

BI2536, a potent and selective inhibitor of polo-like kinase 1, in combination with cisplatin exerts synergistic effects on gastric cancer cells

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Abstract. BI2536 is a highly selective and potent inhibitor of polo-like kinase 1 (PLK1). In this study, we aimed to determine whether BI2536 and cisplatin can synergistically inhibit the malignant behavior of gastric cancer cells. For this purpose, the expression of PLK1 in gastric cancer cells was determined. The effects of BI2536, cisplatin, and the combination of BI2536 and cisplatin on gastric cancer cell viability, invasion, cell cycle arrest and apoptosis were assessed. Furthermore, the expression of cell cycle-regulated proteins was examined. Moreover, the differentially expressed proteins between the SGC-7901 and SGC-7901/DDP (cisplatin-resistant) cells, and the enriched signaling pathways were analyzed by protein pathway array following treatment with BI2536 (IC₅₀) for 48 h. Our results revealed that PLK1 was upregulated in the SGC-7901/DDP (cisplatin-resistant) gastric cancer cells compared with the SGC-7901 cells. BI2536 enhanced the inhibitory effect of cisplatin on SGC-7901 cell viability and invasion. BI2536 induced G₂/M arrest in SGC-7901 and SGC-7901/DDP cells. BI2536 promoted cisplatin-induced gastric cancer SGC-7901/DDP cell apoptosis. It also induced the differential expression of 68 proteins between the SGC-7901 and SGC-7901/DDP cells, and these differentially expressed proteins were involved in

a number of cellular functions and signaling pathways, such as cell death, cell development, tumorigenesis, the cell cycle, DNA duplication/recombination/repair, cellular movement, and the Wnt/ β -catenin and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK)/ribosomal S6 kinase 1 (RSK1) signaling pathways. On the whole, our findings suggest that BI2536 and cisplatin synergistically inhibit the malignant behavior of SGC-7901/DDP (cisplatin-resistant) gastric cancer cells.

Introduction

Gastric cancer is a malignant tumor that is common worldwide and has a poor prognosis (1,2). The 5-year survival rate of patients with gastric cancer is <10% (3). The majority of patients are diagnosed at an advanced stage (4), and few efficacious treatment options are available for patients with this late stage of the disease (5). Surgical therapy combined with adjuvant chemotherapy is the primary treatment option for gastric cancer. It has been demonstrated that the single administration of traditional chemotherapeutic drugs, such as cisplatin and fluorouracil is only 10-20% efficacious in the treatment of gastric cancer (6). Even when combined with new drugs, such as docetaxel, irinotecan and oxaliplatin, the optimum reaction rate is <50% (7). Currently, an early diagnosis coupled with a good treatment strategy is considered an effective approach for the treatment of gastric cancer. The use of biomarkers has been confirmed to be a less invasive method for gastric cancer diagnosis (8). Moreover, targeted therapies for the treatment of gastric cancer have attracted increasing attention (9). However, there is still a lack of effective targeted therapies for the treatment of this disease.

Polo-like kinases (PLKs) are associated with oncogenesis in several types of cancer (10). PLKs exist in 4 isoforms,

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PLK1-4; however, only one of these isoforms, PLK1, is involved in centrosome maturation, chromosome segregation, bipolar spindle formation and cytokinesis execution (11). It has been reported that PLK1 exhibits oncogenic potential in gastric cancer (12). The inhibition of PLK1 following transfection with PLK1 siRNA and folate deficiency have been shown to synergistically inhibit the growth of gastric cancer cell lines (13). Moreover, a high PLK1 expression and DNA aneuploidy have been shown to correlate with a poor prognosis in patients with gastric cancer (14). PLK1 plays a key role in carcinogenesis and represents a promising target in the treatment of cancer (15,16). PLK1 inhibitors have recently emerged as a feasible strategy for the treatment of cancer (11). BI2536 is a highly selective and potent inhibitor of PLK1, which always participates in mitotic progression (17). Preclinical studies have indicated that BI2536 can disrupt spindle assembly, leading to mitotic arrest and the apoptosis of human cancer cell lines (18,19). However, the effects of BI2536 on the regulation of gastric cancer development have not yet been documented, at least to the best of our knowledge.

In the present study, the pivotal roles of BI2536 and cisplatin in regulating gastric cancer cell viability, migration, invasion and apoptosis were investigated. Differentially expressed proteins in gastric cancer cells treated with BI2536 (IC_{50}) for 48 h, as well as the signaling pathways of these differentially expressed proteins were analyzed by protein pathway array (PPA). The aim of this study was to determine whether BI2536 exerts an antitumor effect on gastric cancer and whether it can synergistically inhibit the malignant behavior of gastric cancer cells when used in combination with cisplatin. Our findings may provide new insight into the targeted therapy for this disease.

Materials and methods

Drugs and treatments. BI2536 (cat. no. 50-873-3) and cisplatin (cat. no. 50-901-13218) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and diluted in dimethyl sulfoxide (DMSO) in accordance with the manufacturer's instructions.

Cell culture. The human gastric cancer cell lines, AGS, BGC-823, Hs746T, N87, KATOIII, SGC-7901 and SGC-7901/DDP (a cisplatin-resistant cell line), were obtained from the Molecular Pathology Laboratory at Mount Sinai Medical Center (New York, NY, USA). The BGC-823, SGC-7901 and SGC-7901/DDP cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The AGS cells were grown in Ham's F12 medium. The Hs746T cells were cultured in DMEM containing 10% FBS. The KATOIII cells were maintained in IMDM mixed with 20% FBS. All media contained penicillin (100 U/ml) and streptomycin (100 U/ml), and all cells were cultured at 37°C in a humidified incubator at 5% CO_2 .

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay was used to evaluate cell viability. In brief, the cells (5,000 cells/well) at the logarithmic growth phase were seeded in 96-well plates. Following 24 h of incubation, the cells were treated with various concentrations of cisplatin

(1, 2, 4, 8, 16, 32 and 64 μ M) and BI2536 (1, 2, 4, 8, 16, 32 and 64 nM) for 72 h at 37°C. Subsequently, 20 μ l of MTT solution (5 mg/ml, pH 7.4) were added to each well, followed by incubation of the cells at 37°C for a further 4 h. After terminating the reaction, some of the supernatant was discarded, and 150 μ l of DMSO were added to dissolve the crystals. The absorbance (570 nm) was then measured using a microplate reader (serial no. 155489; Bio-Tek Instruments, Inc., Winooski, VT, USA). Each experiment was performed in triplicate. Furthermore, the half maximal inhibitory concentration (IC_{50}) of cisplatin and BI2536 was further calculated by the modified Kou-type method (20): $IgIC_{50} = X_m - I [P - (3 - P_m - P_n)/4]$, in which X_m indicates Ig maximum dose, I indicates Ig (maximum dose/adjacent dose), P indicates the sum of positive response rate, P_m indicates the largest positive response rate and P_n indicates the smallest positive response rate.

