Identification of Polo-like Kinase 1 as a Potential Therapeutic Target in Anaplastic Thyroid Carcinoma

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Abstract
Anaplastic thyroid carcinoma (ATC) is one of the most aggressive and chemoresistant cancers. The serine/threonine kinase Polo-like kinase 1 (PLK1), a key regulator of multiple steps during mitotic progression, is highly expressed in ATC. Here, we used the BI 2536 PLK1 inhibitor on ATC and nontransformed thyroid follicular cell lines. Our data show that ATC cells are addicted to high levels of PLK1 activity for proliferation, survival, anchorage-independent growth, and tumorigenicity. On treatment with nanomolar doses of BI 2536, ATC cells progressed normally through S phase but died thereafter, directly from mitotic arrest. Immunofluorescence microscopy, immunoblot, and flow cytometry analysis showed that, on PLK1 blockade, ATC cells arrested in prometaphase with a 4N DNA content. Treated ATC cells accumulated phosphohistone H3 and displayed characteristic mitotic (Polo) spindle aberrations. Nontransformed thyroid cells were 3.2- to 18.4-fold less susceptible to BI 2536–induced cell cycle effects compared with ATC cells. These findings identify PLK1 as a promising target for the molecular therapy of ATC. [Cancer Res 2009;69(5):1916–23]

Introduction
Anaplastic (undifferentiated) thyroid carcinoma (ATC), a rare thyroid cancer (2–5% of all cases), ranks among the most lethal human malignancies with a mortality rate above 90% and median survival from diagnosis of approximately 4 to 12 months (1–3). ATC usually presents during the 6th to 7th decade of life as a rapidly enlarging neck mass that extends locally, compresses the adjacent structures, and tends to disseminate to regional nodes and distant sites (1–3). Multimodal therapy, including surgery, chemotherapy, and radiotherapy, does not change the natural course of the disease (1–3).

Several genetic alterations involved in the pathogenesis of ATC are known. Some of them, namely, point mutations in RAS and BRAF (4–8) and point mutations or gene amplification of PIK3CA (9, 10), are found not only in ATC but also in well-differentiated (papillary or follicular) carcinomas, suggesting that ATC may develop secondary to neoplastic progression of well-differentiated thyroid cancers. Conversely, p53 mutations are rarely seen in well-differentiated lesions (<10%), whereas they occur in 67% to 88% of ATC (4, 5). Other mechanisms, besides gene mutation, may functionally impair p53 in ATC (11, 12).

We have recently identified a gene expression signature associated with ATC phenotype, featuring up-regulation of Polo-like kinase 1 (PLK1; ref. 13). PLK1 belongs to the Polo family of serine/threonine kinases, which also includes PLK2, PLK3, and PLK4 (14). PLK1 plays a pivotal role in several G2- and M-phase–related events, namely, centrosome maturation, α-tubulin association with centrosomes and microtubule nucleation, bipolar spindle formation, CDC2 (CDK1)-cyclin B activation, anaphase-promoting complex/cyclosome (APC/C) activation (by direct APC phosphorylation and by inactivation of the APC/C inhibitor Emi1), chromosome segregation, and cytokinesis (14–17). Consistent with its prominent role in G2-M phase, PLK1 expression and activity are low throughout G0, G1, and S phase and then rise in G2 and peak during M phase (15).

PLK1 is overexpressed in several cancer types and its up-regulation often correlates with poor prognosis (14–16). Adoptive overexpression of PLK1 causes cell transformation, whereas its depletion/inactivation leads to mitotic arrest and cell death (18–21). Therefore, PLK1 has been envisaged as a potential target in cancer therapy (17, 22–28).

BI 2536 is a dihydropteridinone compound that inhibits PLK1 activity in an ATP-competitive manner (17, 22). BI 2536 inhibited PLK1 with high potency (IC50, 0.83 nmol/L); it showed a ≥10,000-fold selectivity relative to a panel of 63 other protein kinases and equipotently blocked human, rat, and mouse PLK1 (22). Here, we show that BI 2536 selectively induces prometaphase arrest and mitotic death of ATC cells, suggesting that targeting PLK1 could be pursued as a novel strategy in the treatment of ATC.

