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GSK1070916, a potent Aurora B/C kinase inhibitor with broad antitumor activity in tissue culture cells and human tumor xenograft models

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Abstract

The protein kinases, Aurora A, B, and C have critical roles in the regulation of mitosis and are frequently overexpressed or amplified in human tumors. GSK1070916, is a novel ATP competitive inhibitor that is highly potent and selective for Aurora B/C kinases. Human tumor cells treated with GSK1070916 show dose-dependent inhibition of phosphorylation on serine 10 of Histone H3, a substrate specific for Aurora B kinase. Moreover, GSK1070916 inhibits the proliferation of tumor cells with EC_{50} values of <10 nmol/L in over 100 cell lines spanning a broad range of tumor types. Although GSK1070916 has potent activity against proliferating cells, a dramatic shift in potency is observed in primary, nondividing, normal human vein endothelial cells, consistent with the proposed mechanism. We further determined that treated cells do not arrest in mitosis but instead fail to divide and become polyploid, ultimately leading to apoptosis. GSK1070916 shows dose-dependent inhibition of phosphorylation of an Aurora B–specific substrate in mice and consistent with its broad cellular activity, has antitumor effects in 10 human tumor xenograft models including breast, colon, lung, and two leukemia models. These results show that GSK1070916 is a potent Aurora B/C kinase inhibitor that has the potential for antitumor activity in a wide range of human cancers. [Mol Cancer Ther 2009;8(7):1808–17]

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Introduction

The Aurora kinases comprise three isoforms in mammalian cells, Aurora A, B, and C, and members of this family have been extensively studied in a wide range of different model organisms (reviewed in refs. 1, 2). The protein kinase activity of each member is cell cycle dependent, with their activities gradually increasing at S phase and peaking at G_2-M phase in parallel with elevation of their mRNA and protein expression (3–6). Subsequently, the kinases are degraded by the proteosome upon exit from mitosis through the ubiquitin dependent APC/c pathway (7–9).

Despite a high degree of sequence identity in their catalytic domains, the Aurora kinases exhibit different subcellular localization and functions (1, 2). Aurora A localizes to the duplicated centrosomes and to the spindle poles in mitosis (10). It has been implicated in several processes required for the generation of bipolar spindle apparatus, including centrosome maturation and separation. Aurora C was originally proposed to have a restricted function in meiosis, but more recent findings suggest that it is more closely related to Aurora B with overlapping functions and similar localization patterns (11). Aurora B is a chromosomal passenger protein in complex with at least three other proteins including, inner centromere protein (INCENP), Survivin, and Borealin. Aurora B has been shown to localize to the centromeric regions of chromosomes in the early stages of mitosis but at the onset of anaphase relocates to the microtubules at the spindle equator (12). As the spindle elongates and undergoes cytokinesis, Aurora B accumulates in the spindle midzone and at the site of cleavage furrow ingression before concentrating at the midbody. During mitosis, Aurora B is required for phosphorylation of histone H3 on serine 10 and has been proposed to be important for chromosome condensation. Aurora B has been shown to regulate kinetochore function as it is required for correct chromosome alignment and segregation. Aurora B is also required for spindle checkpoint function and cytokinesis (1, 13, 14).

Aurora B is strongly associated with human malignancies due to frequent overexpression in a number of tumors, including colorectal, prostate, non–small cell lung, thyroid, glioblastoma, and an increased expression level has been reported to correlate with advanced stages of colorectal cancer (15–19). Furthermore, Aurora B has been shown to map to a region of the chromosomal loci known to be tumor-associated amplicons (20, 21). Aurora B kinase therefore represents a promising therapeutic target and several structurally diverse small molecule inhibitors have been reported (22, 23). With the notable exception of AZD1152, which is selective for Aurora B kinase, the majority of these

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compounds are pan-Aurora inhibitors, inhibiting all three Aurora family members.

As Aurora B expression is limited to proliferating cells, the clinical application of Aurora B inhibitors for the treatment of cancer is not expected to suffer from neurotoxicity or other side effects associated with compounds that more generally affect tubulin in nondividing cells. Furthermore, Aurora B inhibitors, unlike classic antimitotics (e.g., taxanes, Vincas, and kinesin inhibitors), do not induce a mitotic arrest but instead result in a failure of cytokinesis leading to cell death. Therefore, inhibitors of Aurora B would be expected to show clinical utility distinct from that of antitubulin compounds as well as other antimitotic drugs known to cause a mitotic arrest.

This report describes a novel, ATP competitive, Aurora B/C kinase inhibitor with potent enzyme and broad cellular activity. Evaluation of GSK1070916 in 161 tumor cell lines of diverse histology shows potent antiproliferative activity with a median EC_{50} value of 8 nmol/L. Consistent with Aurora B activity peaking at mitosis, the antiproliferative effects observed with GSK1070916 are specific for cycling cells, with a >200-fold increase in EC_{50} in primary normal nonproliferating human umbilical vein endothelial cells (HUVEC) compared with proliferating HUVEC cells. Furthermore, GSK1070916 shows dose-dependent inhibition of phosphorylation of an Aurora B–specific substrate in mice and, consistent with its broad cellular activity, has antitumor effects in 10 human tumor xenograft models. These data suggest that small molecule inhibitors of Aurora B kinase in cells with aberrant cell cycle regulation may be a therapeutic strategy applicable to a wide range of tumors in the clinic.

