Sensitivity of Cancer Cells to Plk1 Inhibitor GSK461364A Is Associated with Loss of p53 Function and Chromosome Instability

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Abstract

Polo-like kinases are a family of serine threonine kinases that are critical regulators of cell cycle progression and DNA damage response. Predictive biomarkers for the Plk1-selective inhibitor GSK461364A were identified by comparing the genomics and genetics of a panel of human cancer cell lines with their response to a drug washout followed by an outgrowth assay. In this assay, cell lines that have lost p53 expression or carry mutations in the TP53 gene tended to be more sensitive to GSK461364A. These more sensitive cell lines also had increased levels of chromosome instability, a characteristic associated with loss of p53 function. Further mechanistic studies showed that p53 wild-type (WT) and not mutant cells can activate a postmitotic tetraploidy checkpoint and arrest at pseudo-G1 state after GSK461364A treatment. RNA silencing of WT p53 increased the antiproliferative activity of GSK461364A. Furthermore, silencing of p53 or p21/CDKN1A weakened the tetraploidy checkpoint in cells that survived mitotic arrest and mitotic slippage. As many cancer therapies tend to be more effective in p53 WT patients, the higher sensitivity of p53-deficient tumors toward GSK461364A could potentially offer an opportunity to treat tumors that are refractory to other chemotherapies as well as early line therapy for these genotypes.

Introduction

Plk1 is required for bipolar spindle formation as well as proper spindle assembly and function (1, 2). During the G2-M transition, Plk1 plays a role in centrosome maturation (3) and stimulates the centrosome to nucleate microtubules upon mitotic entry (4). In later stages of mitosis, Plk1 is involved in the activation of components of the anaphase-promoting complex for mitotic exit and in cytokinesis (5, 6). One striking consequence of deregulated Plk1 activity is the formation of aberrant centrosomes and mitotic spindle poles, which are tightly correlated with aneuploidy and chromosomal instability in tumor development (7). Elevated Plk1 levels are found in numerous tumor types including breast cancer (8), colorectal cancer (9), endometrial carcinomas (10), head/neck squamous cell carcinomas (11), non–small cell lung cancer (12), ovarian cancer (13), and pancreatic cancer (14) probably due to increased mitosis in these cancer tissues. Taken together, the critical role of Plk1 in mitosis and its overexpression in cancer makes it an attractive therapeutic target.

GSK461364A is a thiophene amide that inhibits purified Plk1 enzyme in vitro with a Ki of 2 nmol/L and has >100-fold selectivity for Plk1 compared with Plk2 and Plk3 (15). GSK461364A is a potent inhibitor of cell proliferation causing 50% growth inhibition (GI50) below 100 nmol/L in most of the cell lines tested with limited toxicity against human nonproliferating cells. Inhibition of cell cycle progression is concentration dependent with initial delay at G2 phase at high compound concentrations and arrest at M phase at lower concentrations. GSK461364A showed a dose-dependent mitotic arrest in mouse xenografts, which correlated with effects on tumor growth (15). Currently, this compound is in a dose-escalation first-time-in-human trial.

Predictive biomarkers for drug response have been used successfully in the development and subsequent use of cancer therapeutics. Selecting tumors that have specific somatic mutations, which impart greater sensitivity

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to a drug, can increase the overall efficacy of the drug in a population and allow those individuals unlikely to benefit to seek alternative, potentially more effective treatments (16–19). Predictive biomarkers for GSK461364A were identified by comparing the genomics and genetics of a panel of cancer cell lines with the response of the cell lines in a drug washout followed by an outgrowth assay. In this assay, we observed that cell lines with mutations in the TP53 gene tended to be more sensitive to GSK461364A, and that inhibiting the p53 response by RNA silencing conferred increased sensitivity in some p53 wild-type (WT) cells. Furthermore, these more sensitive cell lines also had increased levels of chromosome instability, a characteristic associated with TP53 mutations.