Materials and Methods

Compound. BI 2536, 4-((R)-8-cyclohexyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydro-pteridin-2-ylamino)-3-methoxy-N-(1-methyl-piperidin-4-yl)-benzamide, was provided by Boehringer Ingelheim Austria GmbH. BI 2536 was dissolved in DMSO at a concentration of 10 mmol/L and stored at −80°C. Vehicle alone was used as a control.

Cell cultures. The human ATC cell lines CAL62 (29), SW1736 (30), OCUT-1 (31), 8505C (32), and ACT-1 (33) were grown in DMEM (Invitrogen) containing 10% fetal bovine serum. CAL62 and 8505C were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. OCUT-1 and ACT-1 were a gift of K. Hirakawa and N. Onoda (Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan) and SW1736 cells were a gift of C.H. Heldin (Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden). The Fischer rat-derived untransformed thyroid follicular cell line PC Cl 3 (hereafter “PC”) was grown in Coon’s modified Ham F12 medium supplemented with 5% calf serum and a mixture of six hormones, including thyrotropin (10 milliliters/ml), hydrocortisone (10 mmol/L), insulin (10 μg/mL), apo-transferrin (5 μg/mL), somatostatin (10 ng/mL), and glycyl-histidyl-lysine (10 ng/mL). Sigma-
Aldrich). Normal human thyrocytes were isolated from normal thyroid tissue obtained from a patient who underwent thyroid surgery and cultivated in RPMI 1640 supplemented with 20% fetal bovine serum. Cell proliferation and soft agar colony formation assays were performed according to standard procedures as described in Supplementary Data.

**Protein studies.** Immunoblotting experiments were performed according to standard procedures (Supplementary Data). A list of the antibodies is available in Supplementary Data.

**Immunofluorescence.** Immunofluorescence experiments were performed according to standard procedures (Supplementary Data).

**In vitro PLK1 kinase assays.** The in vitro PLK1 kinase assay was performed as previously described (34). Briefly, exponentially growing cells were solubilized in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 0.5% NP40, 120 mmol/L NaCl] containing a protease inhibitor cocktail (Roche Diagnostics) and a phosphatase inhibitor cocktail (Sigma). Lysates were then incubated with anti-PLK1 monoclonal antibody (Upstate Biotechnology, Inc.); immune complexes were recovered with protein A-Sepharose beads and incubated in the presence of 5 μCi [γ-32P]ATP at 30°C for 10 min with 10 μg of α-casein (Sigma) in 20 μL of PLK1 kinase buffer [20 mmol/L HEPES (pH 7.4), 50 mmol/L/2 mL KCI, 10 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L EDTA, 1 μmol/L ATP]. The reactions were stopped by adding 20 μL of 2× Laemmli buffer. Samples were separated by SDS-PAGE. Gels were dried and visualized by autoradiography or transferred to nitrocellulose and analyzed by autoradiography. Signal intensity was measured at the ImageQuant software.

**Cell cycle analysis.** Cell cycle experiments were performed according to standard flow cytometry procedures. A description is available in Supplementary Data.

**Animal studies.** CAL62 cells (2 × 10⁷ per mouse) were inoculated s.c. into the flank of 6-wk-old female CD1 nu/nu mice (Charles River Laboratories Italia). When tumors reached a volume of ≈ 50 mm³, animals were randomized into treatment and control groups of seven mice each. BI 2536 was dissolved in 0.1 N HCl diluted with 0.9% NaCl, and injected i.v. into the tail vein. The administration volume was 10 mL/kg body weight. Animal studies were conducted according to the Italian regulation for the experimentation on animals. More details are available in Supplementary Data.

**Statistical analysis.** The two-tailed unpaired Student’s t test was used for statistical analysis. All P values were two sided and differences were significant when P < 0.05. Statistical analyses were carried out using the GraphPad Instat software program (version 3.06.3).