Colony formation assay. The cells were digested with 0.25% trypsin and split into individual cells. Subsequently, 50, 100 and 200 cells were seeded into 10-ml culture dishes and maintained under standard culture conditions for 2-3 weeks. When the colonies were visible to the naked eye, the culture dish was washed twice with phosphate-buffered saline (PBS). The colonies were then fixed with 4% paraformaldehyde for 15 min, followed by staining with crystal violet (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 20 min. Under a microscope (Nikon Eclipse TS100; Nikon Instruments, Badhoevedorp, The Netherlands) the colonies that comprised at least 10 cells were counted.

Cell invasion assay. Cell invasion was evaluated using Transwell chambers (8- μ m pore size; Corning Inc., Corning, NY, USA) coated with serum-free RPMI-1640 medium containing Matrigel (Sigma-Aldrich, Shanghai, China). In brief, the SGC-7901 and SGC-7901/DDP cells (5×10^4 cells) were grown in the upper chamber containing medium with 10% FBS, and BI2536 (IC_{10}) and cisplatin (IC_{50}) were then added to treat the cells. The lower chamber was filled with RPMI-1640 medium containing 20% FBS as a chemoattractant. Following incubation for 24 h at 37°C, the non-invading cells were removed using cotton swabs, and the invading cells were stained with 1% crystal violet for 30 min. The invading cells in different fields were then counted using a light microscope (Nikon Model Eclipse TS100LED MV; Nikon Corp., Tokyo, Japan).

Cell cycle analysis. The cells (1×10^5 cells/ml) were collected, washed twice with ice-cold PBS, and fixed with 75% ice-cold ethanol. After washing with ice-cold PBS again, the cells were suspended in 300 μ l of PBS and 20 μ l of RNase A was then added, followed by incubation of the cells for 30 min at 37°C. Subsequently, the cells were stained with 400 μ l of propidium iodide (PI) for 45 min in the dark. Cell cycle analysis at 488 nm was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell apoptosis analysis. Cell apoptosis was assessed by flow cytometry after Annexin V and PI staining (BD Pharmingen, San Diego, CA, USA). In brief, the cells (1×10^6 cells/ml) were harvested and resuspended in 1X Annexin V-binding buffer.

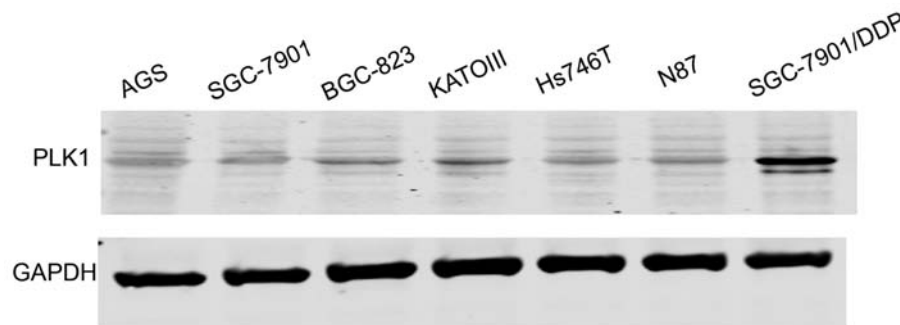


Figure 1. Expression of polo-like kinase 1 (PLK1) in the human gastric cancer cell lines, AGS, SGC-7901, BGC-823, KATOIII, Hs746T, N87 and SGC-7901/DDP.

Subsequently, 5 μ l of Annexin V-FITC was added, and the cells were incubated for 15 min away from light, followed by the addition of 10 μ l of PI and incubation of the cells for 5 min at 4°C. Cell apoptosis was then analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Western blot analysis. The cells were lysed with 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Using a Pierce BCA protein assay kit (Pierce, Rochford, IL, USA), the protein concentration was adjusted to 1 μ g/ μ l. An equal amount of protein extract was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The blots were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were then blocked in 5% non-fat milk in 1X TBST containing 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1% Tween-20 for 1 h. Primary antibodies to PLK1 (1:1,000; cat. no. sc-5585; Santa Cruz Biotechnology, Inc.), p-Cdc2 (1:1,000; cat. no. 9111; Cell Signaling Technology), cyclin B1 (1:1,000; cat. no. sc-594), p-cdc25c (1:1,000; cat. no. sc-327) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000; sc-32233) (all from Santa Cruz Biotechnology, Inc.) were added, followed by incubation of the membranes overnight at 4°C. GAPDH served as an internal control. Subsequently, the membranes were probed with horseradish peroxidase (HRP)-labeled secondary antibodies (1:10,000; cat. no. sc-2370 or sc-2371, Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. After washing with 1X TBST buffer, the bands were detected with a chromogenic substrate using the enhanced chemiluminescence (ECL) method and analyzed using the Quantity One software package (Bio-Rad).

Protein pathway array (PPA) analysis. The cells were lysed with 1X cell lysis buffer, and equal amounts of protein extracts were separated by 10% SDS-PAGE, as described above. The blots were then transferred onto nitrocellulose membranes (Bio-Rad). After blocking in 3% bovine serum albumin (BSA) for 1 h, the membranes were fixed on a western blotting manifold (Mini-PROTEAN II Multiscreen apparatus, cat. no. 170-4017; Bio-Rad) containing 20 channels. A total of 286 protein-specific or phosphorylation-specific antibodies (Table I) were used in the multiplex immunoblot. To each channel (1-19), a mixture of two antibodies dissolved in the blocking buffer was added, followed by incubation of the membranes overnight at 4°C; BSA without any antibody was added to channel 20. Following incubation

with HRP-conjugated secondary anti-rabbit (1:10,000; cat. no. sc-2371) or anti-goat (1:10,000; cat. no. sc-2370) or anti-mouse antibodies (1:10,000; cat. no. sc-2345) (all from Santa Cruz Biotechnology, Inc.) for 1 h, Immun-Star™ HRP Peroxide Buffer and Immun-Star™ HRP Luminol Enhancer (cat. no. 94547; Bio-Rad) were added followed by incubation of the membranes for 4 min. Chemiluminescence signals were then analyzed with the ChemiDoc XRS system (Bio-Rad). The same membranes was then washed twice with 1X TBST buffer and used to detect other primary antibodies, as described above. The signal intensity of each protein was analyzed using Quantity One software 4.5.0 (Bio-Rad). To reduce the variations caused by total protein loading amount, transferring and blotting efficiency, 'global median subtraction' was used to normalize the background subtracted intensity. The normalized expression of each protein = the average intensity of each protein in all samples \times (the signal intensity of each protein/the total intensity of all proteins in the same blot membrane).