Materials and Methods

Cell lines

Cancer cell lines were purchased from the American Type Culture Collection (http://www.atcc.org). Unless otherwise recommended, cell lines were cultured in RPMI 1640 supplemented with a final concentration of 10% fetal bovine serum (FBS), 2 mmol/L GlutaMAX, and 1 mmol/L sodium pyruvate. Genomic DNA was extracted from each line using the Mini DNeasy kit (Qiagen). RNA was isolated from exponentially growing cells by replacing the media with trizol and purifying the RNA using Qiagen RNeasy spin columns (Qiagen). The normal lines human foreskin fibroblasts and human umbilical vascular endothelial cell were purchased from the American Type Culture Collection and only maintained for a few passages. For three cell lines Lovo, P45, and SKMEL5, only transcriptomics data are available but not the “SNP Chip” data. For the rest of the cell lines, the copy number data using Affymatrix SNP Chip and transcriptomics data are both available and have been deposited in ref. 20. Comparison of the GSK 500k SNP chip data with Sanger 10k SNP chip data proved that the cell lines with the same names are genetically identical (>85% identical).

Drug washout/outgrowth assay

Cell lines were seeded into 96 -well plates at a density of 20,000 cells per well. The following day, serially diluted GSK461364A was added to cells and the cells were incubated for 72 hours before the medium was aspirated, and the cells washed four times with 250 μL of RPMI 1640 supplemented with 20% FBS. Cells were additionally washed with PBS without magnesium and trypsinsinized with 10 μL of trypsin/EDTA for 10 minutes at 37°C. The trypsin was neutralized, and the cells suspended with the addition of 250 μL of media containing 10% FBS. The cell suspension was split equally (50 μL/well) into five white duplicate microtitre plates (Nunc, VWR) containing 60 μL of complete media. One of the duplicate microtitre plates was analyzed immediately (T = 0), and the others were analyzed at 24-hour intervals using CellTiter-Glo (CTG, Promega) to assess the number of cells and hence monitor cell proliferation.

Proliferation and GI50 determination

Cell lines were seeded into 384-well microtiter plates. After seeding, the cells were incubated at 37°C in 5% CO2 for 24 hours. GSK461364A was added to each cell line at 10 concentrations with a nontreated control. A zero-time (T = 0) value was read for each cell line. After 72 hours, the medium containing GSK461364A or DMSO control was aspirated from all of the remaining cells and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole and the fluorescent intensity measured using an InCell1000 High Content Analyzer. The percent intensity of the 4′,6-diamidino-2-phenylindole stain at 72 hours, relative to intensity at time zero, was calculated for each concentration of GSK461364A in each of the triplicate wells. The percent intensity values were subsequently used in model 205 of Xfit in Microsoft Excel to calculate GI50. The GI50 was defined as the concentration at which cell density at 72 hours is half of that at time zero (i.e., 50% growth inhibition).

TP53 sequence analysis

Total RNA from cancer cell lines was isolated using a modified Qiagen RNeasy kit (QIAGEN, Inc.) and converted into cDNA using the Roche First Strand cDNA Synthesis kit using oligo dT primers (Roche Diagnostics). All overlapping PCR primers for TP53 gene were designed using the Primer D (in-house developed) software and ordered from IDT (Integrated DNA Technologies, Inc.). PCR primers were tailed with M13 universal sequencing primer sequences (UP & UR). PCRs were carried out using HotstarTaq DNA polymerase (QIAGEN, Inc.). DNA was amplified for 35 cycles at 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 45 seconds followed by 7 minutes of extension at 72°C. PCR products were purified using Agencourt AmPure (Agencourt Bioscience Corp.). Direct sequencing of purified PCR products was done with the AB v1.1 BigDye-terminator cycle sequencing kit (Applied Biosystems), and sequencing reactions were purified using Agencourt CleanSeq reagent kits (Agencourt Bioscience Corp.). Sequencing was done using a Genetic Analyzer 3730XL (Applied Biosystems). All sequence data were assembled and analyzed using the Codon Code Aligner software (CodonCode Corp.), and sequence variants were confirmed by independent PCR amplifications and sequencing. When no cDNA could be amplified or the cDNA sequence data suggested multiple splice forms were present, the TP53 gene was amplified from genomic DNA and sequenced (see Supplementary Table S1 for primer sequences).

