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PLK1 as an oncology target: current status and future potential

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The Polo-like kinases (PLKs) have been investigated as oncology targets for several years; however, only recently have potent inhibitors been described. Here, we report on progress in the clinical validation of the PLKs as antitumor drug targets as well as recent understanding gained regarding their synergistic roles in the context of other molecular defects occurring in tumors. Also relevant to the development of PLK inhibitors as therapeutics are the putative roles of other members of this family as tumor suppressors. The resulting potential drawbacks of non-isoform selective compounds are presented. As an alternative approach to achieving PLK1 specificity, we discuss prospects for developing small molecule inhibitors of the crucial regulatory and subcellular targeting domain containing the Polo-boxes.

Recent developments in the validation of PLK1 as an oncology target

Polo-like kinases (PLKs) have gathered much attention as important elements that regulate cell cycle progression, particularly mitosis. There are four mammalian PLK family members identified thus far: PLK1, PLK2 (aka SNK), PLK3 (aka PRK, FNK and CNK) and PLK4 (aka SAK). More recently, PLK5 has been identified; however, it lacks a kinase domain and does not seem to function in cell cycle regulation [1]. The four are structurally homologous, containing an N-terminal catalytic kinase domain and a C-terminal region composed of 'polo boxes' (only one in PLK4), from which the specificity for their targets is thought to be derived. PLK1 is the most investigated member of the family and has been widely pursued as an oncology target because it is overexpressed in several human tumor types [2–5]. Not only is elevated expression of PLK1 frequently observed, but it also appears to be of prognostic value for pancreatic and gastric carcinoma tumors, among others [6–9]. PLK1 is also overexpressed in human melanomas and PLK1-specific short hairpin (sh)RNA resulted in a significant decrease in the viability of, and increased apoptosis in, melanoma cells without affecting normal human melanocytes [10].

PLK1 is expressed primarily in dividing cells owing to its crucial involvement in numerous mitotic events, including: (i) activation of CDC25c phosphatase, resulting in the removal of inhibitory phosphorylations from CDK1/cyclin B [11]; (ii) progression through mitosis via regulation of microtubule nucleation; (iii) centrosome maturation and kinetochore assembly [12–15]; (iv) regulation of cytokinesis [16,17]; and (v) exit from mitosis through activation of the anaphase-promoting complex [18]. Numerous studies have been published examining the potential of PLK1 as an antitumor drug target, including work with antisense oligonucleotides, small interfering (si)RNA and small molecules [19–21]. The consensus of these studies is that inhibition of PLK1 activity results in a potent antitumor effect both *in vitro* and *in vivo*. The essentiality of PLK1 in normal development and mitosis, however, has raised lingering questions regarding the value of targeting PLK1 in cancer chemotherapy. Here, we review exciting recent developments suggesting that tumor-selective antiproliferative effects are possible, thereby further confirming PLK1 as a valid oncology target.

Mouse knockouts of PLK1 are embryonically lethal, demonstrating that PLK1 has an essential role in normal development [22]. It has also been reported that PLK1-heterozygous mice were susceptible to a threefold increase in spontaneous tumor formation, although PLK1 status was not determined in the tumors from these mice [22,23]. Despite this observation, there is strong body of accumulating evidence that targeting PLK1 can produce tumor-selective antiproliferative effects. Constitutive expression of PLK1 in mammalian cells leads to malignant transformation, suggesting

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that aberrant PLK1 expression is oncogenic [24–26]. A genome-wide RNA interference (RNAi) screen to identify synthetic lethal interactions with mutant Ras identified PLK1 as a promising target (i.e. tumor cells dependent on mutant Ras were sensitized to PLK1 inhibition [27]). The relationship between p53 and PLK1, although complex, also suggests cancer-specific targeting is possible in tumors expressing mutant p53. In one important study, p53-deficient cancer cells were shown to be considerably more sensitive to PLK1 knockdown than were cancer cells with functional p53 [28]. At the same time, nontransformed cells were not significantly affected by PLK1 knockdown unless p53 was concomitantly depleted, which then resulted in sensitization [28]. Further evidence was obtained in a study of the depletion of PLK1 in isogenic pairs of cell lines and demonstrated that Plk1 depletion preferentially induced mitotic defects and prevented cell survival in the p53 mutant cell lines [29]. Several studies have identified mechanistic links that p53 and PLK1 negatively regulate each other. For example, PLK1 has been shown to bind to and inhibit physically the transcriptional and pro-apoptotic functions of p53 [30,31]. It has also been shown to regulate p53 stability via interactions with murine double minute 2 (MDM2) [32] and Topors [33], both of which are E3 ligases that target p53 for degradation. Thus, oncogenic PLK1 can drive the dysregulation of wild-type p53. Conversely, wild-type p53 acts to repress PLK1 expression [34] and has been shown to regulate PLK1 gene expression during G2/M [35]. Thus, mutant p53 leads to elevated PLK1 levels and a loss of proliferative control. These studies show that oncogenic PLK1 and mutated p53 negatively influence the other during tumorigenesis, suggesting a potential co-association in multiple tumor types irrespective of what happens first (e.g. p53 loss or PLK1 activation).