Statistical analysis. All *in vitro* experiments were repeated 3 times and PPA was performed twice. All measurement data are expressed as the means \pm SD. The differences between groups were calculated using the Student's t-test or one-way ANOVA. Further comparison between groups was performed using a Tukey post-hoc test. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Unsupervised hierarchical clustering analysis was performed using the BRB ArrayTools Software V3.3.0. The significant pathway for the differentially expressed proteins was analyzed using Ingenuity Pathway Analysis (IPA) software. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PLK1 is upregulated in SGC-7901/DDP gastric cancer cells. As shown in Fig. 1, PLK1 was upregulated in the SGC-7901/DDP (cisplatin-resistant) gastric cancer cells compared with the SGC-7901 cells. Thus, we further explored the function of the PLK1 inhibitor, BI2536, in gastric cancer cells.

BI2536 enhances the inhibitory effects of cisplatin on the viability and colony-forming ability of the SGC-7901/DDP cells. As shown in Fig. 2A and B, cisplatin and BI2536 significantly inhibited the viability of the 7 gastric cancer cell lines in a dose-dependent manner. The highest chemosensitivity to cisplatin was observed in the BGC-823 and SGC-7901

Table I. List of antibodies included in the protein pathway array.

Antibodies specific for phosphorylation				
p-AKT (Ser473)	p-ERK5	p-p44/42 MAPK (Erk1/2)	p-PKCα/βII	p-STAT3
	(Thr218/Tyr220)	(Thr202/Tyr204)	(Thr638/641)	(Ser727)
p-β-catenin (Ser33/37/Thr41)	p-FAK (Tyr397)	p-p53 (Ser392)	p-PKCδ (Thr505)	p-STAT5 (Tyr694)
p-CDC2 (Tyr15)	p-GSK-3α/β (Ser21/9)	p-p70 S6 kinase (Thr389)	p-PTEN (Ser380)	
p-c-Jun (Ser73)	p-JNK(G-7)	p-P90RSK (Ser380)	p-Rb (Ser780)	
p-CREB (Ser133)	p-Met (Tyr1234)	p-PDK1 (Ser241)	p-Rb (Ser807/811)	
p-eIF4B (Ser422)	p-p38 MAPK (Thr180/Tyr182)	p-PKCα (Ser657)	p-Smad1/5 (Ser463/465)	
Antibodies specific for non-phosphorylation				
14-3-3 β	cSHMT	HER2/ErbB2	MMP-13	Rap1
α-tubulin	CTGF	HES1	MSR	Reg IV
ADAM8	CTLA-4	HGF	MTA1	RHAMM
ADAM10	CUL-1	HIF-1α	MTHFD1	RhoA
ADH	CX3CR1	HIF-2α	MTHFD2	Ribosomal protein L6
AIM2	Cyclin B1	HIF-3α	MTHFR	RIP
Akt	Cyclin D1	Hint	NALP1	RUNX3
ALG-2	Cyclin E	HMG-1	N-cadherin	SK3
Annexin I	Cytokeratin 5	HNF-3α	NFATc1	SLUG
ASCL1	Cytokeratin 18	HoxC11	NF-κB p50	Smad4
ASC-R	Cytokeratin 19	H-Ras	NF-κB p52	Smad7
ATF-1	DACH1	HSL	NF-κB p65	Snail
Aurora A/AIK	DARPP-32	HSP27	NHERF-2	SOD-1
Autotaxin	DDB2	HSP70	Nkx-3.1	SPAK
Axin	DHFR	Hsp90	nm23-H1/2/3	SRC-1
β3-tubulin	Dnmt1	ICAM-1	NMT1	Stat1
β-catenin	DPYD	IDO	NOS2	Stat3
Bad	DRG1	IFN-γ	Notch4	SUGT1
Bak	E2A	IGFBP5	NQO1	Survivin
Bax	E2F1	IGF-Irβ	ODC	Syk
Bcl-2	E-cadherin	IL-1β	OPN	Tak1
Bcl-6	Eg5	IL-3Rα	p14	Tau
Bcl-xL	EGFR	IL-6	p16	TCF-1
BECN1	eIF4B	IL-8	p27	TDP1
BID	Endoglin	IL-8RA	P2X7	TFIIH p89
BMP-2	ENT1	IL-11	p38α/β	TGF-β
Calpain 2	Ep-CAM	IL-18	p44/42 MAPK (Erk1/2)	TIMP-3
Calpastatin	EphB2	Integrin α4	P504S	TIP30
Calretinin	Epo	IRF-1	p53	TIRAP
CaMKKα	ERCC1	ITF	p63	TNF-R2
CARD12	ERα	Jagged1	p73	TNFα
Caspase-1	ERβ	JAK2	Pannexin-1	tPA
Cathepsin B	E-Selectin	JNK1	Patched	TRAF6
CD10	Factor XIII B	KAI1	Pax-2	TS
CD33	FAH	Keratin 10	PC2	tsg101
Cdc2 p34	FAS	KiSS-1	P-cadherin	TTF-1
Cdc25B	FEN-1	KLF6	PCNA	Twist
Cdc25C	FGF-8	K-Ras	PDEF	Tyro3
Cdc42	FGFR-4	LKB1	PEDF	uPA

Table I. Continued.

Cdk2	FKHR	LSD1	PERK	uPAR
Cdk4	FLIPS/L	L-Selectin	PKC α	VAP-1
Cdk6	Flt-3/Flk-2	Lyn	PKC ϵ	V-ATPase H
Cdx2	FOXM1	Maspin	PLK	VCAM-1
c-Fms/CSF-1R	FT α	MAT II β	PRL-3	VEGF
Chk1	FUS/TLS	MDM2	PSCA	Vimentin
c-IAP2	Fusin	Mesothelin	PSM	VSV-G
CKR-7	Galectin-3	MetAP-2	PSTPIP1	Wnt-1
Clusterin	GLP-1R	MetRS	PTEN	WT1
COL1A2	Glutamine synthetase	MGr1-Ag	Rab 7	XIAP
Connexin 43	GSTP1	MMP-2	Raf-B	YB-1
Cox-2	HCAM	MMP-7	RAGE	
CREB	HDAC1	MMP-9	RANKL	

