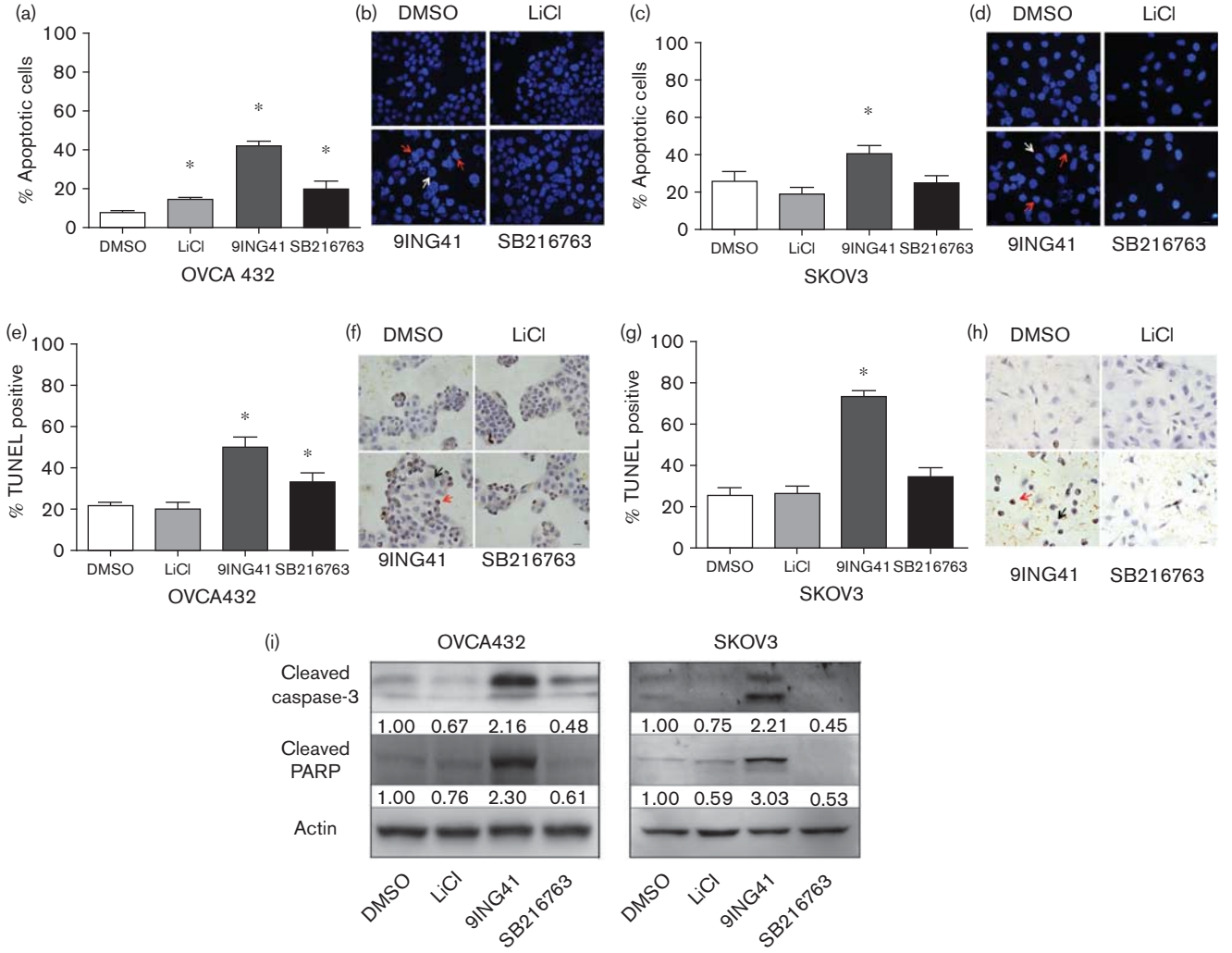
All values are expressed as the mean ± SEM. The Student *t*-test was used to assess differences between compound and negative control samples assuming two-tails. *P*-values of less than 0.05 were considered statistically significant. GraphPad Prism 4.02 was used to calculate IC_{50} values.