The above studies show by using siRNA approaches that PLK1 targeting in p53-defective cells might provide cancer-specific targeting and provide mechanistic rationale, but can this be exploited pharmacologically with small molecule inhibitors? To this end, a recent study examined potential biomarkers that are predictive for the efficacy of the PLK1-selective inhibitor, GSK461364A (Fig. 1) [36,37]. These were identified through comparative genetics measuring the proliferation of a panel of human cancer cell lines treated with GSK461364A. The results showed that cell lines with decreased expression or having p53 mutations had increased sensitivity to GSK461364A. Cellular mechanistic studies showed that p53 proficient but not mutant cells can activate a post-mitotic checkpoint and arrest at a pseudo-G1 state subsequent to inhibitor treatment and that downregulation of wild-type p53 increased its antiproliferative activity [37].

The ATP-based inhibitor of PLK1, BI2536 (Fig. 1), is potent against a large panel of cancer cells, regardless of p53 status [21]. However, the hypothesis that p53-defective cancer cells can be selectively sensitized to PLK1 inhibition by BI25360 has been proposed more recently [38]. Specifically, it was posed that, in the absence of wild-type p53, the viability of stressed cells depends on functional PLK1. Indeed, it was observed that treating cells with ionizing irradiation induced a G1 arrest in the wild-type p53 cells, which then protected the cells from the toxic effects of BI2536. By contrast, the mutant p53 cells remained sensitized to BI2536. Furthermore, compounds that upregulate p53, such as the Nutlin class of MDM2 binders, also provided a protective effect for wild-type p53 cancer cells. When tumor xenografts were examined, BI2536 alone caused regression of p53-deficient xenografts, whereas Nutlin-3 did not reduce the efficacy of BI2536 [38]. Most interestingly, the inclusion of Nutlin-3 protected the mice from neutropenia induced by BI2536, which is the dose-limiting toxicity (DLT) seen in human trials (discussed below). The clinical implications of these observations are profound, because they suggest that combining PLK1 inhibitors with ionizing radiation or other traditional therapeutics that induce a p53-dependent G1 arrest would protect normal cells from toxicity associated with PLK1 inhibition. Collectively, the studies summarized above suggest that specific tumors with increased PLK1 expression and nonfunctional p53 can be sensitized to PLK1 inhibition, thus providing a therapeutic window for tumor selectivity. Much research will be needed to identify the best combination therapies, that is, which other agents should PLK1 inhibitors be paired with for optimal therapeutic benefit. In addition, because many cancer therapies require wild-type p53 for efficacy through the induction of apoptosis, sensitization of p53-deficient tumors toward PLK1-targeted compounds might have therapeutic benefit where resistance to conventional therapeutic regimens is observed.

BI2536 and other clinical PLK inhibitors: current status and what has been learned so far

A significant body of data on the clinical evaluation of PLK1 inhibitory compounds has been generated during the past few years. This includes both phase I and II studies on BI2536 [39–41], GSK461364 [42], ON 01910.Na [43–45] and HMN-214 [46,47]. Whereas BI2536 and GSK461364 are ATP-competitive inhibitors, the mechanism for the latter two compounds on PLK1 activity has not yet been completely established and these compounds have been shown to have non-ATP-competitive behavior. In addition, they are known to be nonselective for the PLK family and, therefore, present a more ambiguous picture in terms of attributing toxicities to on-target inhibition.