Materials and Methods

GSK1070916 Compound

GSK1070916 is a novel, azaindole derived, Aurora B/C kinase inhibitor synthesized at GlaxoSmithKline. The discovery and synthesis of GSK1070916 will be described in a separate article.[4]

Cellular Mechanistic pHH3 Assay

Cells were plated in 96-well plates in the recommended growth media containing 10% fetal bovine serum (FBS). The following day, 2-fold serial dilutions of GSK1070916 were added to the cells and incubated for 2 h at 37°C in 5% CO_2. The cells were then lysed and lysate transferred to 96-well plates coated with a pan-Histone antibody (Chemicon-MAB3422) and incubated at 4°C overnight. After washing, phospho-Histone H3 Serine10 antibody (Cell Signaling) in 1% nonfat milk in PBS containing 0.05% Tween 20 (PBST) was added for 5 h at room temperature, followed by PBST washes and incubation with horseradish peroxidase–conjugated anti-rabbit antibody (Cell Signaling) in 1% nonfat milk/PBST for an additional 1 h at room temperature. Following incubation, the plates were washed thrice with PBST and BM Chemiluminescence ELISA peroxidase substrate POD (Roche) added and the plates read. Values from the wells with no cells were subtracted for background correction and the data plotted as the percent of the DMSO-treated control samples using Microsoft Excel XLfit4 software.

Table 1. Intracellular inhibition of Aurora B kinase in human tumor cell lines after treatment with GSK1070916

Inhibition of pHH3-S10 by GSK1070916A		
Tumor type	Human cell line	EC_{50} (μmol/L)
Colon	Colo205	0.008
Breast	MX-1	0.012
Colon	HCT116	0.020
Lung	A549	0.046
Cervical	HeLa	0.118

Cellular Proliferation Assays

Cells were plated in 96-well plates in the recommended growth media and incubated at 37°C in 5% CO_2 overnight. The following day, the cells were treated with serial dilutions of GSK1070916. At this time, one set of cells was treated with CellTiter-Glo (Promega) for a time equal to 0 (T = 0) measurement. Following a 6- to 7-d incubation with compound, cell proliferation was measured using the CellTiter-Glo reagent according to the manufacture's recommended protocol. As inhibition of Aurora B induces endomitosis, the degree of which differs depending on the cell type, an extended compound treatment time was required to accurately reflect the effects on cell viability across a large panel of cell lines. For analysis of cell viability, values from wells with no cells were subtracted for background correction and the data plotted as a percent of the DMSO-treated control samples using Microsoft Excel XLfit4 software. The EC_{50} values represent the concentration of GSK1070916 where 50% maximal effect is observed.

Nonproliferating Cellular Assays

Nontumorgenic HUVECs (Lonza) were plated under proliferating and nonproliferating conditions in 96-well plates in endothelial growth medium (EGM-2; Lonza), with 2% FBS and supplements. To establish the nonproliferating cell conditions, primary HUVEC cells were seeded at 35,000 cells in a half area 96-well tissue culture plate, 4 d before compound treatment. The next day, fresh media was added, followed by a change in media one day before compound treatment. Growth arrest was confirmed under these conditions using fluorescence-activated cell sorting cell cycle analysis (data not shown). For cycling cell conditions, HUVEC cells were seeded at 500 cells per 96-well 1 d before compound treatment. At the time of treatment, 2-fold serial dilutions from 20000 nmol/L to 0.15 nmol/L of GSK1070916 were added and the plates incubated at 37°C, in 5% CO_2. After 6 d, media was removed and 10 μL of CellTiter-Glo reagent (Promega) added to the nonproliferating half area plates and 20 μL added to the proliferating cell plates for 30 min at room temperature. Values from wells

[4] Adams N, et al. Manuscript in preparation.

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with no cells were subtracted for background correction, and the data were plotted as a percent of the DMSO-treated controls using Microsoft Excel XLfit4 software.

Cell Cycle Analysis

HCT116 (colon carcinoma) and A549 (non–small cell lung carcinoma) tumor cells were seeded in 96-well plates in DMEM containing 10% FBS. The following day, serial dilutions of GSK1070916 were added and the plates incubated for 24, 48, and 72 h at 37°C in 5% CO_2. At the end of compound treatment, the cells were processed for cell cycle analysis using a detergent-trypsin method (24). All steps were done at room temperature while slowly shaking. The propidium iodide–stained samples were analyzed for DNA content using an Automated Micro Sampler (Cytek) on a FACScan flow cytometer (BD Biosciences). Cell cycle profiles were determined by analyzing the FL2-Area histograms using FlowJo software (Tree Star). DNA content was divided into five regions—sub-2N DNA, 2N DNA, >2N <4N DNA, 4N DNA, and >4N DNA—and the percentage of cellular events in each of the five areas was quantified.

Western Blot Analysis

Colo205 (colon carcinoma) cells were plated in 100-mm dishes in RPMI containing 10% FBS. The following day, 10, 100, and 1000 nmol/L GSK1070916 were added to the cells. The resulting final concentration of DSMO was 0.01% for all wells. Cells treated with DMSO alone or 100 nmol/L Staurosporine were included as negative and positive controls, respectively. After a 24 or 48 h exposure to compound, the cells were lysed in Lysis Buffer (Cell Signaling) with additional protease inhibitors (Roche) and the lysate analyzed by Western blot analysis using antibodies specific for PARP (BD Biosciences) and Caspase 3 (Biomol) overnight at 4°C. After washing, the blots were incubated with secondary antibodies [Alexa Fluor 680 Goat anti-Mouse IgG (Molecular Probes) and IRDye 800 Sheep anti-Rabbit IgG (Rockland)] for 1 h at room temperature, followed by imaging using the LI-COR Odyssey Infrared Imaging System.