**Results**

**BI 2536–mediated PLK1 inhibition reduces growth and viability of ATC cells.** We used ATC cell lines CAL62, SW1736, OCUT-1, 8505C, and ACT-1. These cells overexpress PLK1 (data not shown; ref. 13) and have varied patterns of chromosome aberrations and oncogene mutations (30): SW1736, OCUT-1, and 8505C harbor the BRAF V600E mutation (32), whereas CAL624 and ACT-15 cells exhibit the KRAS G12R and the NRAS Q61K mutations, respectively. All the ATC cells have p53 gene alterations: 8505C, CAL624, and ACT-15 have p53 gene mutations; SW1736 and OCUT-1 are negative for p53 expression (13, 31).

To verify whether PLK1 overexpression in aggressive thyroid carcinoma cells was paralleled by an increase in its activity, PLK1 protein was immunoprecipitated from equal amounts of protein lysates obtained from exponentially growing CAL62 and SW1736 cells and from a normal thyroid primary culture; precipitates were subjected to in vitro kinase assay using α-casein as a substrate. As shown in Fig. 1A, higher (＞10-fold) PLK1 activity was detected in ATC cells compared with normal thyrocytes.

Next, we tested the effect of PLK1 inactivation on ATC cell growth and viability. Cells were treated with BI 2536 over a 1,000-fold concentration range (starting at 0.1 nmol/L) and counted every day. Vehicle alone was used as a control. Figure 1B shows the average result of three independent experiments. BI 2536 treatment caused a significant (P < 0.0001) growth impairment of the cancer cell lines. At 72 hours of treatment, EC₅₀ values were 1.4, 2.3, 3.2, 5.4, and 5.6 nmol/L for CAL62, OCUT-1, SW1736, 8505C, and ACT-1 cells, respectively. Cell growth impairment was most likely the result of both growth arrest (due to G2-M-phase stall, see below) and reduced cell viability. Compared with vehicle control, after 72 hours of treatment with 10 nmol/L BI 2536, a reduced fraction of viable ATC cells (16.7%, 59.1%, 66.8%, 68.8%, and 75.6% for CAL62, OCUT-1, 8505C, ACT-1, and SW1736, respectively) was detected by trypan blue exclusion assay (Fig. 1C). To analyze BI 2536 effects on nontransformed cycling thyroid cells, we used normal thyroid primary culture cells and thyroid follicular PC cells. BI 2536 showed an EC₅₀ value for the growth of normal thyroid (25.8 nmol/L) and PC cells (17.8 nmol/L) that was, respectively, 4.6 to 18.4-fold and 3.3- to 13-fold higher compared with ATC cells (P < 0.0001; Fig. 1B). In line with this, 72 hours of treatment with 10 nmol/L BI 2536 failed to produce any significant reduction in the proportion of viable normal thyroid cells (Fig. 1C). At in vitro kinase assays, levels of PLK1 inhibition in untransformed PC and in normal thyroid cells were, respectively, similar to or higher than those of CAL62 cells. At the 1 nmol/L submaximal inhibitory BI 2536 concentration, residual PLK1 activity was 48.5% in CAL62, 52.6% in PC, and 28.1% in normal thyroid cells. This excluded a difference in PLK1 inhibitory potency as the cause for the different susceptibility to the compound of ATC versus nontransformed cells (Fig. 1D).