The phosphorylation-specific antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), except for p-PKC α (Ser657) which was from Upstate Biotech (Lake Placid, NY, USA), and p-Met (Tyr1234), p-c-Jun kinase (G-7) and p-FAK (Tyr397) which were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The non-phosphorylation-specific antibodies, including Stat1, HER2/ErbB2, β -catenin, p44/42 mitogen-activated protein kinase [MAPK; extracellular signal regulated kinase (Erk)1/2], Akt, Notch4, eIF4B, NF- κ B p50, cAMP responsive element binding, estrogen receptor α , Bcl-xL, RIP, aurora A/AIK, matrix metalloproteinase (MMP)-9 and Snail were purchased from Cell Signaling Technology; X-linked inhibitor of apoptosis (XIAP) and glycogen synthase kinase (GSK) were from BD Biosciences (San Jose, CA, USA); transforming growth factor (TGF)- β was from R&D Systems (Minneapolis, MN, USA); Hsp90 was from Enzo Life Sciences (Farmingdale, NY, USA); hypoxia-inducible factor (HIF)-2 α was from Novus Biologicals (Littleton, CO, USA); cytokeratin 18 was from Dako Corp. (Carpinteria, CA, USA); fumarylacetoacetate hydrolase (FAH) was from Proteintech Group (Chicago, IL, USA); keratin 10 was from Covance Research Products (Berkeley, CA, USA); G protein of vesicular stomatitis virus was from Abcam (Cambridge, MA, USA); the other antibodies were from Santa Cruz Biotechnology, Inc.

cells, the IC₅₀ values of which were 2 and 6 μ M, respectively. The least chemosensitivity to cisplatin was exhibited by the Hs746T and SGC-7901/DDP cells, the IC₅₀ values of which were 30 and 60 μ M, respectively. Notably, BI2536 (IC₁₀) significantly enhanced the inhibitory effects of cisplatin on the viability of the gastric cancer cells, particularly by improving the chemosensitivity of SGC-7901/DDP to cisplatin (Fig. 2C). Therefore, a colony formation assay was then performed using the SGC-7901 and SGC-7901/DDP cells in order to verify the effects of BI2536 and cisplatin on cell viability. As shown in Fig. 2D and E, BI2536 (IC₅) alone did not inhibit colony formation compared with the controls (P>0.05); however, cisplatin (IC₁₀) significantly inhibited colony formation (P<0.05), particularly in the SGC-7901 cells (P<0.01). Following co-treatment with BI2536 (IC₅) and cisplatin (IC₁₀), the results revealed that BI2536 (IC₅) significantly enhanced the inhibitory effects of cisplatin on the colony-forming ability of the SGC-7901/DDP cells (P<0.01), but not that of the SGC-7901 cells (P>0.05).

BI2536 enhances the inhibitory effects of cisplatin on the invasive ability of the SGC-7901/DDP cells. We further determined the effects of BI2536 and cisplatin on gastric cancer cell invasion (Fig. 3). The results revealed that BI2536 (IC₁₀) did not inhibit the invasive ability of the SGC-7901 and SGC-7901/DDP cells (P>0.05), although cisplatin (IC₅₀) significantly inhibited the invasive ability of the cells (P<0.05). Moreover, following treatment with a combination of BI2536 (IC₁₀) and cisplatin (IC₅₀), only the inhibitory effects

of cisplatin on the invasiveness of the SGC-7901/DDP cells, but not that of the SGC-7901 cells (P>0.05), were enhanced (P<0.01).

BI2536 significantly induces G₂/M arrest in the SGC-7901/DDP cells. In the cell cycle analysis, the SGC-7901 cells were treated with 1, 5 and 10 nM BI2536 for 72 h, and the SGC-7901/DDP cells were treated with 5, 10 and 20 nM BI2536 for 24 h. The results of flow cytometry revealed that BI2536 significantly induced G₂/M arrest in both the SGC-7901 and SGC-7901/DDP cells (P<0.05) (Fig. 4A and B). We further determined the expression of key proteins involved in the G₂/M cell cycle, including p-Cdc2, cyclin B1 and p-Cdc25C by western blot analysis (Fig. 4C and D). We found that PLK1 expression was not significantly altered following treatment with various concentrations of BI2536 in both the SGC-7901 and SGC-7901/DDP cells (P>0.05). Notably, compared with the control group, BI2536 treatment resulted in the decreased expression of p-Cdc25C and in the increased expression of p-Cdc2 and cyclin B1 in the SGC-7901/DDP cells in a dose-dependent manner (P<0.01) (Fig. 4D), while the expression levels of these proteins exhibited no significant changes in the SGC-7901 cells (P>0.05).

BI2536 promotes cisplatin-induced SGC-7901/DDP cell apoptosis. Flow cytometry was also performed to determine the effects of BI2536 on gastric cancer cell apoptosis. Following treatment with various concentrations of BI2536 for 24 h, the proportions of SGC-7901 and SGC-7901/DDP cells