Immunofluorescent Microscopy

A549 cells were seeded in four-chamber slides (Lab-Tek II), in DMEM with 10% FBS, overnight. The next day, the cells were either DMSO treated or treated with GSK1070916 at 4, 12, 110, or 330 nmol/L and incubated for 24 h at 37°C in 5% CO_2. The final concentration of DSMO was 0.01% for all wells. After treatment, the cells were washed with PBS and fixed with 1% paraformaldehyde in PBS for 10 min, followed by permeabilization with 0.1% triton-X-100 in PBS with 5% goat serum for 30 min and then sequentially stained with antibodies. The cells were first stained with pericentrin antibody (Abcam) at room temperature for 1 h, followed by a 1-h incubation with Oregon green–labeled anti-rabbit IgG (Molecular Probes). α-Tubulin antibody (Molecular Probes), followed by Cy3-labeled anti-mouse IgG (Jackson Immuno Research), was then added, followed immediately by staining of DNA with 4',6-diamidino-2-phenylindole (DAPI; Pierce) for 45 min at room temperature. All antibodies were diluted in PBS with 5% goat serum. The slides were then mounted in antifade solution (ProLong Antifade; Molecular Probes) and the cells evaluated by fluorescent microscopy.

In vivo Pharmacodynamic Analysis

Female athymic nude mice (three mice per dosing group) with HCT116 xenograft tumors (≥250 mm^3) were dosed i.p. with 25, 50, or 100 mg/kg GSK1070916 in 2% Cremophor EL, 2% N,N-dimethylacetamide, and 96% acidified water (pH 5.0). The animals were dosed i.p. as the pharmacokinetic studies showed no oral bioavailability for this compound with the formulation used in these studies. Vehicle-treated tumors were included as controls. At 0.5, 1, 2, 4, 5, 12, 24, 48, and 72 h after dosing, tumors were harvested and evaluated for pHH3-S10 activity and GSK1070916 concentrations and blood samples for GSK1070916 concentrations as described below. Freshly harvested tumor samples were minced and suspended in CelLytic MT tissue lysis buffer (Sigma) containing protease inhibitor cocktail and DNase I (Roche) at 1 gram of tumor per 10 mL of lysis buffer. The tumor samples were further processed through a 50-μm Medicon filter (BD Biosciences). Tumor tissue lysates were then assayed for Histone H3 serine 10 phosphorylation using the pHH3-S10 immunoassay as described above.

In vivo Drug Concentration Determination

GSK1070916A was isolated from mouse blood (diluted 1:1 v/v with water) and tumor homogenate (diluted 1 gram of tumor to 10 mL of lysis buffer as described above) by protein precipitation with 95:5 (v: v) acetonitrile/10 mmol/L ammonium formate (pH 3.0) and quantified by LC/MS/MS as described (25). The Aria TX2 chromatographic system (Cohesive Technologies; Thermo Scientific) was coupled with a Sciex API 4000 (Applied Biosystems) without any postcolumn split. The lower limit of quantification was 10.0 ng/mL for both blood and tumor homogenate. The upper limit of quantification was 2500 ng/mL for blood, and 1000 ng/mL for tumor homogenate.

Efficacy in Tumor Xenografts

Tumors were initiated by injection of tumor cell suspensions (A549, SW620, HCT116, H460, MCF-7, HL60, K562) or tumor fragments (Colo205) s.c. into nude (A549, SW620, HCT116, H460, MCF-7, HL60, and Colo205) or severe combined immunodeficient (SCID; K562) mice. When the tumors reached a volume of 80 to 200 mm^3, the mice were randomized into groups of 5 to 10 mice per group. GSK1070916 was administered at 25, 50, or 100 mg/kg once daily for 5 consecutive days-on, 2 d-off, schedule for two (Colo205 and HL60) or three (A549, SW620, HCT116, H460, MCF-7, K562) cycles. Tumors were measured twice weekly using calipers and tumor volume calculated using the following equation:

$$\text{Tumor Volume}(mm^3) = (\text{length} \times \text{width}^2) \times 0.5$$

Complete regressions were defined as tumor volume measurements <14 mm^3 for three consecutive measurements. Partial regressions were defined as tumor volume measurements ≤50% of initial tumor volume for three consecutive measurements. All animal studies were done in

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compliance with federal requirements, GlaxoSmithKline policy on the Care and Use of Animals, and related codes of practice.

For the SCID (HL60) and NOD-SCID (MV-4-11) mouse leukemia models, the mice (n = 10 per group) were treated i.p. with GSK1070916 at 12.5, 25, or 50 mg/kg or 12.5, 25, or 40 mg/kg, respectively. GSK1070916 was given once daily for 5 consecutive days with 2 d off, for two (HL60) or three (MV-4-11) cycles of treatment, starting 16 d after i.v. implantation via tail vein injections of HL60 cells and 21 d after implantation of MV-4-11 cells. The study end point was death or moribundity due to progression of the MV-4-11 or HL60 leukemia.

Results

Kinase Activity of GSK1070916

GSK1070916 potently inhibits Aurora B/INCENP and Aurora C/INCENP kinases with apparent inhibition constant (K_i*) values of 0.38 ± 0.29 nmol/L and 1.45 ± 0.35 nmol/L, respectively, but is less potent against Aurora A/TPX2 with a K_i* value of 492 ± 61 nmol/L.[5] The selectivity of GSK1070916 was evaluated against a panel of 328 protein kinases, and of these only five in addition to Aurora B and C were identified with IC_{50} values of <100 nmol/L; FLT1, TIE2, SIK, FLT4, and FGFR1 (IC_{50} values of 42, 59, 70, 74, and 78 nmol/L, respectively).[5]

Cellular Activity of GSK1070916

To measure intracellular inhibition of Aurora B kinase activity, an ELISA was developed to determine the levels of histone H3 serine10 phosphorylation (pHH3-S10), a specific Aurora B substrate. In A549 human lung tumor cell lysates, potent, dose-dependent inhibition of pHH3-S10 was observed with average EC_{50} of 46 nmol/L. GSK1070916 was also evaluated in an additional panel of tumor cell lines and was shown to inhibit the phosphorylation of HH3-S10 in all cell lines with average EC_{50} values ranging from 8 to 118 nmol/L (Table 1).