Results

Inhibition of GSK3β blocks ovarian cancer cellular proliferation

Nine GSK3β inhibitors were tested from chemical variants of a maleimide that were shown to have selectivity and higher inhibition of GSK3β compared with SB216763 using in-vitro kinase assays [13]. The inhibitors were screened against two serous ovarian cancer cell lines, OVCA432 and SKOV3, for their ability to slow proliferation after 96 h. OVCA432 cells are a more epithelial serous cell type with cuboidal shape and mutant p53 expression, whereas SKOV3 cells are a p53-null serous cell line with fibroblastic, invasive character-

Fig. 1



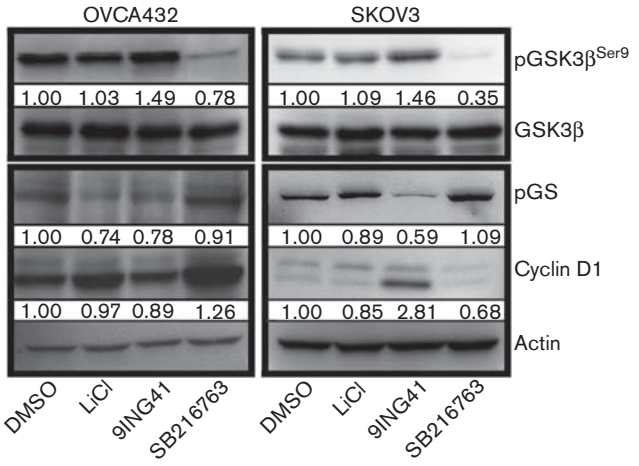
(a and c) Induction of cellular apoptosis by GSK3βi. OVCA432, and SKOV3 cells were treated with 0.1% DMSO, 50 μmol/l LiCl, 5 μmol/l 9ING41, and 25 μmol/l SB216763 for 24 h and stained with DAPI. DAPI-stained cells exhibiting condensed, pyknotic, or fragmented nuclei were representative of apoptotic cells. (b and d) Representative DAPI-stained OVCA432 (b) and SKOV3 (d) cells. White arrow indicates 'healthy cells,' and red arrow indicates 'apoptotic cells.' Scale bar 20 μm. (e and g) OVCA432 and SKOV3 cells were treated with GSK3βi for 24 h and then stained for TUNEL-positive apoptotic cells. TUNEL-positive cells are stained brown. (f and h) Representative TUNEL-stained OVCA 432 and SKOV3 (h) cells. Black arrow indicates 'healthy cells' and red arrow indicates TUNEL-positive cells. All data represent the average percentage of apoptotic cells ± SEM in three fields from three or more independent experiments. *Significantly different from DMSO $P<0.05$. Scale bar 20 μm. (i) Cleaved caspase-3 and cleaved PARP protein expression from OVCA432 and SKOV3 cell lines treated for 24 h with GSK3βi. The densitometry value from triplicate experiments is shown below each band. DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; GSK3βi, glycogen synthase kinase 3 β inhibitor; LiCl, lithium chloride; PARP, poly-ADP ribose polymerase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

istics. The IC_{50} values for the drugs compared with the commercially available inhibitor SB216763 are reported in Table 1. Of the novel inhibitors, four were consistently more active than SB216763 in both cell lines. Overall, 9ING41 was the most cytotoxic in both cell lines and was chosen as the candidate for further evaluation. On the basis of IC_{50} values taken from logarithmic doses spanning five concentrations, the optimal concentrations for in-vitro assays were determined.