A phase I study of ON 01910.Na (Fig. 1) was conducted to establish DLTs and determine the preliminary pharmacokinetics and pharmacodynamics (PK/PD) of this compound [44]. Treated patients had solid tumors that were refractory to standard therapy and ON 01910.Na was administered in a dose escalation up to 4370 mg. The maximum tolerated dose (MTD) was found to be 3120 mg and, therefore, was applied to six assessable patients. Observed toxicities upon treatment with ON 01910.Na included skeletal, abdominal or tumor pain and nausea, with hematological events being infrequent and mild. Pharmacodynamic responses to ON 01910.Na included that of a patient with refractory ovarian cancer who remained progression free for 24 months.

HMN-214 (Fig. 1), an oral prodrug of HMN-176, acts on the PLK1 pathway and, although it affects the subcellular localization of PLK1, its exact mechanism of action and specificity is currently unknown. To determine the safety profile and pharmacokinetics of HMN-214, 33 patients with cancer were treated using a continuous dosing schedule every 28 days [46]. DLTs were observed at 10 mg/m^2/day and included severe myalgia and/or bone pain syndrome and hyperglycemia. A single DLT of grade 3 bone pain was observed at 8 mg/m^2/day, which was then considered to be the MTD. An indication of antitumor efficacy in the treated population was evidenced by stable disease in 7 out of 29 patients. In