PLK1 protein level and kinase activity rise in G2 and peak during M phase, and PLK1 is involved in regulating these phases (14–17). In keeping with this, the dramatic effects resulting from PLK1 depletion/inhibition occur during mitosis (19–21) when its activity is most required. Indeed, among the cell lines tested, normal thyroid primary culture cells were both the less sensitive to BI 2536 and the slowest growing ones (0.87 population doublings in 72 hours; Fig. 1B), suggesting a correlation between growth rate and susceptibility to PLK1 inhibition. However, this did not apply to all cell lines. In fact, despite showing lower PLK1 expression (13) and activity (Fig. 1D, inset), nontransformed PC (3.2 population doublings in 72 hours) displayed a growth rate that was comparable with that of ATC (from 1.2 to 3.7 population doublings in 72 hours) cells (Fig. 1B); this suggests that factors other than proliferation rate may influence susceptibility to PLK1 inhibition. Indeed, with lower potency with respect to PLK1 (IC₅₀, 0.83 nmol/L), BI 2536 is known to inhibit PLK2 (IC₅₀, 3.5 nmol/L) and PLK3 (IC₅₀, 9.0 nmol/L) as well (17, 22). This prompted us to consider that, regardless of the low nanomolar concentrations used in our experiments, effects on these two kinases might contribute to the different sensitivity of the cells to the compound. Such possibility was ruled out by quantitative reverse transcription-PCR experiments, showing only mild differences in PLK2 and PLK3 expression between the different cells and no correlation with their sensitivity to BI 2536 (Supplementary Fig. S1A), and by small interfering RNA (siRNA) experiments in which only silencing of PLK1, but not PLK2 or PLK3, was able to cause a significant (P < 0.0001) growth impairment of CAL62 cells (Supplementary Fig. S1B and C).

1 Catalogue of Somatic Mutations in Cancer at http://www.sanger.ac.uk/genetics/CGP/cosmic.
2 T.C. Nappi et al., unpublished data.
BI 2536 inhibits ATC cell anchorage-independent proliferation and tumor growth. We tested whether BI 2536 inhibited the ability of CAL62 cells to proliferate in vitro in semisolid medium and in vivo in mice xenografts. BI 2536 (5 nmol/L) abrogated almost completely CAL62 colony formation in soft agar (>76-fold compared with vehicle control) as shown in Fig. 2A. For animal studies, BI 2536 or vehicle was administered i.v. to immunodeficient nu/nu mice that had developed CAL62 xenograft tumors of ~50 mm³ volume. As shown in Fig. 2B, after two cycles of 50 mg/kg BI 2536 given twice weekly, treated mice had significantly smaller tumors compared with control mice ($P < 0.001$), with a treatment versus control value of 9%.

BI 2536 induces prometaphase arrest and mitotic death of ATC cells. To investigate the mechanisms of cell growth and viability reduction on BI 2536 treatment, exponentially growing ATC cells were treated with BI 2536 at the lowest effective concentration (as estimated by the cell growth assays in Fig. 1) and cell cycle profile was analyzed by flow cytometry, immunofluorescence microscopy, and immunoblot. Vehicle was used as a negative control. As shown in Fig. 3A, starting at 12 hours of treatment, 5 nmol/L (CAL62) and 10 nmol/L (8505C, ACT-1, OCUT-1, and SW1736) of BI 2536 increased...
the 4N DNA fraction in ATC cells. This was followed at 36 and 48 hours by the appearance of a sub-G1 peak. At 48 hours, sub-G1 fractions in BI 2536–treated compared with vehicle-treated cells were 20% versus 0.67% for CAL62, 16.72% versus 1.26% for 8505C, 14.8% versus 2.2% for ACT-1, 12% versus 2.31% for OCUT-1, and 6.15% versus 1.7% for SW1736. Cell fractions with a 4N DNA peak were hardly detectable, indicating that the treatment did not cause reduplication of unseparated sister chromatids after failure of cytokinesis (see also below; Fig. 3A). The presence of a residual 2N DNA fraction in some BI 2536–treated ATC cell lines was attributed to their slower doubling time (Fig. 1B) rather than G1- or S-phase arrest (see also below). None of the aforementioned effects could be seen in PC cells on incubation with 10 nmol/L BI 2536 (Fig. 3A). In these cells, only treatment with ≥50 nmol/L concentrations of the compound gave results similar to those observed in ATC cells (Supplementary Fig. 5A).