Inhibition of GSK3β induces cellular apoptosis

To investigate possible mechanisms for inhibition of proliferation, apoptosis analyses on OVCA432 and SKOV3 cells were performed (Fig. 1a–d). LiCl and SB216763 were chosen as positive controls, and 9ING41 was used based on its potency in the cell growth assays. In OVCA432 cells, 50 μmol/l LiCl, 5 μmol/l 9ING41, and 25 μmol/l SB216763 induced apoptosis. In SKOV3 cells, only 5 μmol/l 9ING41 induced apoptosis compared with

Fig. 2



Protein expression in OVCA432 and SKOV3 ovarian cancer cells after treatment with GSK3βi for 24 h. Western blots were analyzed with antibodies specific for phospho-glycogen synthase kinase 3β (pGSK3β), phospho-glycogen synthase (pGS), and cyclin D1. GSK3β and actin antibodies were used as internal controls. Western blots were quantified with densitometry (values below each band). All data represent the average of three or more experiments.

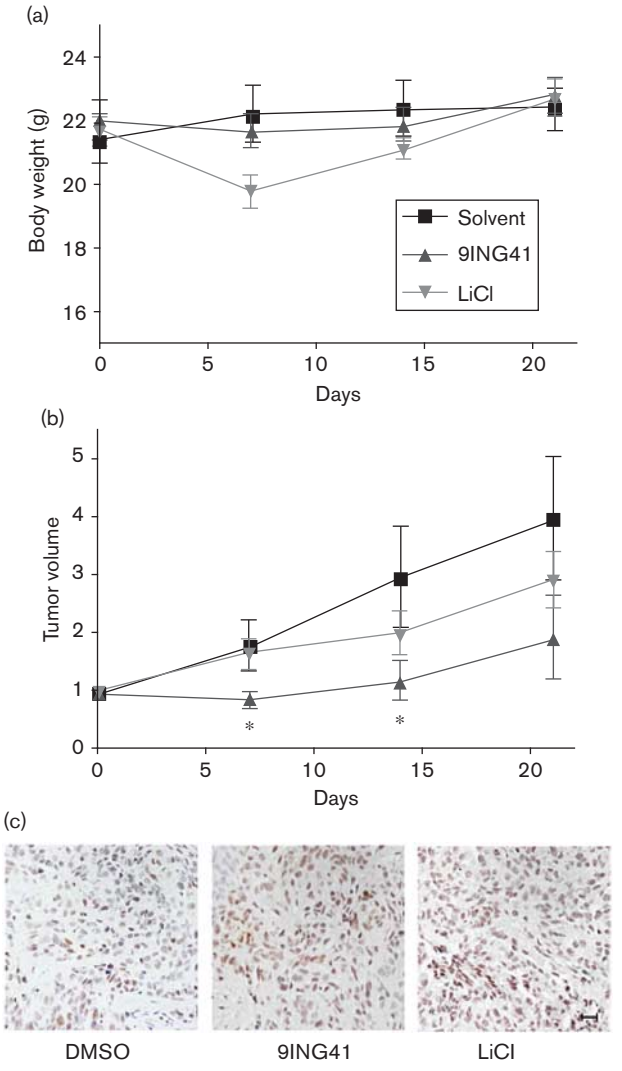
DMSO control. Much higher doses of LiCl have the ability to induce apoptosis as demonstrated previously [3].

To confirm that the cells were undergoing apoptosis, TUNEL staining was performed. Similar to DAPI analysis, 9ING41 significantly increased apoptosis in OVCA432 cells as compared with the negative control, DMSO (Fig. 1e,f). In addition, 9ING41 significantly increased apoptosis in SKOV3 as compared with DMSO (Fig. 1g,h). To establish the mechanism of apoptosis downstream of GSK3βi, caspase-3 cleavage was measured (Fig. 1i). Western blot analyses showed an increase in cleaved caspase-3 in OVCA432 and SKOV3 cells treated with GSK3βi 9ING41. Finally, PARP cleavage was investigated (Fig. 1i). The cleaved product of PARP was detected in both cell lines when treated with 9ING41, indicating that the cells were undergoing apoptosis.