Copy number analysis

All the cancer cell lines were analyzed using Affymetrix 500K SNP Chip (Affymetrix, Inc.). In addition, 20 unique lymphoblastic cell lines composing part of a human genomic diversity panel (obtained from Coriell Institute; ref. 21) were assayed and used as a reference panel. Probes were mapped to human genome build 35 (June 2004). For each probe, a log2 copy number ratio was obtained.
measured from raw data derived from the scanned image of the array. Probe-wise ratios were calculated for the single channel Affymetrix chips by comparing the "perfect match" intensities with the range of intensities seen in the reference chip set using a method similar to that of the dChip software package (22). Subsequently, every assay was normalized using the assumption that the median copy number was diploid. Finally, the standard circular binary segmentation (23) was used to reduce probe-wise noise and identify copy number transitions. Low-level gains (three to four copies) and monosomies were identified by segment scores of 0.25 to 0.65 and −0.25 to −0.7, whereas focal amplifications (gains of more than approximately five copies) and homozgyous losses were recognized by scores of >0.65 and <−0.7 respectively, thresholds that are based on previously published calibrations (24). These data are available at ref. 20.

RNA expression analysis

A transcript profile was generated for each cell line using RNA from the cell lines and Affymetrix U133_2plus microarrays following the Affymetrix protocol (Affymetrix). These data are available at ref. 20.

Expression Taqman

MDM2 mRNA expression was determined by real-time Q-PCR using a custom assay for NRD1 (see Supplementary Table S2 for primer and probe sequences) to normalize the expression of MDM2 across the cell lines. The data were collected using the Applied Biosystems Prism 7900HT Sequence Detection System and analyzed with SDS v2.1 and Excel (Microsoft Corp.).

Cell viability assay after p53 knockdown

The TP53 mRNA was knocked down using a functionally validated small interfering RNA (siRNA) duplex (SI02655170, Qiagen). Cells were transfected with the p53 siRNA using HiPerfect following the manufacturer’s procedures (Qiagen) 5 to 6 hours after plating. After 24 hours of incubation at 37°C, the transfection medium was aspirated and replaced with fresh media containing GSK461364A at 30 or 300 nmol/L. After a further 72 hours, the cells were washed three times with RPMI 1640 containing 20% FBS before adding RPMI 1640 with 10% FBS with no inhibitor. Cell viability was assayed after 72 hours using the CTG assay (Promega) for A549 or MTT assay (Roche) for NCI-H460 following the manufacturer’s instructions.

RNA interference, cell synchronization, and immunoblotting

For cell synchronization, A549 and PL45 cells (seeded at 2 × 10^5 cells/mL in six-well dishes) were treated with 2 mmol/L thymidine, incubated for 20 hours, then washed thoroughly and repeatedly with DPBS (+Ca, Mg) before adding fresh media. After an 8-hour release, cells were again treated with thymidine for an additional 20 hours of incubation. Finally, cells were washed as before, released in fresh media, and after 2 hours, GSK461364A was added to a final concentration of 250 nmol/L. At various times after thymidine release, cells were harvested and lysed in radioimmunoprecipitation assay buffer [with 1× EDTA-free protease inhibitor tablets (Roche), Phosphatase inhibitor cocktails 1 and 2 (Calbiochem)]. Forty micrograms of each lysate were run on a 4% to 12% gel (NuPage), transferred to a polyvinylidene difluoride membrane, and sequentially probed with antibodies to p53 (Calbiochem OP43L), Cyclin E (BD 51-1459GR), phospho-Histone-H3 (S10; Upstate 06-570), p21 (Upstate 05-345), Cyclin B1 (Upstate 05-373), and glyceraldehyde-3-phosphate dehydrogenase (Covance MMS-580S). For RNA silencing studies, A549 cells were transfected in suspension with 50 nmol/L siRNA motif smartpools (Dharmacon) for p21, p53, or nontargeting controls, using Lipofectamine 2000. Transfected cells were seeded at <2 × 10^5 cells/mL in 10-cm dishes in OptiMem with 5% FBS. After 24 hours, GSK461364A was added to a final concentration of 250 nmol/L. At various times posttreatment, cells were harvested, lysed, and lysates were immunoblotted as above.

DNA content analysis

Cells were fixed in chilled 70% ethanol for 2 hours, washed with PBS, and stained using propidium iodide (Molecular Probes) solution containing 20 μg/mL propidium iodide, 0.2 mg/mL RNase A, and 0.1% Triton X-100 in PBS. Data were acquired on a FACScalibur and analyzed with the FlowJo software package (Tree Star, Inc.).