The effect of GSK1070916 on cell proliferation was determined using a panel of 161 tumor cell lines. Exponentially growing cells were treated with GSK1070916 and the cell viability determined using the CellTiter-Glo luminescent assay. Potent effects on cell viability, spanning numerous tumor types from both solid and hematologic, were observed. Greater than 100 cell lines generated EC_{50} values of ≤10 nmol/L, with a median EC_{50} of 8 nmol/L (Table 2).

To assess the specificity of GSK1070916 for cycling cells, the Aurora inhibitor was evaluated for its effect on the viability of normal nonproliferating human cells HUVECs and compared with normal proliferating HUVEC cells. The results show that although GSK1070916A inhibits proliferating cells with an EC_{50} of 19 nmol/L, only minimal

Table 2. Antiproliferative activity after treatment with GSK1070916 in human solid and hematologic tumor cell lines

Cell line origin	Cell line	EC_{50} (nmol/L)
Colon	SW48	2
	Colo 201	2
	SW480	3
	WiDr	3
	Colo205	7
	RKO E6	7
	RKO	7
	LoVo	7
	HCT-116	8
	SW620	8
	HT29	10
	SW1417	19
	DLD-1	28
	HCT-8	38
	Colo 320HSR	92
	Colo 320DM	96
	NCl-H630	127
Bone	U20S	34
Breast	SK-BR-3	4
	MDA-MB-453	6
	MX-1	8
	MDA-MB-231	11
	MDA-MB-468	12
	MCF-7	13
	T47D	13
Cervial	HELA	13
Head & neck	HN5	10
Kidney	A498	33
	786-O	57
Liver	Hep-3B	12
Ovarian	OVCAR-3	3
	A2780	5
	OVCAR-4	11
	SKOV3	12
	OVCAR-8	30
Pancreatic	AsPC-1	2
	Mia PaCa	3
	BxPC3	3
	PANC-1	13
Prostate	PC3	11
	LNCAP	12
	DU145	15
Skin	SK-MEL-2	2
	A375P	7
	SK-MEL-28	7
	SK-MEL-5	8
	SW954	11
Uterine	HEC-1-B	4
Lung	NCI-H358	4
	A549	7
	NCI-H157	8
	MV522	13
	NCI-H460	14
	NCI-H1299	23
	NCI-H1155	40

(Continued on the following page)

[5] Anderson K, Lai Z, McDonald OB, Stuart DJ, Nartey EN, Hardwicke MA, Newlander K, Dhanak D, Adams N, Patrick D, Copeland RA, Tummino PJ, Yang J. Biochemical characterization of GSK1070916, a potent and selective inhibitor of Aurora B and Aurora C kinases with an extremely long residence time. Biochem J 2009;420:259–65.

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Table 2. Antiproliferative activity after treatment with GSK1070916 in human solid and hematologic tumor cell lines (Cont'd)

Cell line origin	Cell line	EC_{50} (nmol/L)
Myeloma	HUNS1	9
	RPMI8266	11
	SKO 007	352
	U266B1	422
B-cell Lymphoma	CRO-AP2	3
	CRO-AP5	3
	SR	3
	NU-DUL-1	4
	MHH-PREB-1	4
	OCI-LY-19	4
	SU-DHL-16	4
	Pfeiffer	4
	U-937	5
	SU-DHL-5	5
	SU-DHL-4	5
	SU-DHL-6	6
	HT	7
	JM1	7
	Farage	7
	MC/CAR	9
	RL	10
	DB	11
	SC-1	12
	DOHH-2	14
	Toledo	15
	SU-DHL-10	15
	ARH-77	15
	BC-1	18
	BC-3	27
	BCP-1	31
	RC-K8	38
	BC-2	75
	REC-1	77
Burkitt lymphoma	ST486	2
	EB-2	2
	Raji	3
	GA10	4
	Daudi	5
	Jiyoye	5
	CA-46	5
	NAMALWA	6
	NC-37	7
	EB-1	7
	EB-3	7
	P3HR-1	8
	MC116	8
	1A2	9
	HS-Sultan	14
	DG-75	18
Hodgkin lymphoma	HD-MY-Z	4
	Hs 445	5
	RPMI 6666	10
	L-428	31
	TO 175.t	54
B-ALL	JVM-3	2
	SUP-B15	2
	NALM-6	2
	KARPAS-231	3
	SEM	3
	RCH-ACV	4
	CESS	5
	Kasumi-2	6
	TANOUE	1478
AML	PLB-985	1
	NOMO-1	2
	CCRF-SB	2
	OCI-AML2	4
	OCI-AML3	4
	ML-2	5
	THP-1	5
	MV-4-11	7
	HL-60	10
	F-36P	10
	NB-4	11
	M-07e	13
	OCI-M1	20
	GDM-1	21
	BDCM	26
	CMK	27
	KG-1	32
	HEL 92.1.7	60
CML	EM-2	4
	EM-3	4
	BV173	5
	KCL-22	5
	KU812	8
	K562	13
	MEG-01	44
T-ALL	ALL-SIL	2
	MOLT-16	3
	HSB-2	3
	CML-T1	3
	MOLT4	3
	Jurkat	3
	CTV-1	4
	SKW-3	5
	MOLT-3	5
	CEM/C1	6
	CCRF-CEM	7
	JRT3-T3.5	9
	DND-41	787
T-cell lymphoma	MJ	7
	HuT 78	14
	HH	18
Chronic B-cell Leukemia	MEC-1	10

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; T-ALL, T-cell acute lymphoblastic leukemia.

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effects were observed on nonproliferating HUVEC cells with an EC_{50} of 3900 nmol/L (data not shown).