GSK3β inhibitors block downstream signal transduction

To understand whether inhibition of GSK3β was directly responsible for cellular death, downstream targets of GSK3β were analyzed using western blots (Fig. 2). A serine 9 phospho-specific antibody for GSK3β was used to probe cell lysates collected from OVCA432 and SKOV3 after being treated with 0.1% DMSO, 50 µmol/l LiCl, 5 µmol/l 9ING41, and 25 µmol/l SB216763 for 24 h. 9ING41 increased phosphorylation of the inhibitory residue GSK3β^{ser9} in OVCA432 and SKOV3 cells when normalized to total GSK3β. GSK3β phosphorylates

Fig. 3



(a) Average body weights of animals treated with solvent, 9ING41, and LiCl. (b) Tumor progression in an SKOV3 xenograft injected intraperitoneally with GSK3βi. All data represent the average ± SEM of four independent animals treated with DMSO/0.85% saline solvent (9 : 7), 9ING41 (40 mg/kg), or LiCl (340 mg/kg) every 2 days for 21 days. Tumor volumes were calculated on first day palpable and then as a ratio over time compared with initial volume. *Significantly different from control $P<0.05$. (c) Immunohistochemical analysis of activated cleaved caspase 3 in xenograft tumors dissected from mice after treatment. Scale bar 10 µm. DMSO, dimethyl sulfoxide; GSK3βi, glycogen synthase kinase 3 β inhibitor; LiCl, lithium chloride.

glycogen synthase as one of its substrates. Therefore, the phosphorylation status of glycogen synthase was evaluated after treatment with compounds. Phosphorylation of glycogen synthase was significantly inhibited by 9ING41 in both cell lines demonstrating inhibition of GSK3β. Finally, expression of cyclin D1 was evaluated because this is a target gene of TCF/lymphoid enhancer-binding factor transcription following inhibition of GSK3β and stabilization of β-catenin. Cyclin D1 expression increased in SKOV3 cells treated with 9ING41.

Table 2 Plasma levels for 9ING41

	C[a] (ng/ml)	
Time (h)	9ING41 (i.v.) 10 mg/kg	9ING41 (p.o.) 400 mg/kg
0.083	1390	4.7
0.167	1410	18.2
0.5	1190	156
1	690	540
2	367	820
4	139	934
6	77	340
8	41	123
24	6.7	8.3

i.v., inravenous; p.o., oral.
[a]Blood was collected from three rats per group, per time point.

Table 3 Pharmacokinetic parameters[a] of 9ING41 in rats

Compound	C_o (ng/ml)	$T_{1/2}$ (h)	AUC (h ng/ml)	CL (ml/h/kg)	V (l/kg)	F (%)
9ING41 (i.v.) 10 mg/kg	1390	4.85	2740	3690	26.2	
9ING41 (p.o.) 400 mg/kg	1040	4.0	4950	1 32 600	810	4.5

AUC, area under the concentration–time curve; CL, systematic clearance; C_o, plasma concentration at $T=0$; $T_{1/2}$, apparent elimination half-life; F, bioavailability; i.v., intravenous; p.o., oral; V, volume of distribution.
[a]Mean value of three animals.

GSK3β inhibitors slow ovarian xenograft tumor growth

To validate these in-vitro findings, an in-vivo xenograft experiment was performed using SKOV3 cells. Animals were given an i.p. injection of either LiCl, 9ING41, or solvent (DMSO : saline) every other day for 21 days [15]. SKOV3 cells were selected on the basis of previous experiments that suggested that LiCl could reduce tumor burden in nude mice and because it is the most commonly used ovarian cancer cell line for xenografts [3]. Animal body weight was measured every other day, and no significant differences were found, suggesting limited toxicity from treatment with either drug (Fig. 3a). When the tumor volume was calculated and compared with the initial volume, the 9ING41-injected animals had significantly smaller tumor volumes at 7 and 14 days, suggesting that inhibition of GSK3β slows tumor growth (Fig. 3b). By the end of the study, all of the tumor volume ratios were similar. The tumors were excised and analyzed for protein expression by immunohistochemistry. Paraffin-embedded excised tumors were immunohistochemically stained with cleaved caspase-3 antibody. The presence of brown diaminobenzidine-stained nuclei marked tumor cells undergoing apoptosis (Fig. 3c).