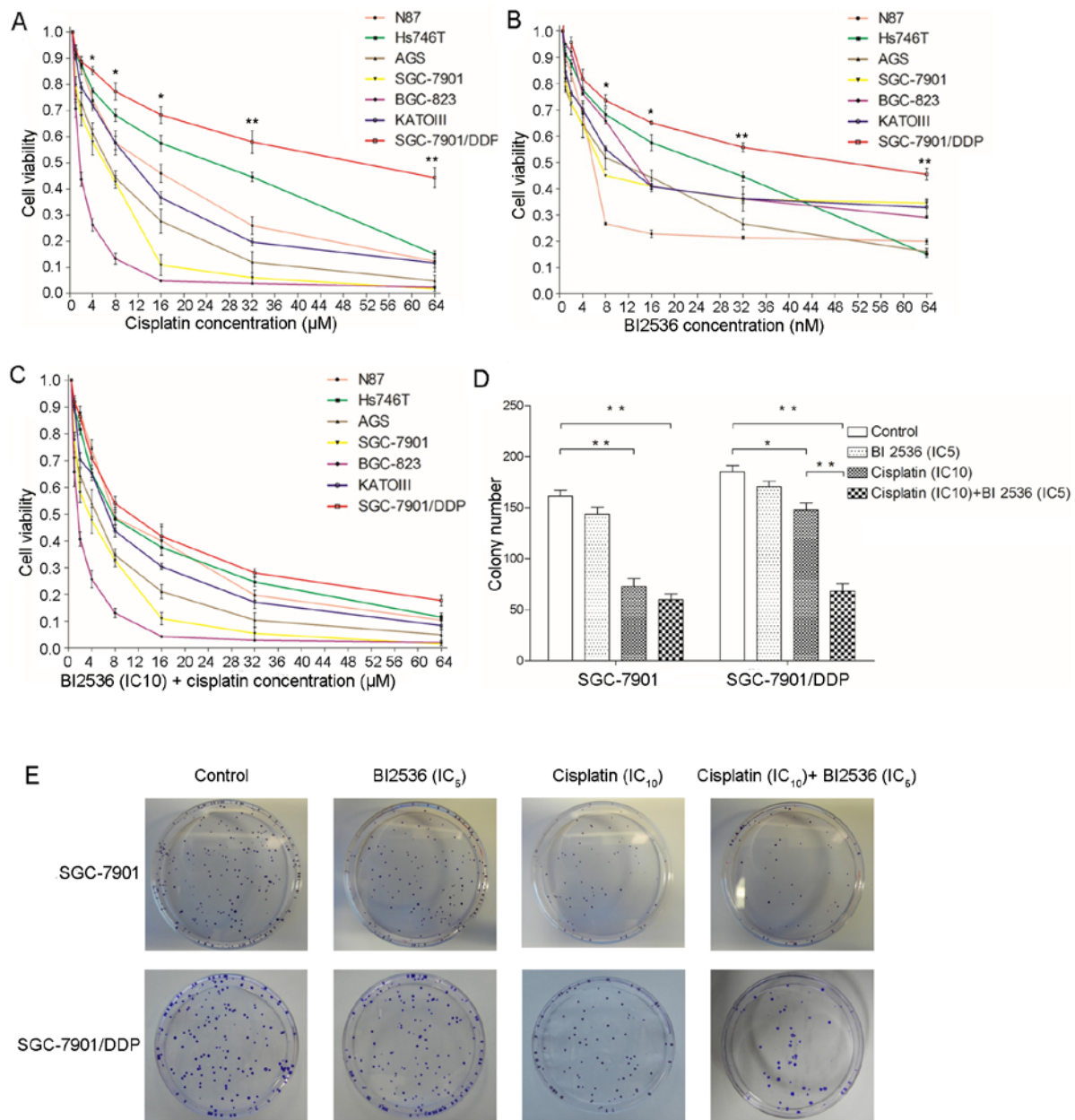


Figure 2. BI2536 enhances the inhibitory effects of cisplatin on gastric cancer cell viability. (A) Effects of cisplatin on AGS, BGC-823, Hs746T, N87, KATOIII, SGC-7901 and SGC-7901/DDP cell viability. (B) Effects of BI2536 on the viability of the above-mentioned 7 gastric cancer cell lines. (C) Effects of the combination of cisplatin and BI2536 on the viability of the above-mentioned 7 gastric cancer cell lines. (D and E) Colony formation assay revealed the effects of BI2536 and cisplatin on SGC-7901 and SGC-7901/DDP cell viability. Error bars indicate the means \pm SD and the symbol * indicates a statistically significant difference (* P <0.05 and ** P <0.01).

undergoing early apoptosis were all significantly increased (P <0.05) (Fig. 5A and B). Furthermore, we found that cisplatin significantly induced SGC-7901 and SGC-7901/DDP cell apoptosis when used in combination with BI2536 (IC₂₀) (P <0.05) (Fig. 5C and D). Notably, BI2536 (IC₂₀, 20 nM) significantly promoted cisplatin-induced SGC-7901/DDP cell apoptosis (P <0.05) (Fig. 5D).

BI2536 induces the differential expression of signaling proteins between the SGC-7901 and SGC-7901/DDP cells. We applied PPA analysis to analyze the differentially expressed proteins between the SGC-7901 and SGC-7901/DDP cells following treatment with BI2536 (IC₅₀) for 48 h. We found that 68 proteins were differentially expressed when compared

with the controls (Fig. 6A). IPA analysis also revealed that the differentially expressed proteins induced by BI2536 treatment were involved in many cell functions and signaling pathways, such as cell death, cell development, tumorigenesis, the cell cycle, DNA duplication/recombination/repair, cellular movement, and in the Wnt/ β -catenin and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK)/ribosomal S6 kinase 1 (RSK1) signaling pathways (Fig. 6B).

Discussion

Cisplatin is a common and effective anticancer drug; however, its use is limited due to its related side-effects, such as renal, gastrointestinal and neurological toxicities (21). Therefore, to

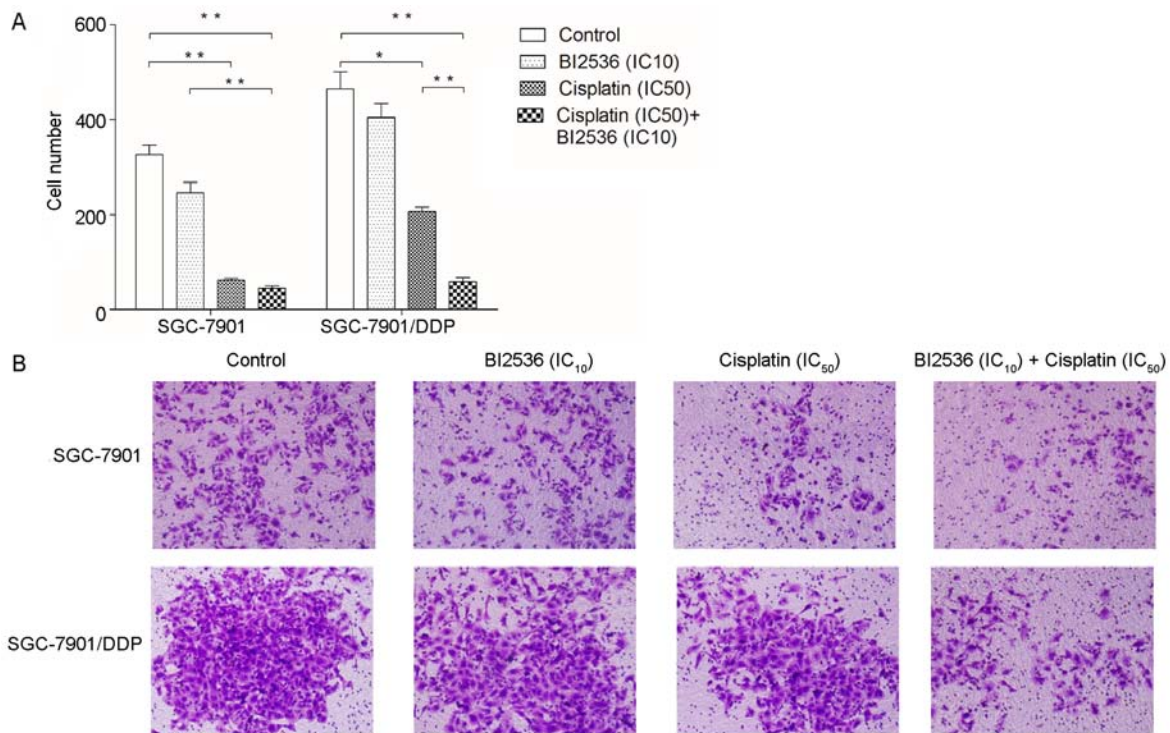


Figure 3. BI2536 enhances the inhibitory effects of cisplatin on gastric cancer SGC-7901/DDP cell invasion. (A) Quantitative results of the number of invading SGC-7901/DDP and SGC-7901 cells. (B) Transwell assay revealed the invading SGC-7901/DDP and SGC-7901 cells. Error bars indicate the means \pm SD and the symbol * indicates a statistically significant difference (* P <0.05 and ** P <0.01).