Results

Analysis of cancer cell line response to Plk1 inhibitor, GSK461364A, using a drug washout/outgrowth assay

As an i.v. delivered compound, it is anticipated that GSK461364A will be dosed intermittently with periods of high compound exposure followed by drug holidays leading to periods of low to undetectable blood concentration of the inhibitor. An in vitro drug washout followed by outgrowth assay was developed to mimic this intermittent dosing schedule in which cell viability after temporary exposure to Plk1 inhibitor GSK461364A was measured. Cell lines that were capable of resuming growth following a 3-day exposure to high-concentration (≥1 μmol/L) GSK461364A were considered resistant, whereas sensitive cell lines were defined as those capable of resuming growth only at low compound concentrations, defined as concentration below their respective GI50s. Throughout this article, cellular response qualified as “sensitive” or “resistant” will refer to the cellular response to GSK461364A in the washout/outgrowth assay because with standard proliferation assay under constant drug exposure, most of the cell lines seemed to be responsive (GI50s in Table 1). An example of resistant (NCI-H460) and sensitive (NCI-H596) cell lines using this assay are depicted in Fig. 1. Using this assay, 18 cell lines...
Table 1. The TP53 status of the cell lines with response to GSK461364A determined by a compound washout/outgrowth assay

<table>
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<th>Tissue</th>
<th>Cell line</th>
<th>GI50</th>
<th>Response</th>
<th>TP53 status</th>
<th>TP53 mutation</th>
<th>TP53 mutation dominant negative</th>
<th>Reported occurrence</th>
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NOTE: The TP53 status for each cell line was determined based on three sets of data: COSMIC database (27), direct sequencing of cDNA or genomic DNA, and level of TP53 mRNA expression from the Affymetrix expression array analysis (Supplementary Table S3; ref. 20). The dominant-negative status was sourced from the IARC (28) database and the reported occurrence was based on the Universal Mutation Database (32). GI50 from a 72-h proliferation assay under constant drug exposure are also listed. Abbreviation: HFF, human foreskin fibroblasts.
were classified as resistant, and 25 were classified as sensitive to transient exposure to GSK461364A (Table 1). Five cell lines showed outgrowth at concentrations <1 μmol/L but higher than their respective GI50, so the response of those cell lines were deemed intermediate (Supplementary Table S3).

**TP53 mutation status correlates with sensitivity to Plk1 inhibitor GSK461364A in the drug washout/outgrowth assay**

RNAi studies on Plk1 suggested that p53-deficient cells are more sensitive to Plk1 depletion (25, 26). TP53 status was collected for each of the cell lines with washout/outgrowth assay data. The TP53 mutation status for each cell line was determined based on three sets of data (Supplementary Table S3): COSMIC database (27), direct sequencing of cDNA or genomic DNA, and level of TP53 mRNA determined from the Affymetrix expression array analysis (20). Where no TP53 mRNA was detected, the TP53 status was considered “null.” Data analysis showed that cell lines that are sensitive have a higher incidence of TP53 mutations or are more likely to be TP53 null compared with the cell lines that are resistant (P = 0.00078; Fisher’s Exact test; Table 1; Fig. 2A). As a single predictor of response, TP53 status has a positive and negative predictive value of 79% and 80%, respectively (sensitivity, 88%; specificity, 67%).

Apart from TP53, sensitivity in the washout/outgrowth assay does not correlate with the mRNA expression level of the target PLK1 itself (P > 0.05; t test; Supplementary Table S3; Supplementary Fig. S1). Unlike AURKA, DNA amplification of PLK1 gene is rarely reported in the tumors. Indeed, among the cell lines tested, only one cell line has a low copy number gain (Supplementary Table S3). As a recent article using a synthetic lethal siRNA library screen discovered that inhibition of a mitotic pathway involving Plk1 resulted in the subsequent death of Ras mutant cells (29), we also examined sensitivity correlation with KRAS/BRAF mutations. We collected the KRAS and BRAF mutation status for the cell lines with washout/outgrowth data and found that the sensitivity of the cell lines in this assay is not significantly associated with KRAS or BRAF mutation status (P > 0.05; Fisher’s Exact test) when comparing KRAS status or BRAF status separately or combining both mutations (Supplementary Table S3; Supplementary Fig. S1).