Bioavailability and pharmacokinetic study

To determine whether the inhibitors could be developed as in-vivo drug therapies for ovarian cancer, a series of metabolic clearance and pharmacokinetics studies were conducted. Although in-vivo efficacy was detected for a reduction in tumor growth, higher doses would likely result in more effective chemotherapy. Therefore, the maximum dose that could be administered *in vivo* was determined. To establish the maximum tolerated dose (MTD) and potential toxic effects, 9ING41 was administered orally at 100 or 500 mg/kg to three male and three female Sprague–Dawley rats, and adverse effects were monitored throughout the testing period (8 days). These studies showed that 9ING41 was well tolerated, and no differences between animals treated with the compound and those treated with the control were observed with regard to any of the following parameters: mortality/morbidity, clinical observations, body weights, clinical pathology, and gross necropsy findings (data not shown). As no adverse effects were seen, the MTD is considered to be 500 mg/kg in rats.

To determine the bioavailability and plasma pharmacokinetics of 9ING41, male Sprague–Dawley rats were administered the compound at doses of 10 mg/kg (intravenously) and 400 mg/kg (orally). The mean plasma drug levels for the compound evaluated are summarized in Table 2. Pharmacokinetic analysis of the plasma level data (Table 3) indicated that the bioavailability of 9ING41 was 4.5%, and the volumes of distribution were ≥ 26 l/kg, suggesting that this drug was well distributed to the tissues. The elimination half-life was 4.0–4.85 h.

The inhibition potential of 9ING41 on the hERG potassium channel was assessed to determine cardiac side effects. In this assay, the hERG channel was overexpressed in U2OS cells, and activity was evaluated by measuring the permeability of the potassium channel to thallium and specific thallium dye. The Food and Drug Administration criterion for defining a drug as hERG positive is $IC_{50} < 1$ μmol/l. The potency of 9ING41 to inhibiting the hERG channel is low (IC_{50} 3.8 μmol/l).

Discussion

The Wnt pathway has been implicated in many cancers, including ovarian cancer; however, very few studies have used chemical inhibitors of this pathway for possible cancer therapies [6,19]. In advanced stages of ovarian cancer, an increased level of GSK3β is detected [20,21]. The overexpression of GSK3β increased proliferation of ovarian cancer cell lines, indicating that this protein has a unique role in ovarian cancer. SKOV3 was growth inhibited by LiCl and SB216763, but 9ING41 was significantly better at slowing cellular proliferation in both SKOV3 and OVCA432 [3]. In this study, the novel inhibitor of GSK3β, 9ING41, induced apoptosis. Cleaved PARP and activated caspase-3 were upregulated, indicating that GSK3βi induced apoptosis in two serous ovarian cancer cell lines. The absence of cleaved PARP and activated caspase-3 with LiCl and SB216763 could be dose and potency related. The downstream target of GSK3β, glycogen synthase, was phosphorylated less in response to 9ING41 compared with solvent control, suggesting that the drug was capable of blocking the

target kinase intracellularly. Cyclin D1 expression was different between the cell lines. Some have reported that cyclin D1 expression is decreased with inhibition of GSK3β [3,22,] whereas others have reported an increase in TCF-dependent activities, which correlates with an increase in apoptosis when GSK3β is inhibited in two different immortalized ovarian surface epithelium cell lines [14]. We have observed an increase in cyclin D1 expression in SKOV3 cells treated with 9ING41, but there was no increase in cellular proliferation. In fact, there was a significant increase in apoptosis in SKOV3 cells when treated with 9ING41. Our data coincide with the previous observation in which inhibition of GSK3β increases cytosolic β-catenin levels that, upon nuclear translocation, activates TCF transcription, which causes apoptosis of OSE [14]. Overall, the growth inhibition observed is not associated with changes in cell cycle. Although both LiCl and SB216763 are known GSK3β inhibitors, the compounds had different effects on OVCA432 and SKOV3 ovarian cancer cells. LiCl is a noncompetitive inhibitor that binds many different targets in the cell. Although both 9ING41 and SB216763 inactivate GSK3β kinase activity by competitively binding to the active ATP site, 9ING41 also enhanced GSK3β^{ser9} inhibitory phosphorylation [3,15].