improve the antitumor efficacy of cisplatin and reduce cisplatin-induced side-effects, further studies are warranted in order to aid the development of small-molecule drugs. In the present study, we combined the PLK1 inhibitor, BI2536, with cisplatin to treat gastric cancer cells and to determine whether BI2536 and cisplatin can synergistically inhibit the malignant behavior of gastric cancer cells. The results revealed that BI2536 enhanced the cisplatin-induced inhibitory effects on SGC-7901/DDP cell viability and invasion. BI2536 induced G₂/M arrest in the SGC-7901/DDP cells by decreasing the expression of p-Cdc25C and increasing the expression of p-Cdc2 and cyclin B1. BI2536 promoted cisplatin-induced SGC-7901/DDP cell apoptosis. Moreover, BI2536 induced the differential expression of 68 proteins between the SGC-7901 and SGC-7901/DDP cells, and these differentially expressed proteins were involved in several cell functions and signaling pathways, such as the Wnt/ β -catenin and MEK/ERK/RSK1 signaling pathways.

In many anticancer treatments, the G₂/M checkpoint is an effective target site for molecular targeted therapy and chemotherapy sensitization (22,23). There are data to suggest that mammalian PLK1 plays a regulatory role at the cell cycle G₂ checkpoint (24,25). PLK1 has been implicated in mitotic entry via the activation of Cdc25C (26). PLK1 has also been identified as a target that can sensitize cells to traditional chemotherapeutic drugs in the treatment of cancer (27,28). In addition, a high degree of G₂/M arrest induced by PLK1 inhibition has been found to be associated with radiosensitization in various cancer cell lines (29). The combination of MS275 and BI2536 has been shown to synergistically inhibit cell growth and to induce G₂/M phase arrest in A549 non-small cell lung cancer cells (30). Gleixner *et al* demonstrated that the inhibitory effect of BI2536 on CML cell growth was

associated with mitotic arrest, particularly G₂/M arrest, and consecutively resulted in apoptosis (31). In this study, BI2536 enhanced the cisplatin-induced inhibitory effects on SGC-7901 cell viability and invasive ability. BI2536 induced G₂/M arrest in the SGC-7901/DDP cells by decreasing the expression of p-Cdc25C and increasing the expression of p-Cdc2 and cyclin B1. BI2536 promoted cisplatin-induced SGC-7901/DDP cell apoptosis. Taken together, we speculate that the combination of cisplatin and BI2536 can synergistically inhibit cell growth, induce G₂/M phase arrest, and consecutively induce the apoptosis of SGC-7901/DDP cells.

Furthermore, we applied PPA analysis to examine the differentially expressed proteins between the SGC-7901 and SGC-7901/DDP cells following treatment with BI2536 (IC₅₀) for 48 h. A total of 68 proteins were found to be differentially expressed, which were involved in signaling pathways, such as the Wnt/ β -catenin and MEK/ERK/RSK1 signaling pathways. It has been reported that Wnt/ β -catenin signaling plays a key role in regulating the self-renewal of gastric cancer stem cells, and salinomycin treatment may be used for the treatment of gastric cancer by targeting Wnt/ β -catenin signaling (32). The inhibition of the Wnt/ β -catenin pathway by niclosamide has been shown to result in decreased cellular proliferation and increased cell death in gastric cancer (33). In addition, ERK/RSK1 activation by growth factors can delay the cell cycle at the G₂ phase, thus reducing mitotic aberrations and maintaining genomic integrity (34). Notably, PLK1 is involved in mitotic arrest via the inhibition of the MEK/ERK/RSK1 cascade (35). Although the association between BI2536 and the Wnt/ β -catenin or MEK/ERK/RSK1 signaling pathways has not yet been verified experimentally, our results provide an important indication pertaining to BI2536 likely promoting