Although the deletions and insertions that cause frame shift changes most likely lead to the loss of p53 function, missense mutations that cause single amino acid changes may leave some aspects of p53 function intact. It was reported that more than half of the rare TP53 mutations still display significant p53 activity and p53 protein (30). The most detrimental point mutations result in a dominant-negative phenotype leading to a loss of p53 function (31). These dominant-negative mutations are also most frequently observed in human cancers. The functional status of individual TP53 mutations was sourced from the IARC (28) and the Universal Mutation Database (32) databases to further refine the association of the sensitivity of cell lines to GSK461364A and the mutation status of TP53. The close examination revealed that most of the sensitive cell lines are either null for TP53 or carry point mutations that cause frameshifts or have been documented as “dominant-negative,” which are most detrimental...
to p53 function. However, among the resistant cell lines, only PC3 is null and DLD1 carries a dominant-negative mutation (Table 1).

Lovo, MCF7, and ZR75-1 are the only cell lines WT for p53 that remain sensitive to GSK461364A. Because the cellular function of p53 can be suppressed by alternative mechanisms such as the overexpression of MDM2 (33), we examined MDM2 mRNA levels in all cell lines. We observed that these three cell lines had significantly higher MDM2 mRNA expression when compared with MDM2 levels in cell lines that are either WT p53 and resistant or mutant p53 and sensitive to GSK461364A in the drug washout/outgrowth assay (Fig. 2B). These data suggest that p53 protein function might be compromised by high MDM2 levels in these three sensitive p53 WT cell lines.

Cell lines sensitive to GSK461364A by drug washout/outgrowth assay show more chromosome instability

A genome-wide scan was done to identify specific DNA copy number changes that are associated with a GSK461364A sensitivity phenotype. A histologically diverse panel (high proportion of lung and breast cancer cell lines) of 24 cancer cell lines (12 sensitive and 12 resistant lines; Supplementary Table S4) were assessed for DNA copy number changes using microarray-based methods. Overall, more DNA copy number alterations were observed for sensitive cell lines compared with resistant cell lines (Fig. 3A). As expected, large (>25 Mb) copy number alterations were prevalent in this panel of cancer cell lines. In many cases, this encompassed entire chromosomal arms (e.g., 8q+). Although specific large gains/losses were not predictive of GSK461364A response, the general frequency of gains/losses was higher in the 12 sensitive lines when compared with the resistant lines (P = 0.0413; t test; Supplementary Table S5; Fig. 3B). This higher level of chromosome instability in sensitive lines is consistent with previous observations that associate loss of p53 function with chromosome instability (34, 35).

GSK461364A treatment of p53 WT but not mutant cells activates a tetraploid G1 checkpoint following sustained mitotic arrest

To investigate the mechanism linking p53 status and sensitivity to GSK461364A by drug washout/outgrowth assay, a p53 WT and resistant cell line A549, and a p53 mutant and sensitive cell line PL45 were synchronized by a double thymidine block, released, and 2 hours later were treated with GSK461364A. Protein lysates were made at different time points after drug treatment and were examined for expression levels of the cell cycle markers Cyclin B1, phosphorylated histone H3 at serine-10 (pHH3), Cyclin E, p53, as well as p21 (Fig. 4A). Cells from duplicated plates were simultaneously harvested, fixed, and analyzed by fluorescence-activated cell sorting for cell cycle phase determination (Fig. 4B). GSK461364A induced comparable mitotic arrest in both cell lines evidenced by the accumulation of Cyclin B1 followed by the accumulation of pHH3 (at 12–32 h after thymidine release). Following mitotic arrest, both cell lines underwent aberrant mitotic exit in a state of tetraploidy (32–48 h), termed mitotic slippage, indicated by the decrease of both pHH3 and Cyclin B1 without an increase in 2N cells that would indicate successful chromatin segregation and cytokinesis (Fig. 4B). Following drug treatment, A549 cells showed increased levels of p21 (12–72 h), reflecting p53 activation, and after slipping from mitosis, the cells were apparently arrested in a tetraploid G1 phase suggested by the sustained level of 4N DNA and accumulation of Cyclin E (48–72 h). As expected for PL45 that harbors a mutant p53, GSK461364A caused no detectable induction of p21 expression. Following mitotic slippage, PL45 cells showed no accumulation of Cyclin E, indicating absence of a tetraploid G1 arrest.