Overexpression of GSK3β, as demonstrated by independent studies in ovarian cancer, conveys a growth advantage that, when blocked, allows for apoptosis and slower tumor growth [3,9]. *In vivo*, 9ING41 slowed tumor growth after 7 and 14 days. Although tumor volume did not remain significantly lower for the entire xenograft, metabolic clearance suggests that modification to the chemical structure to improve bioavailability, or dosing animals with a higher concentration of drug, might be able to improve the in-vivo antitumor activity. Although the maximum tolerance study was conducted on male rats, the lack of toxicity detected in nude female mice, based on body weight, implies that 9ING41 would not be toxic if tested in higher concentrations and suggests that further studies should be conducted to confirm the use of GSK3βi for serous ovarian cancers. These novel GSK3βi were initially synthesized to potentially treat a variety of conditions including neurodegeneration and cancer. The MTD studies used the oral route of administration to account for first-pass liver metabolism effects on serum concentration. For the in-vivo analysis of tumor xenografts, the dose and i.p. administration was chosen on the basis of previous studies evaluating subcutaneously grafted thyroid cancers [15]. Toxicity was not detected from either route of administration. As the nonselective inhibitor LiCl has been used for decades to treat neurological conditions, the likelihood that giving a GSK3β inhibitor would increase cancer risk has not been substantiated [23]. Interestingly, LiCl was reported to significantly slow SKOV3 tumor growth [3]. However, the previous study mixed the drugs and cells together before subcutaneous injection and therefore might have selectively induced apoptosis before the xenograft could properly form. This method could mimic a model for early-stage ovarian cancer or postcytoreductive surgery as the tumor was established. In this study, tumors were allowed to grow until palpable before the animals were systemically injected i.p. with the drugs. LiCl was not able to significantly reduce tumor growth *in vivo*, indicating the necessity for a novel and more selective GSK3βi, such as 9ING41.

The function of the GSK3β, APC, axin, and β-catenin complex in the human ovary and in human ovarian cancer is not fully understood [24]. In normal OSE, β-catenin seems to be stabilizing cell junctions and is mostly located at the cell surface [14,25]. Expression of mutant β-catenin or introduction of small interfering RNA against β-catenin was previously demonstrated to reduce apoptosis in ovarian cancer cells [14]. A significant increase in β-catenin and GSK3β has been detected in ovarian cancer as compared with normal cells without β-catenin nuclear localization [20,21,26]. Inhibition of GSK3β causes a reduction in N-cadherin, and this may provide a mechanism for reduced cellular proliferation and slower tumor growth *in vivo* [12,27].

The GSK3β chemical inhibitor 9ING41 is a novel drug capable of slowing proliferation by inducing apoptosis and reducing tumor volume *in vivo*. Although deregulation of many members of the Wnt pathway has been demonstrated in a variety of tumors, blocking GSK3β slowed tumor growth *in vitro* and *in vivo* by inducing apoptosis in ovarian cancer cells. Owing to the low toxicity associated with currently approved GSK3βi, such as LiCl, combined with lack of body weight changes in the current xenograft, these compounds merit additional studies to determine whether they might be a reasonable therapeutic approach for serous cancers. In summary, novel GSK3βi are more potent cytotoxic compounds for ovarian cancer cells as compared with commercially available inhibitors and may function to slow tumor growth by inducing apoptosis.

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Conflicts of interest

There are no conflicts of interest.

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