The accumulation of cells with a 4N DNA content is indicative of a cell cycle block in either G2 or M phase. To distinguish between the two possibilities, BI 2536–treated or DMSO-treated cells were either stained with anti-phosphohistone H3 by immunofluorescence or analyzed by immunoblot for cyclin B1 levels. PLK1 protein levels were also examined as surrogate G2-M phase marker. The percentage of mitotic (phosphohistone H3 positive) cells was increased in BI 2536–treated ATC, but not PC cells, as soon as 12 hours after treatment, peaking at 24 hours and decreasing thereafter (Fig. 3B). Both cyclin B1 and PLK1 protein levels underwent analogous changes on BI 2536 treatment, with a peak at 24 or 36 hours and a decline at 24 to 36 hours or 36 to 48 hours depending on the cell line (Fig. 3C). These findings indicated that the compound caused M-phase arrest of ATC cells. Concentrations ≥50 nmol/L were required to elicit similar responses in PC cells (Supplementary Fig. S2 and C).

The reduction of phosphohistone H3–positive cells and cyclin B1 levels at the most onward time points could be due to death of ATC cells directly from mitosis; alternatively, on the 4N block, cells may attempt to start a novel cycle and reduplicate their genome. As mentioned above, this second hypothesis could be ruled out given the absence of >4N DNA peaks at flow cytometry. On the other hand, caspase-3 cleavage fragments appeared in ATC cell lines treated with BI 2536 before or at mitosis exit (caspase B1 degradation; Fig. 3C). In the highly proliferative CAL62 cells (3.7 population doublings in 72 hours; Fig. 1B), which displayed a major reduction of cyclin B1 levels after 36 hours of treatment, cleaved caspase-3 fragments were detectable as soon as 24 hours after incubation with BI 2536 and peaked at 36 hours. At the other end of the spectrum, in the less rapidly growing SW1736 cells (1.14 population doublings in 72 hours; Fig. 1B), which showed more modest reduction of cyclin B1 after 36 hours of treatment, caspase-3 fragments showed a milder and delayed increase. Taken together, these findings suggest that, on PLK1 inhibition, ATC cells rapidly undergo a deadly faith directly from mitosis and that, among different ATC cells, those with the highest proliferative rate tend to be the most susceptible to PLK1 inhibition.

To further characterize the cell cycle effects of PLK1 inhibition, we synchronized CAL62 cells at the G1-S transition by double thymidine block; cells were released in the presence of 5 nmol/L BI 2536 or vehicle and analyzed by flow cytometry and immunoblot at different time points. As shown in Fig. 4A, BI 2536–treated cells progressed normally through S phase and then arrested in G2-M (at 11 hours). Sub-G1 DNA fractions became detectable after 29 to 48 hours from release (Fig. 4A). Instead, cells released in control medium normally progressed through S and G2-M and then reentered a new G1 phase (Fig. 4A). Western blot analysis showed consistent results. In vehicle-treated cells, cyclin B1 was down-regulated after 24 hours from release as cells had exited mitosis (Fig. 4B), whereas in cells treated with BI 2536, cyclin B1 down-regulation was delayed until 35 hours (Fig. 4B). In treated cells, cleaved caspase-3 fragments were clearly detectable after 24 hours of treatment, again suggesting that cell death may occur directly from mitosis. Moreover, despite a reduction of cyclin B1 levels at the latest time points, BI 2536–treated cells failed to down-regulate geminin (Fig. 4B), an event that is required to start a new DNA duplication cycle (35). This indicates that treated cells remained unlicensed for replication, ruling out the possibility of reduplication of undivided cells.
BI 2536 induces characteristic mitotic spindle aberrations in ATC cells. Depletion or inactivation of PLK1 has been reported to induce characteristic mitotic spindle aberrations in several cancer cells (14–17, 19, 21, 22, 24). To further prove that BI 2536 effects in ATC cells were mediated by PLK1 inhibition, we asked whether compound treatment phenocopied PLK1 knockdown (13). Exponentially growing CAL62 and SW1736 cell lines were treated with BI 2536 (5 and 10 nmol/L, respectively), collected at different time intervals, and stained for immunofluorescence with anti-α-tubulin to visualize mitotic spindle and 4′,6-diamidino-2-phenylindole (DAPI) to visualize DNA. Consistent with the results obtained by anti-phosphohistone H3 staining, both ATC cell lines showed an accumulation of mitotic cells in a prometaphase state starting at 12 hours and peaking at 24 hours; in treated cells, anaphase and cytokinesis figures were virtually absent (Fig. 5A). No increase in the mitotic fraction could be seen in control PC cells on treatment with 10 nmol/L BI 2536, and all the mitotic phases were normally represented (Fig. 5A).