RNA silencing of p53 response increases sensitivity to GSK461364A and weakens postmitotic tetraploidy checkpoint

To validate the association of loss of p53 function with sensitivity to GSK461364A, a functionally validated siRNA
against p53 was transfected into NCI-H460 or A549 cells (both are p53 WT, resistant). After transfection, the cells were incubated with GSK461364A for 72 hours; the compound was subsequently washed out; and cell viability was measured 72 hours following washout. Compared with cells with nonsilencing control siRNA, knockdown of p53 significantly enhanced the sensitivity of the cells to GSK461364A in preventing outgrowth (Fig. 5A). The

Figure 3. Sensitive cell lines carry more overall chromosome instability compared to resistant cell lines. A, genome wide DNA copy number profiles for sensitive and resistant lines listed in Supplementary Table S4. Dark regions, alteration frequencies in the resistant lines; red and green regions, sensitive lines. Several regions can differentiate these groups (arrows). Overall, sensitive cell lines carry more DNA copy number alterations. B, number of large (>25 mb) DNA copy number changes in sensitive versus resistant cancer cell lines.

Figure 4. Plk1 inhibition by GSK461364A causes prolonged mitotic delay, aberrant mitotic exit, and p53 activation. A, A549 and PL45 cells were synchronized with a double-thymidine block technique, released for 2 h, treated with 250 nmol/L GSK461364A, and harvested at different time points. GSK461364A induced comparable G2-M arrest (accumulation of Cyclin B1 followed by accumulation of pH3) followed by aberrant mitotic exit (decrease of both pH3 and Cyclin B1) in both cell lines. A549 cells activate p53 response (elevated levels of p21) and postmitotic accumulation of Cyclin E at 48 and 72 h, suggesting G1 tetraploidy checkpoint arrest. No significant induction of p21 or accumulation of Cyclin E is observed for PL45. B, analysis of DNA content by propidium iodide staining confirms that synchronized cells treated with GSK461364A arrest with 4N DNA and fail to successfully segregate chromatin in 2N daughter cells at the time of biochemical mitotic exit.
total number of cells on assay end was less at 30 or 300 nmol/L drug treatment compared with no drug treatment, due to the reduced cell numbers present for outgrowth following washout (GI50s of 3-d drug exposure for NCI-H460 and A549 were 9.2 and 9.9 nmol/L, respectively).

To test whether p53/p21 activation upon GSK461364A treatment was essential for the postmitotic tetraploid G1 arrest observed in A549, cells were transfected with siRNA targeting p53, p21, or a nonsilencing (NS) control treated with GSK461364A, and cell lysates prepared at multiple time points were immunoblotted for markers of p53 response and cell cycle. In all cases, GSK461364A treatment caused mitotic arrest (accumulation of Cyclin B1 and pHH3 at 12–24 h posttreatment) and mitotic slippage (decrease in Cyclin B1 and pHH3 at 36–48 h without cytokinesis; Fig. 5B). Increased p53 and p21 were evident in NS cells, and by 72 hours, cells showed Cyclin E accumulation indicating tetraploid G1 arrest. In contrast, both p53 and p21 silenced cells exhibited reduced p21 expression and reduced Cyclin E accumulation, indicating a weakened tetraploid G1 arrest. p21-silenced cells showed further indication of reentering cell cycle as suggested by the reappearance of Cyclin B1 protein at 72 hours (Fig. 5B). These results suggest that loss of p53 and p21 response can abrogate a postmitotic tetraploidy checkpoint normally triggered in cells that survive mitotic slippage. In this case, p53 WT cells can arrest at pseudo-G1 after prolonged GSK461364A treatment and escape apoptosis, whereas the p53-deficient cells may resume cell cycle encountering further rounds of mitotic injury. Overall, this suggests one explanation for the association between p53 loss of function and sensitivity to GSK461364A.