To investigate the mechanism leading to decreased cell viability, the effects on the cell cycle in tumor cell lines treated with GSK1070916 were evaluated by fluorescence-activated cell sorting analysis. In A549 cells, a dose-dependent decrease in the 2N DNA content and increases in 4N, >4N, and sub-2N (cell death) DNA compared with DMSO-treated cells were observed (Fig. 1A). The increase in >4N DNA is indicative of a failure of cytokinesis resulting in polyploidy, a phenotype consistent with Aurora B inhibition (1, 13, 14, 26–28). Furthermore, as the percentage of >4N DNA–containing cells decreased over time, a concomitant increase in the percentage of sub2N DNA cells was detected. These results suggest that with increased time the polyploid cells loose viability.

To determine if the cell death observed after treatment with GSK1070916 involved the apoptotic pathway, two markers of apoptosis, Caspase-3, and PARP cleavage, were investigated. Colon tumor (Colo205) cells were treated with GSK1070916 at 10, 100, or 1000 nmol/L for 24 or 48 hours, and lysates were analyzed by Western blot analysis using antibodies specific for Caspase-3 and PARP. GSK1070916 induced apoptosis as measured by Caspase-3 and PARP cleavage by 24 hours at concentrations as low as 10 nmol/L (Fig. 1B).

To investigate the precise mitotic alterations, mitotic structures after treatment with GSK1070916 were investigated

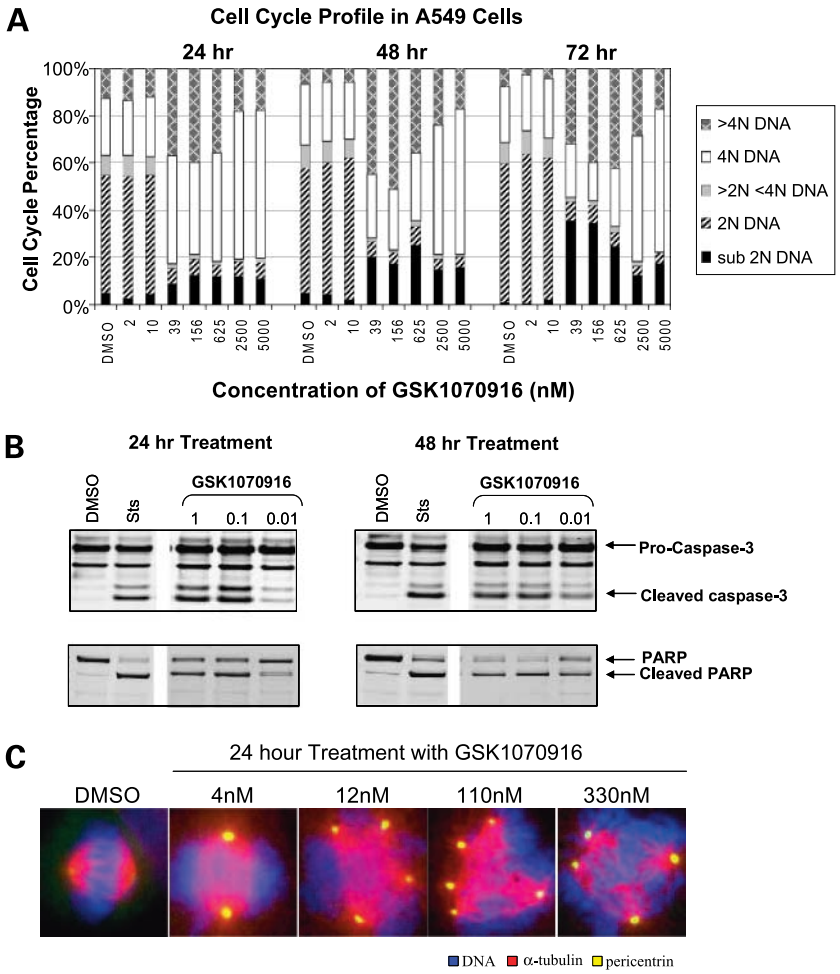

Figure 1. Cellular phenotype after treatment with GSK1070916. **A,** cell cycle distribution after treatment with GSK1070916. The cell cycle profile was evaluated after 24, 48, or 72 h GSK1070916 treatment by fluorescence-activated cell sorting analysis in A549 cells. A dose-dependent decrease in the 2N DNA content and increases in 4N, >4N, and sub-2N (cell death) DNA compared with DMSO-treated cells were observed. **B,** induction of Caspase-3 and PARP cleavage by GSK1070916. Colo205 cells were treated with 0.01, 0.1, and 1 μmol/L of GSK1070916, 0.1 μmol/L of staurosporine (*Sts*) or DMSO for 24 or 48 h. Lysates were analyzed by Western blot analysis using antibodies specific for Caspase-3 and PARP. GSK1070916 induced apoptosis as measured by Caspase-3 and PARP cleavage. **C,** mitotic phenotype of GSK1070916-treated A549 tumor cells by immunofluorescent staining. A549 cells were treated with 4, 12, 110, or 330 nmol/L of GSK1070916 or DMSO for 24 h and stained with α-tubulin antibody (*red staining*) to visualize mitotic spindles, pericentrin antibodies for centrosomes (*yellow staining*), and 4′,6-diamidino-2-phenylindole stained for DNA (*blue staining*). Normal metaphase mitotic structures were observed in the DMSO-treated control cells. Treatment with increasing concentrations of GSK1070916 resulted in unorganized chromosomes as well as the formation of malformed bipolar and multipolar spindles with multipolar spindles being the predominant phenotype.