A characteristic phenotype in inhibitor-treated ATC cells, but not PC cells, was the presence of aberrant monopolar (Polo) spindles, a hallmark of PLK1 depletion/inactivation (Fig. 5A). Such Polo spindles are monoastral microtubule arrays nucleated from a single organizing center and containing unseparated centrosomes circled

Figure 3. Cell cycle effects of BI 2536 in ATC cells. A, the indicated cell lines were treated with 5 nmol/L (CAL62) and 10 nmol/L (8505C, ACT-1, OCUT-1, SW1736, and PC) of BI 2536 or vehicle (NT) and DNA content was analyzed by flow cytometry at different time points. B, CAL62 and SW1736 ATC cells or control PC cells, grown on glass coverslips, were treated with BI 2536. At the indicated time points, cells were fixed, stained with anti-phosphohistone H3 (p-H3; Ser10) and analyzed by fluorescence microscopy. The percentage of positive cells represents the average of three experiments in which at least 500 cells were counted. Insets, representative micrographs. C, CAL62 and SW1736 cells, treated with BI 2536 or vehicle (NT), were lysed at the indicated time points and subjected to immunoblot with anti-cyclin B1 (cycB1) and anti-PLK1 and anti-cleaved caspase-3 antibodies (which detect only the p17 and p19 cleaved caspase-3 fragments). Tubulin levels are shown for normalization. Data are representative of at least three different experiments.
by a ring of condensed chromosomes (15). Monopolar spindles seem to occur secondary to a defect in timely organization of microtubule asters at prophase followed by the instantaneous polymerization of mitotic microtubules in a monopolar aster only after nuclear envelope breakdown (17). They occurred in BI 2536–treated ATC cells along with other aberrant spindle phenotypes, such as multipolar spindles and spindles without a clear spatial organization (disorganized) that accumulated with time (Fig. 5A and B). After 36 hours of treatment, albeit only very few mitotic cells could be seen on coverslips, virtually 100% of them had an abnormal spindle. Moreover, after 36 and 48 hours of treatment, in parallel with the reduction of phosphohistone H3–positive cells and cyclin B1 levels and with increased levels of caspase-3 fragments, most of the remaining ATC cells either showed an apoptotic nuclear morphology or harbored decondensed/partially condensed nuclei with gross morphologic aberrations (Fig. 5A). These are hallmarks of the so-called mitotic catastrophe, a form of cell death taking place from within mitosis and resulting in either apoptosis or necrosis (36). In nontreated cells, a similar effect could only be obtained with compound doses ≥50 nmol/L (Supplementary Fig. S2D).

Discussion

Targeting key components of the mitotic machinery has been envisaged as a possible strategy in cancer treatment. Among them, PLK1 has emerged as a promising candidate (15, 16). PLK1 depletion/inhibition reduces survival of several cancer cells and inhibits tumor growth in vivo in xenograft models (19–27). It
is feasible that, based on the specific role of PLK1 in mitosis, its inhibition may avoid some of the side effects associated with current antimitotic agents (such as taxanes and Vinca alkaloid derivatives) that, by targeting microtubules, affect many critical cellular processes (e.g., axonal transport) unrelated to mitosis (15, 16).

Here, we took advantage of the high potency and selectivity of BI 2536, a compound able to inhibit PLK1 activity at low nanomolar concentrations, to exploit PLK1 requirement of ATC cells. ATC cells displayed high PLK1 activity and addiction to high concentrations of functional PLK1 for faithful bipolar spindle assembly and mitosis, being dependent on PLK1 for proliferation, survival, anchorage-independent growth, and tumor development. Concentrations as low as 5 to 10 nmol/L of BI 2536 were sufficient to inhibit PLK1 and cause prometaphase arrest with characteristic spindle aberrations. This caused mitotic entrapment and subsequent mitotic cell death, a process resulting from aberrant mitosis and that can occur through necrosis or apoptosis (36).