**Discussion**

The prospective selection of patients for treatment with targeted agents based on the genetic profile of their tumors has proven to be an effective strategy in improving therapeutic response rates. In addition, biomarkers derived from *in vitro* tumor models have been shown to have predictive values in the clinic. For example, imatinib selectively kills cells with the activated BCR-ABL gene fusion *in vitro* (36), whereas lapatinib selectively inhibits proliferation of ERBB2-overexpressing cells (17). We showed in this article that loss of p53 function and chromosome instability are predictive for *in vitro* sensitivity to the Plk1 inhibitor GSK461364A in a drug washout/oungrowth assay. This observation suggests a hypothesis for testing in the clinical development of agents targeting Plk1 and may eventually guide a targeted therapeutic approach.

Although standard proliferation assays can be effective for quantifying the response of cell lines to targeted agents (e.g., ref. 37), the high rate of proliferation of tumor cells *in vitro* confounds such analysis of those compounds targeting mitosis. As expected, compounds...
targeting Plk1 almost universally inhibit proliferation in vitro (15). This is unsurprising because Plk1 is required in almost all aspects of mitosis (38) and cells in culture are generally rapidly dividing. An alternative approach to identify tumor cells that respond to Plk1 inhibition was to measure cellular outgrowth after drug washout. This approach resulted in a wider range of responsiveness in a panel of cancer cell lines (Supplementary Table S3). More importantly, we believe that this drug washout/outgrowth assay is likely a more relevant metric for in vivo efficacy for GSK461364A as this compound is dosed intermittently as continuous i.v. infusion for a short period of time followed by a longer period of drug holiday.

Cell lines that have lost p53 expression or carry mutated p53 were more likely to be sensitive to GSK461364A in a drug washout/outgrowth assay ($P = 0.00078$; Fisher's Exact test; Fig. 2A). There is no significant difference for p21 expression level between the two groups ($P > 0.05$; Fisher's Exact test; Supplementary Table S3). A minority of cell lines (3 of 25; 12%) was responsive to GSK461364A while harboring WT p53. Elevated mRNA level of MDM2 (Fig. 2B) in these three cell lines (MCF7, Lovo, and ZR75-1) suggests that the multiple mechanisms that cancer cells use to inactivate p53 pathway may affect sensitivity to GSK461364A.

In the drug washout/outgrowth assay, we established that p53-deficient cells were more sensitive to Plk1 inhibitor. p53 function is affected to different degrees depending on the type of mutation it harbors. The mutations most detrimental to p53 function are those resulting in loss of p53 protein (null) and the dominant-negative mutations (31). Of the 27 TP53 non-WT cell lines tested in the drug washout/outgrowth assay, six unpredictably showed a resistant profile. Closer examination of the TP53 status in these cell lines revealed that four of these six cell lines (HN3, MALME3M, SKMEL28, and NCI-H727) carry TP53 mutations that have not been reported as dominant-negative and have been shown to occur in low frequencies in cancer (Supplementary Table S3), suggesting that the effect of these mutations on p53 function may be incomplete inactivation. In contrast, sensitive cell lines were predominantly null for p53 or carried dominant-negative mutations.

Cancer genome instability is often characterized by the manifestation of large genomic copy number alterations that can range from ~25 Mb to an entire chromosome arm in size (39). The persistent accumulation of DNA copy number alterations has been correlated with TP53 mutations in several human tumor types, such as lung (35) and breast (34) cancers. As expected, considering p53 status, increased rates of large DNA copy number alterations were associated with the cell lines more sensitive to GSK461364A ($P = 0.0413$; $t$ test; Fig. 3). Using a leave-one-out validation approach, the expression pattern of 70 genes predictive of copy number instability in cancer cell lines (40) further delineated cell lines sensitive to GSK461364A by washout/outgrowth assay (success rate = 72%; $P < 0.05$). This correlation substantiates the observation that higher rates of DNA alterations associate with GSK461364A-sensitive response in vitro. At a functional level, it is likely that cells lacking a p53 response are unable to arrest at the various cell cycle checkpoints ($G_1$, $G_2$, postmitotic) that would either prevent injury or allow repair, resulting in accumulated heritable DNA copy number alteration. Thus, a nondiploid phenotype is manifested more frequently in those harboring mutations of p53.