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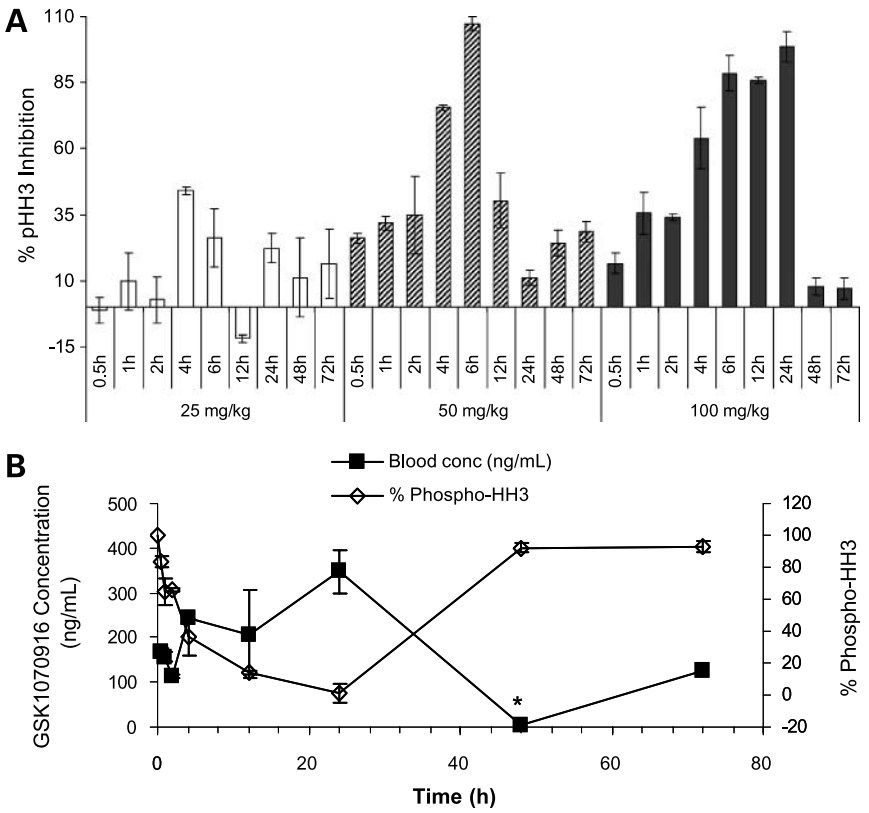

Figure 2. Pharmacodynamic effects and concentrations of GSK1070916. GSK1070916A was administered i.p. as a single dose to nude mice with subcutaneous HCT116 tumors at 25, 50, or 100 mg/kg. **A,** at the indicated times, pHH3-Ser10 levels in tumors were determined. GSK1070916 inhibited HH3-S10 phosphorylation in a dose-dependent manner at all three doses. **B,** after a single i.p. dose of 100 mg/kg of GSK1070916, drug concentrations were measured in blood and HCT116 tumor tissues. Blood concentrations of >170 ng/mL of GSK1070916 correlated with sustained decrease (>60%) in HH3-S10 phosphorylation for up to 24 h. *, the concentration of GSK1070916 in the blood at 48 h was below the lower limit of quantification in this assay. A value of 5 ng/mL that is one half the lower limit of quantification was therefore used to not over represent the concentration in the graph. The levels of GSK1070916 in tumor samples after a single administration were below the level of detection in this study.

in A549 cells by immunofluorescent microscopy. As shown in Fig. 1C, normal metaphase mitotic structures were observed in the DMSO-treated control cells. Treatment with increasing concentrations of GSK1070916 resulted in unorganized chromosomes as well as the formation of malformed bipolar and multipolar spindles (12, 110, and 330 nmol/L), with the latter being the predominant phenotype.

In Vivo Activity of GSK1070916

To investigate the pharmocodynamic effect of GSK1070916 *in vivo*, phosphorylation of HH3-S10 was measured in tumor lysates. In nude mice implanted with human colon tumor (HCT116) xenografts, a single dose of GSK1070916 administered i.p. inhibited HH3-S10 phosphorylation in a dose-dependent manner (Fig. 2). After a single i.p. dose of 100 mg/kg of GSK1070916, drug concentrations were measured in blood and HCT116 tumor tissue (Fig. 2B). Blood concentrations of >170 ng/mL of GSK1070916 correlated with sustained decrease (>60%) in HH3-S10 phosphorylation for up to 24 hours (Fig. 2B). The levels of GSK1070916 in tumor samples after a single administration were below the level of drug detection in this study.

GSK1070916 was evaluated for its ability to inhibit the growth of various tumor xenografts in nude mice. As shown in Fig. 3, repeated i.p. administration (5 consecutive days with 2 days off for up to three cycles) produced antitumor activity in all models evaluated with regressions (complete or partial) in 4 of 8 tumor types [lung, A549; colon, HCT116; acute myelogenous leukemia (AML), HL60; and chronic myelogenous leukemia, K562], stable disease in 3 of 8 (colon, Colo205; lung, H460; and breast, MCF-7), and tumor growth delay in 1 of 8 tumor types (colon, SW620). Daily administration of GSK1070916 was well-tolerated with <15% body weight loss, except where noted (Fig. 3).

GSK1070916 was evaluated for antitumor activity in two human leukemia mouse models including an AML (HL60) SCID and an AML (MV-4-11) NOD-SCID model. A dose-dependent increase in the median survival times was observed in both models after i.p. dosing in the GSK1070916-treated animals compared with the vehicle-treated mice (Fig. 4).

Discussion

Cancer is a disease characterized by uncontrolled proliferation of abnormal cells. Modulation of atypical cell cycle

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regulation would therefore be a valuable therapeutic strategy for many tumor types. RNA interference, gene knockout, and antibodies targeting Aurora B show a critical role for Aurora B in coordinating microtubule dynamics, chromosome movement, spindle checkpoint, and cytokinesis (14, 26–28). Furthermore, mRNA and protein levels of Aurora B are frequently elevated in cancers including colon, prostate, glioblastoma, non–small cell lung, and thyroid (15–19). The evidence linking Aurora overexpression and malignancy has generated significant interest in the development of small molecule inhibitors.