These effects are hallmarks of PLK1 inhibition, suggesting that the compound is exerting its effects in ATC cells by blocking PLK1 and not other targets (15–17). Besides inhibiting PLK1, BI 2536 also affects the activities of PLK2 and PLK3, albeit at higher concentrations (17, 22). However, PLK2 and PLK3 expression levels did not correlate with ATC cell sensitivity to BI 2536, and PLK2 and PLK3 depletion failed to reduce ATC cell growth, excluding any contribution of these two kinases to BI 2536–induced growth and viability impairment in ATC cells. Moreover, PLK2 and PLK3 are thought to function in G1 and S phases of the cell cycle, which were not significantly affected by BI 2536 treatment of ATC cells. Interestingly, polyploidy, a major concern when targeting kinases involved in mitosis/cytokinesis as it may promote tumorigenesis (16, 37), was not detected, and the possibility of reduplication was ruled out. Instead, this potentially harmful phenomenon was reported with Aurora B inhibitors that silenced the mitotic spindle checkpoint, causing precocious mitotic exit and polyploidy (16).

Importantly, untransformed thyrocytes proved to be less sensitive to BI 2536, suggesting the possibility of achieving a therapeutic window in vivo. In fact, the threshold of PLK1 activity to sustain a normal mitotic cycle was lower in nontransformed cells, where proliferation and viability impairment was observed only at high doses of BI 2536. Although in the case of primary human normal thyroid cells this was clearly correlated to reduced proliferation rate, this was not the case for nontransformed PC thyroid follicular cells. Beyond the influence of growth rate, the reason for the different PLK1 requirement between normal and cancer cells remains unknown. Several reports have indicated that the p53 status may be a genetic determinant of susceptibility to PLK1 inhibition: p53 protein is stabilized in PLK1-depleted cancer cells (20, 21) and p53 depletion enhances the sensitivity of various

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**Figure 5.** Mitotic spindle defects in BI 2536–treated ATC cells. Exponentially growing PC, CAL62, and SW1736 cells were treated with BI 2536 (10, 5, and 10 nmol/L, respectively) or vehicle (NT) and stained by immunofluorescence with anti-α-tubulin (red) and DAPI (cyan) at different time points. Representative micrographs are reported in A and percentages of mitotic cells with monopolar (Polo), multipolar, or grossly disorganized mitotic spindles are reported in B. Values refer to the average of triplicate experiments in which at least 300 mitotic cells were counted. A, of note is the aberrant nuclear morphology shown by ATC cells after 36 to 48 h of treatment.
cell types to PLK1 knockdown (21, 38). Along this line, all the ATC cell lines used in this study either harbor p53 gene mutations or are negative for wild-type p53 expression. Intriguingly, OCUT-1 and SW1736 cells that do not express p53 showed a milder increase in cleaved caspase-3 levels on PLK1 inhibition compared with the other ATC cells that instead harbor a mutant p53 protein. Moreover, we observed that growth of PC cells stably expressing a V143A dominant-negative p53 was significantly (P < 0.001) compromised by 72 hours of BI 2536 (10 nmol/L) treatment or 48 hours of siRNA-mediated PLK1 depletion (>3- and 16.5-fold, respectively) compared with native PC cells (which harbor wild-type p53; Supplementary Fig. S3). Thus, one possibility is that p53 function may be required to survive PLK1 depletion and the frequent impairment of p53 would make ATC cells particularly susceptible to PLK1 inhibition.

Whatever the mechanism, our findings suggest that PLK1 might be exploited as a molecular target in ATC therapy and BI 2536, which has already progressed into phase I clinical trials in patients with advanced solid tumors (28), or other specific PLK1 inhibitors could be examined in clinical studies for the treatment of such deadly human cancer.

Disclosure of Potential Conflicts of Interest

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