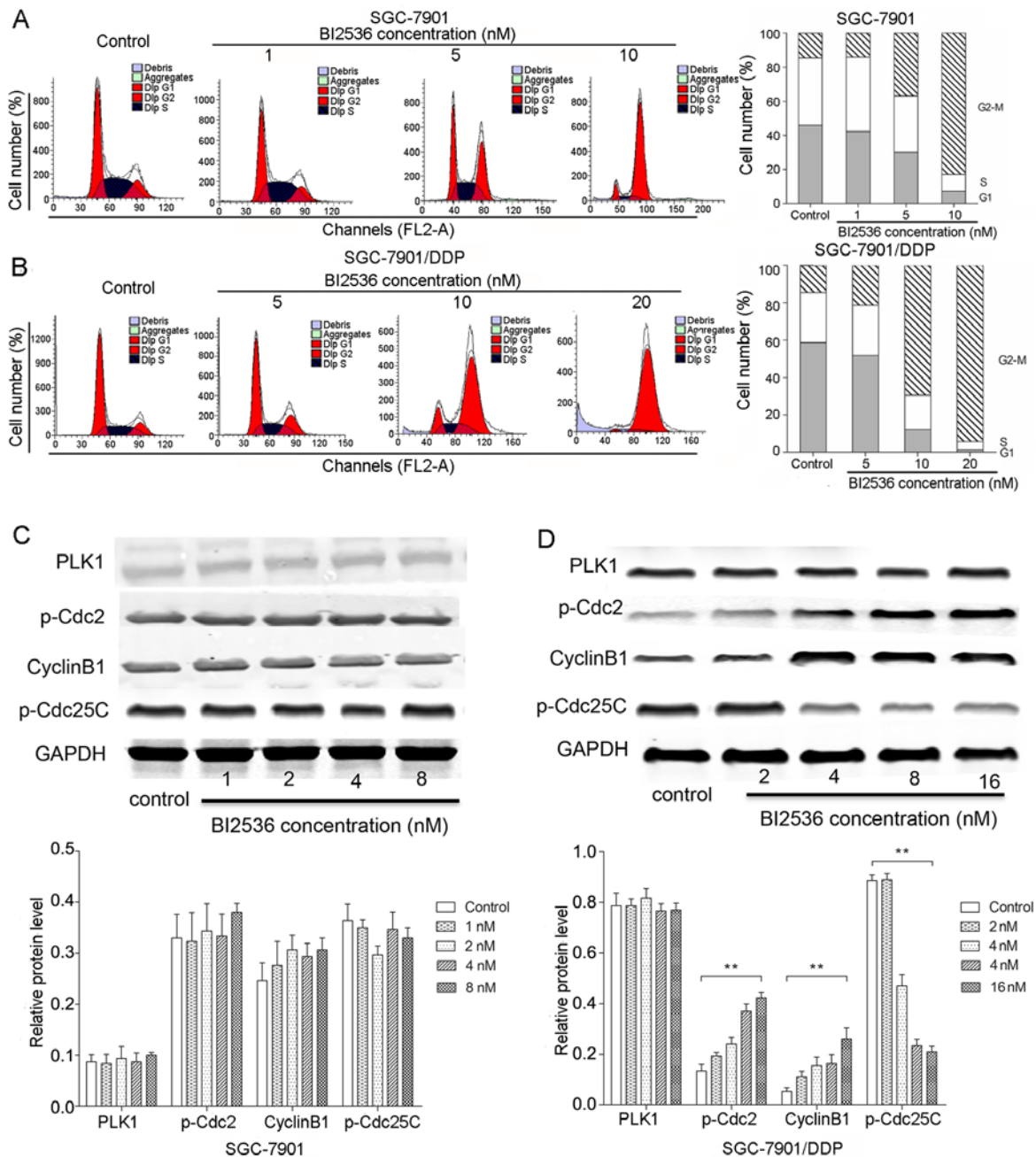


Figure 4. BI2536 induces G₂/M arrest in SGC-7901 and SGC-7901/DDP gastric cancer cells. (A and B) Flow cytometry demonstrated that BI2536 significantly induced G₂/M arrest in the SGC-7901 and SGC-7901/DDP cells. (C and D) The expression of key proteins involved in the G₂/M cell cycle, including p-Cdc2, cyclin B1 and p-Cdc25C was examined by western blot analysis. Error bars indicate the means \pm SD and the symbol * indicates a statistically significant difference (** $P < 0.01$). PLK1, polo-like kinase 1.

the chemotherapeutic sensitivity of SGC-7901/DDP cells to cisplatin via the involvement of the Wnt/ β -catenin or MEK/ERK/RSK1 signaling pathways.

The strengths of our study were that BI2536 and cisplatin synergistically inhibited the malignant behavior of the SGC-7901/DDP (cisplatin-resistant) gastric cancer cells, which may provide a broader perspective for improving the chemotherapeutic sensitivity of cancer cells to cisplatin. Despite the clear strength of our study, however, some limitations merit further consideration. Firstly, there were no significant effects of BI2536 treatment alone on cell viability, migration and apoptosis, which limited the clinical application of BI2536. Secondly, the synergistic effects of BI2536 and

cisplatin were not verified using gastric cancer primary cells or an *in vivo* xenograft model of SGC7901 and SGC7901/DDP cells. Further research is still required in order to verify the synergistic interaction between BI2536 and cisplatin in gastric cancer primary cells. Thirdly, we did not analyze PLK1 expression according to the information of the The Cancer Genome Atlas (TCGA) and Cancer Cell Line Encyclopedia (CCLE) databases. Further studies are required to investigate the role of PLK1 in SGC7901 and SGC7901/DDP gastric cancer cells using siRNA-mediated gene knockdown. Fourthly, signaling pathways were only analyzed by PPA. The expression of Wnt/ β -catenin and MEK/ERK/RSK1 signaling pathway-related proteins were not determined by qPCR or