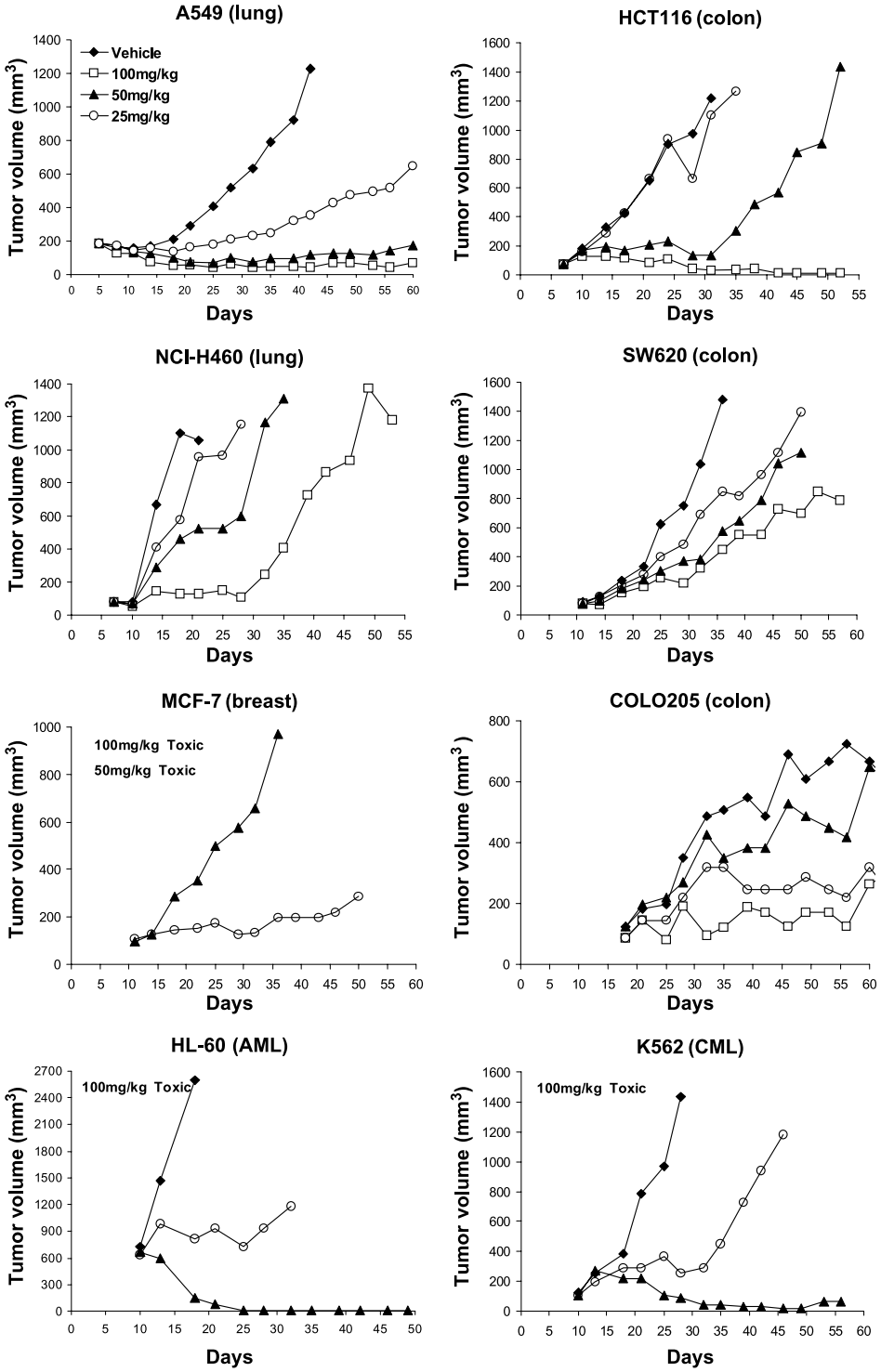

Figure 3. Antitumor activity of GSK1070916A *in vivo*. GSK1070916 was administered at 25, 50, or 100 mg/kg once daily for 5 consecutive d-on, 2 d-off schedule for two (Colo205 and HL60) or three (A549, SW620, HCT116, H460, MCF-7, K562) cycles. Toxicity is shown for each tumor line where observed.