To study the mechanism of action linking p53 mutation and Plk1 inhibitor sensitivity by washout/outgrowth assay, cell cycle markers were assessed on synchronized cells following GSK461364A treatment. Regardless of p53 mutation status, levels of Cyclin B1, pH3, and DNA content confirm that GSK461364A resulted in prolonged mitotic arrest followed by aberrant mitotic exit with tetraploidy, termed mitotic slippage. We observe that in GSK461364A-resistant/p53 WT cells (A549), p21 levels accumulated at mitotic arrest, leading cells that survive mitotic slippage to a p53/p21-dependent postmitotic checkpoint arrest in a tetraploid pseudo-G1 phase characterized by elevated Cyclin E. In contrast, GSK461364A-sensitive/p53 mutant cells (PL45) showed comparable mitotic arrest and mitotic slippage, but failed to undergo postmitotic checkpoint arrest following treatment with GSK461364A. We further confirmed that RNA silencing of either p53 or p21 in A549 cells can significantly reduce the accumulation of Cyclin E in GSK461364A treated A549 cells, suggesting an abrogation of the postmitotic checkpoint (Fig. 5B). These results are concordant with previous studies showing that p53 can prevent endoreduplication following mitotic failure whereas cells lacking p53 fail to arrest at a postmitotic checkpoint (41–43). This raises the possibility that the p53 WT cells injured by prolonged Plk1 inhibition may have a survival advantage by exiting the cell cycle to further arrest in pseudo-G1 phase. In contrast, p53 functionally deficient cells treated with a Plk1 inhibitor would fail to exit the cell cycle, undergoing compounding rounds of mitotic injury leading to death through mitotic catastrophe caused by repeated aberrant mitosis.

The observation that transient exposure of Plk1 inhibitor GSK461364A has greater antiproliferative/survival effect in p53-deficient tumors is consistent with earlier studies on Plk1. Two independent RNAi studies both showed that the elimination of Plk1 function preferentially reduced the survival of cells with mutant p53 (25, 26). There are also reports that anaplastic thyroid carcinoma (ATC), which has high frequency of p53 mutation (70–90%) and carries chromosomal instability signature, is profoundly sensitive to Plk1 inhibition by both siRNA knockdown study (44) and compound study (45). Interestingly, Aurora kinase inhibitor VX-680 was also shown to preferentially kill cancer cells with compromised p53 function, and a similar mechanism of weakened pseudo-G1 tetraploidy checkpoint in p53-defective cells was proposed (46). While preparing this article, a new study by Sur et al. (47) was published reporting that
upon UV irradiation or nutlin treatment, cells without WT p53 were more sensitive to Plk1 inhibitor than cells with WT p53. However, without irradiation or Nutlin, in the in vitro standard proliferation assay, isogenic lines with different p53 background seemed to have similar GI50 to the Plk1 inhibitor. This is consistent with what we observed using a standard 72-hour proliferation assay in that most of the cancer cell lines seemed to be sensitive irrespective of p53 background (see GI50 in our Table 1), and differences in outgrowth potential were only observable upon drug washout. Although our study suggests that a Plk1 inhibitor as single agent might work better in cancers that have lost p53 function, it is very appealing to consider that a Plk1 inhibitor may also combine well with other agents particularly in the same p53 mutant background tumors as suggested by Sur et al.

Loss of p53 function mostly only occur in cancer cells, whereas normal cells express WT p53 protein. The positive association of loss of p53 function with drug response raises the possibility that Plk1 inhibitors can selectively eliminate cancer cells whereas the cycling normal cells would be protected by WT p53. In addition, many cancer therapies tend to be more effective in p53 WT patients (48). The sensitivity to GSK461364A toward p53-deficient tumors compared with that of WT p53 tumors could potentially offer an opportunity to treat tumors that are refractory to chemotherapies as well as early-line therapy for these genotypes. With the caveat of being a preclinical study, the results warrant testing the hypothesis in the clinic by applying these biomarkers (sequencing TP53, evaluating p53 expression profile, or profiling genomic instability) with the goal of improving identification of potentially responsive patients to Plk1 inhibitors.

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Sensitivity of Cancer Cells to Plk1 Inhibitor GSK461364A Is Associated with Loss of p53 Function and Chromosome Instability

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