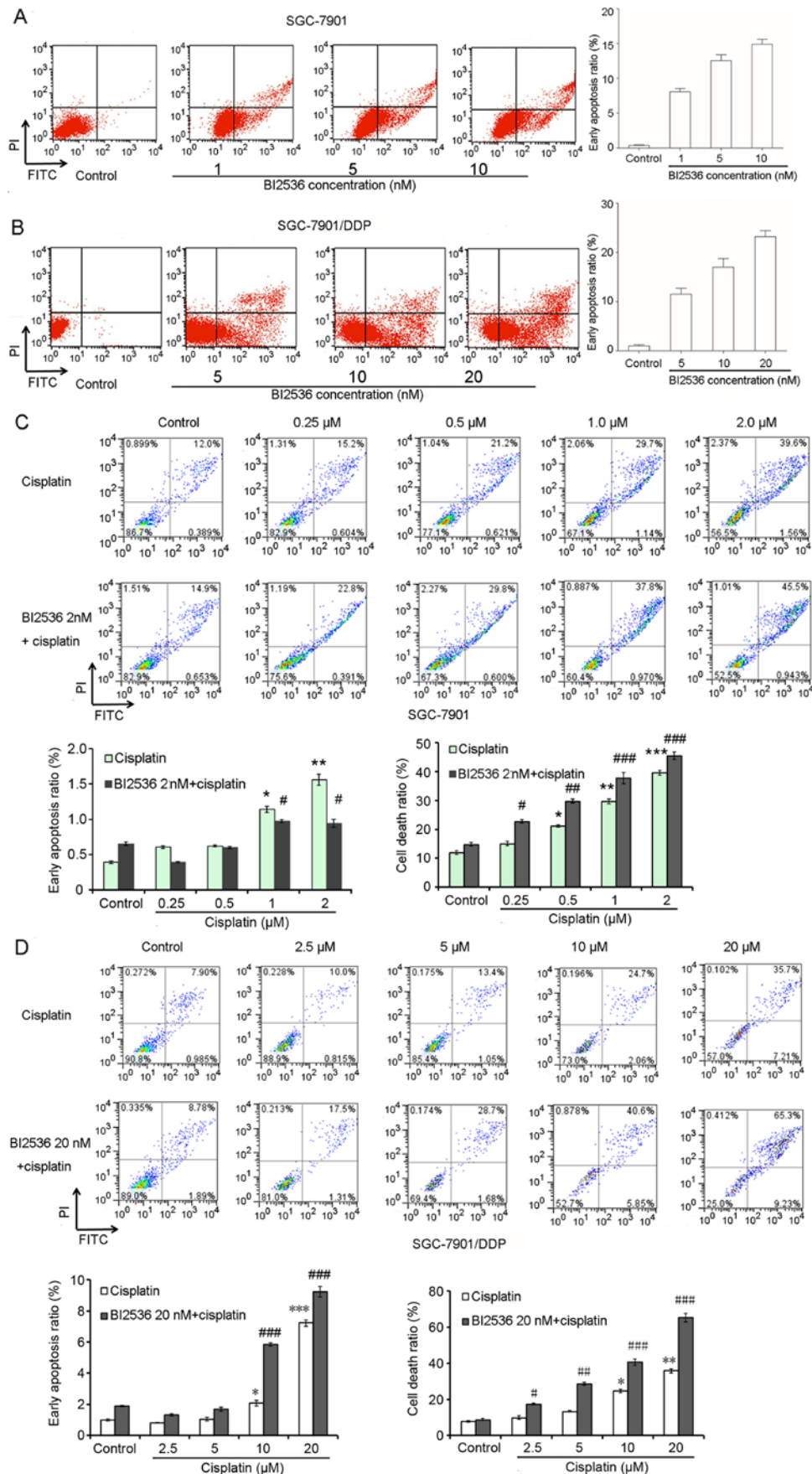


Figure 5. BI2536 promotes cisplatin-induced SGC-7901/DDP gastric cancer cell apoptosis. (A and B) Flow cytometry demonstrated the effects of BI2536 on SGC-7901 and SGC-7901/DDP cell apoptosis. (C) Flow cytometry demonstrated the effects of the combination of various concentrations of cisplatin (0, 0.25, 0.5, 1 and 2 μ M) and BI2536 (2 nM) on SGC-7901 and SGC-7901/DDP cell apoptosis. (D) Flow cytometry demonstrated the effects of the combination of various concentrations of cisplatin (0, 2.5, 5, 10 and 20 μ M) and BI2536 (20 nM) on SGC-7901 and SGC-7901/DDP cell apoptosis. Error bars indicate the means \pm SD, and the symbols * and # indicate a statistically significant difference compared with the corresponding control group. * p <0.05, ** p <0.01 and *** p <0.001.

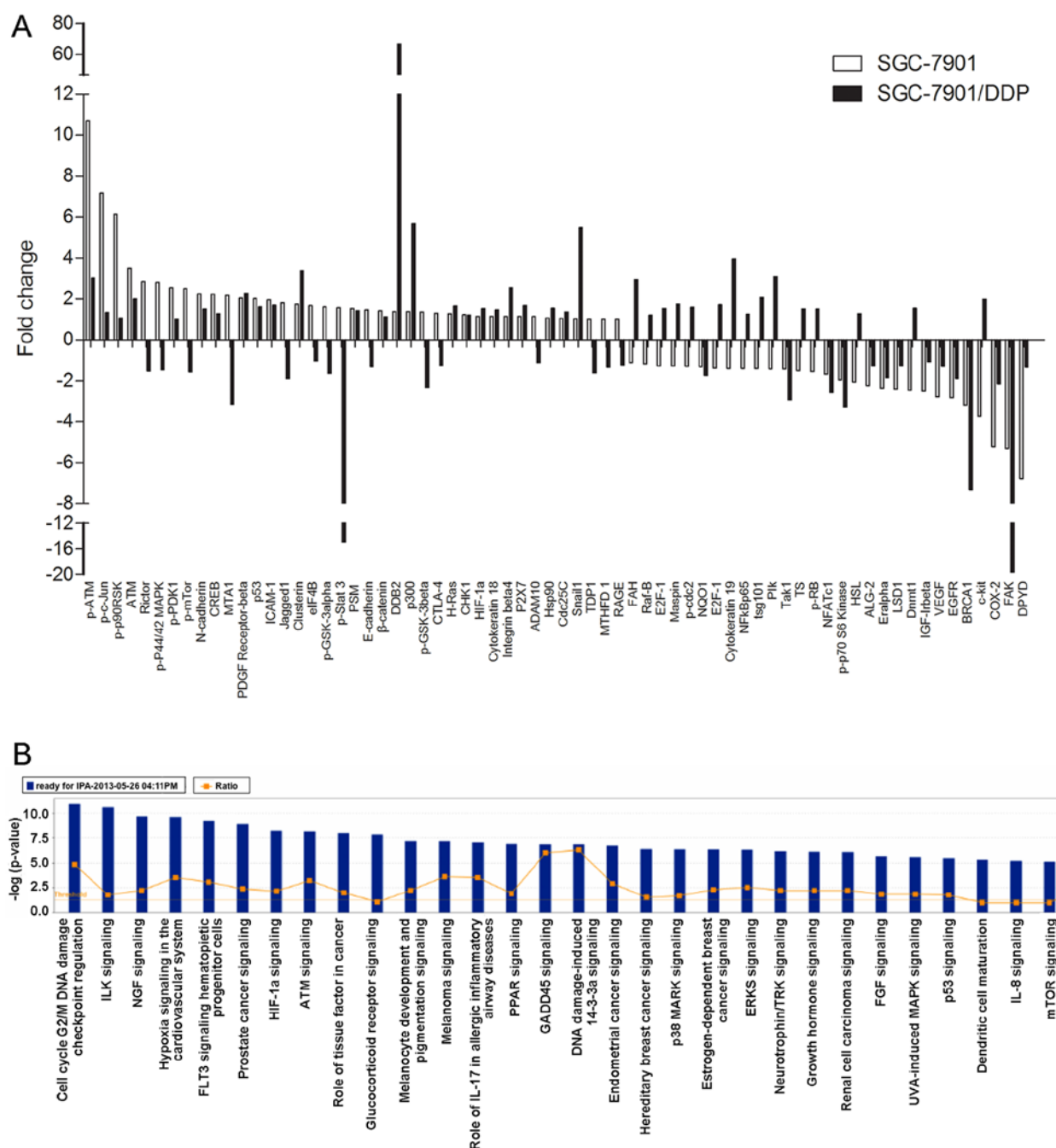


Figure 6. BI2536 induces the differential expression of signaling proteins between the SGC-7901 and SGC-7901/DDP cells, as determined by protein pathway array. (A) Proteins differentially expressed between the SGC-7901 and SGC-7901/DDP cells following treatment with BI2536 (IC_{50}) for 48 h. (B) Ingenuity Pathway Analysis (IPA) analysis revealed the cellular functions and signaling pathways enriched by the differentially expressed proteins induced by BI2536.

western blot analysis in treated samples. Fifthly, we only used MTT assay to determine changes in cell viability, which only monitored the ATP-dependent metabolic activity. To better detect the synergistic effects of BI2536 and cisplatin on cell proliferation, BrdU DNA proliferation assay should also be performed to monitor the number of cellular divisions and DNA synthesis. Finally, we only analyzed the differentially expressed proteins between the SGC-7901 and SGC-7901/DDP cells following treatment with BI2536 (IC_{50}) for 48 h. The key mechanisms involved in the combined effects of BI2536 and cisplatin treatment in regulating the malignant behavior of gastric cancer cells remain largely unknown. Therefore, further studies are still required in order to verify our observations.

In conclusion, the findings of the present study suggest that BI2536 and cisplatin synergistically inhibit the malignant behavior of SGC-7901/DDP (cisplatin-resistant) gastric cancer cells. BI2536 may enhance the chemotherapeutic sensitivity of SGC-7901/DDP cells to cisplatin via the involvement of the Wnt/ β -catenin or MEK/ERK/RSK1 signaling pathways. The development of a PLK1 inhibitor may thus be an effective strategy for the treatment of gastric cancer.

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Competing interests

The authors declare that they have no competing interests.

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