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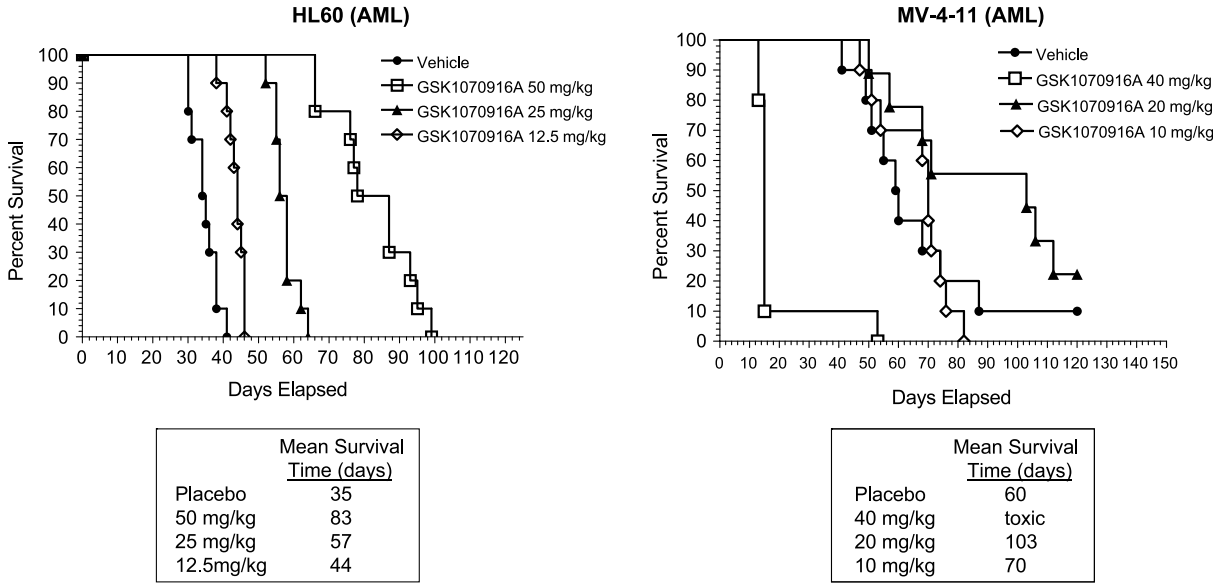

	Mean Survival Time (days)
Placebo	35
50 mg/kg	83
25 mg/kg	57
12.5mg/kg	44

	Mean Survival Time (days)
Placebo	60
40 mg/kg	toxic
20 mg/kg	103
10 mg/kg	70

Figure 4. Antitumor activity of GSK1070916 in human leukemia mouse models. For AML, SCID (HL60), and NOD-SCID (MV-4-11) mouse leukemia models, the mice (n = 10 per group) were treated i.p. with GSK1070916 at 12.5, 25, or 50 mg/kg or 10, 20, or 40 mg/kg, respectively. GSK1070916 was given once daily for 5 consecutive d with 2 d off, for two (HL60) or three (MV-4-11) cycles of treatment. A dose-dependent increase in the median survival times was observed after i.p. dosing in the GSK1070916 treated animals compared with the vehicle-treated mice.

The majority of reported small molecule Aurora inhibitors do not distinguish between enzyme isoforms (22, 23). To date, the only Aurora B selective inhibitor to be tested in the clinic is AZD1152. Interestingly, nonselective inhibitors of Aurora kinases induce a cellular phenotype characteristic of Aurora B inhibition, indicating that inactivation of Aurora B kinase may bypass the requirement for Aurora A (28). This raises the possibility that a more selective compound may be more desirable in that it may be associated with less potential toxicity.

In this report, we describe the cellular and *in vivo* activity of a novel, ATP competitive azaindole, GSK1070916. The compound selectively inhibits Aurora B/INCENP (K_i* = 0.38 ± 0.29 nmol/L) and Aurora C/INCENP complexes (K_i* = 1.45 ± 0.35 nmol/L) over AuroraA/TPX2 complex (K_i* = 492 ± 61 nmol/L). Interestingly, Anderson et al.[5] have observed that the inhibition of Aurora B and Aurora C is time dependent with dissociation half lives of >480 and 270 minutes, respectively. The slow enzyme off-rate distinguishes GSK1070916 from two other Aurora clinical compounds, MK0457 (VX-680) and AZD1152, which have dissociation half-lives of <30 minutes. This slow enzyme off-rate may confer a selective advantage for GSK1070916, such as less frequent dosing and perhaps greater tolerance.

To assess inhibition of cellular Aurora B by GSK1070916, we measured the phosphorylation status of an Aurora B–specific substrate, histone H3 serine-10 (pHH3-S10) in A549, non–small cell lung tumor cells. Treatment of cells with GSK1070916 resulted in dose-dependent inhibition of pHH3-S10. To expand these results, pHH3-S10 levels were monitored in a small cell line panel, including breast, colon, and cervical cell lines. Potent pHH3-S10 inhibition was observed in all tumor types after a 2-hour treatment with GSK1070916. To further characterize the cellular phenotype of GSK1070916, the cell cycle and detailed mitotic structures were analyzed. Treatment with GSK1070916 led to a dose- and time-dependent increase in 4N DNA and >4N DNA and sub-2N DNA containing cells. This is consistent with cells entering mitosis, failing to divide, and ultimately leading to cell death. The observed cell death was a result of apoptosis, as indicated by Caspase-3 and PARP cleavage. Consistent with the role of Aurora B kinase in mitosis and cytokinesis, cells treated with GSK1070916 showed abnormal mitotic structures with multipolar spindles being the predominant phenotype.

In agreement with the inhibition of cellular Aurora B in multiple tissue culture cell types, GSK1070916 showed antiproliferative effects in a diverse range of tumor cell line lines including solid and hematologic cell lines with a median EC_{50} of 8 nmol/L. Consistent with Aurora B activity peaking at mitosis, the antiproliferative effects observed with GSK1070916 are specific for cycling cells, in that a >200-fold increase in EC_{50} value is observed on primary normal nonproliferating HUVECs compared with proliferating HUVEC cells.

In vivo administration of GSK1070916 resulted in a dose-dependent inhibition of a direct marker of Aurora B activity (pHH3-S10), in advanced s.c. tumors, indicating a pharmacodynamic response in mice. Subsequent studies using a daily administration for 5 consecutive days schedule with GSK1070916 showed antitumor activity in a broad spectrum of human tumor xenografts in mice including tumor regressions in 4 of 8 subcutaneous models. Additionally, a dose-dependent increase in the median survival times was observed in two human leukemia mouse models. Overall,

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the cellular and *in vivo* profile of GSK1070916 is consistent with a selective Aurora B/C kinase inhibitor. Based on its favorable *in vitro* and *in vivo* activity and developability profile, GSK1070916 is being progressed to clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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