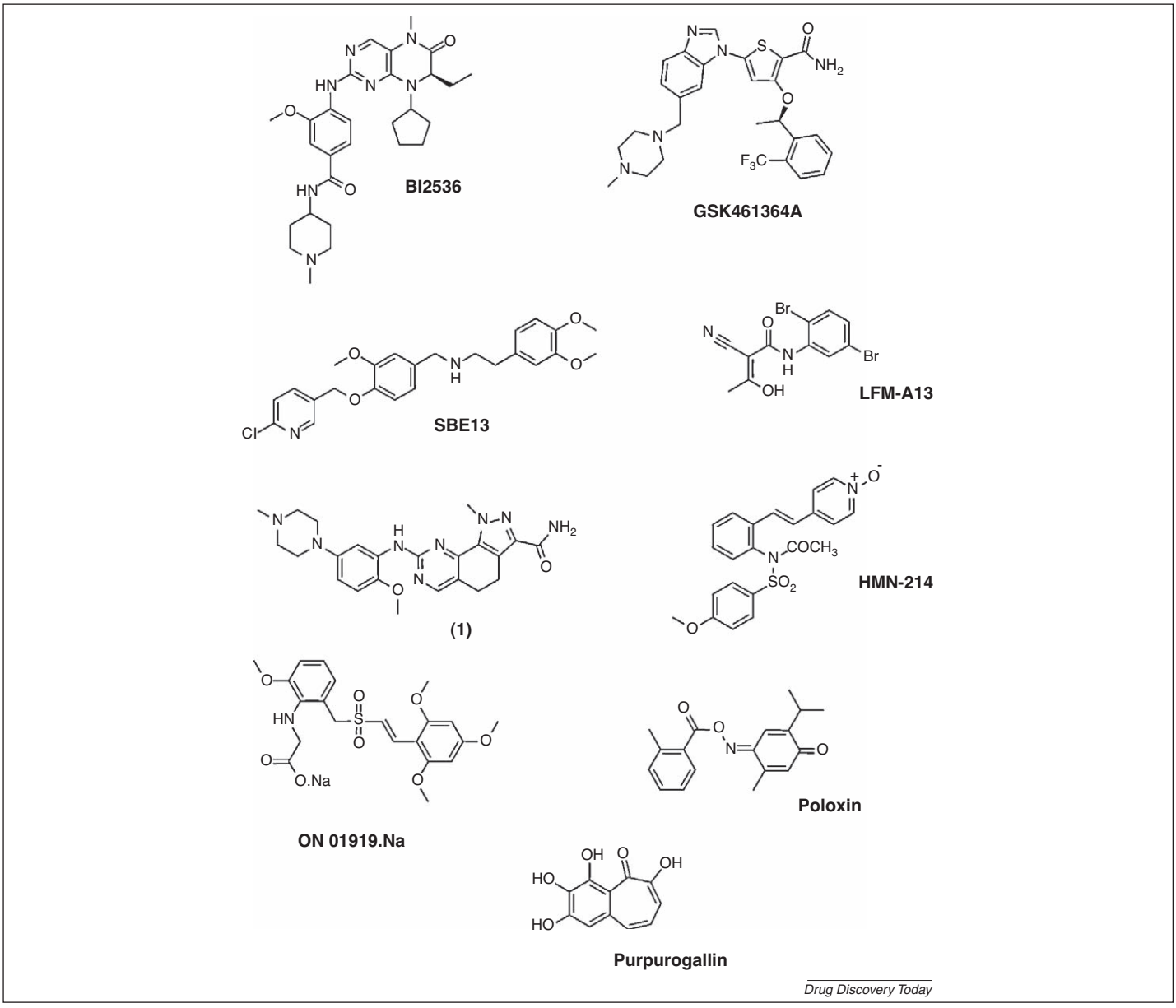

FIGURE 1

Chemical structures of PLK1 ATP-competitive and PBD inhibitors in preclinical and clinical development.

addition, one heavily pretreated patient with breast cancer had stable disease for a 6-month period.

BI2536 is an exquisitely potent and selective inhibitor of the Polo-like kinase family, with the primary target being PLK1 [21,48]. This compound provided insights into the phenotypic outcome of PLK1 inhibition and showed that cells treated with BI2536 are delayed in prophase but enter prometaphase after import of nuclear Cdk1–cyclin B into the nucleus. In addition, treated cells lack prophase microtubule asters and form monopolar spindles that are unstably attached to kinetochores. BI2536 prevents PLK1 localization at kinetochores and centrosomes and, when added to metaphase cells, leads to spindle collapse. It was the first PLK1 inhibitor to enter clinical trials and, therefore, a significant body of clinical data has been accumulated to date. The first phase I trial to be reported on BI2536 examined safety and PKs in 40 patients after administration of single intravenous doses of BI2536 (25–250 mg) [49]. The MTD was indicated to be 200 mg, with reversible neutropenia observed as the DLT and the most frequent adverse event at the MTD (nausea, fatigue and anorexia being mild to moderate adverse events). Minor antitumor responses were observed in approximately 25% of the study group.

A second phase I trial has recently been described with similar findings to the above study [39]. This open-label, dose-escalation study investigated the MTD, safety, efficacy and PKs in two treatment schedules in 44 and 26 patients with advanced solid tumors. Grade 3 and 4 neutropenia was observed in approximately one-third of patients, and other minor adverse events included gastrointestinal issues and fatigue. Stable disease in 14 of the patients treated using the days 1 and 8 dosing schedule was the overall response. In terms of PK studies, it was found that plasma concentrations of BI2536 increased dose proportionally with the average terminal half-life being 50 h. A further study examined

the clinical effects during optimization of the dosing schedule over the range of 25–250 mg over three different administration schedules in 104 patients [50]. These trial results showed that, when BI2536 is given as a 200-mg single administration, cycle duration can be reduced from 3 to 2 weeks, and an acceptable risk of neutropenia was observed.

Recently, the potential for BI2536 as an effective cancer drug has been further tested in phase II clinical trials. The first published study investigated the efficacy, safety and PKs of two dosing schedules in 95 patients with relapsed stage IIIB/IV non-small cell lung cancer [41]. The study group was randomized and given BI2536 via IV administration on day 1 (200 mg) or days 1–3 (50 or 60 mg) for a 21-day treatment course. Only four patients had a partial response, with observed median progression free survival of 8.3 weeks and 7 weeks, respectively. Median overall survival was 28.7 weeks and, similar to the phase I studies, a grade 4 neutropenia occurred in one-third of patients and nonhematological events included fatigue and nausea. Two deaths by pulmonary hemorrhage and sepsis were considered drug related. The overall conclusion of this study was that BI2536 monotherapy has modest efficacy and favorable safety in patients with relapsed non-small cell lung cancer.

A second phase II trial recently published involved a multicentric study in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma [40]. The study group included 76 patients who generally were young, had significant progressive disease and had also recently completed other relevant systemic treatments. BI2536 200–250 mg was given via IV on day 1 every 3 weeks and patients received a median number of two cycles (four in ovarian cancer). Adverse events included grade 3–4 neutropenia in most patients, and thrombocytopenia, anemia and pain as minor adverse effects in the trial group. Disappointingly, no confirmed objective responses were observed, leading to the overall conclusion that BI2536 displays limited antitumor activity according to the design of the trial in the five tumor types included. However, a positive result in the subset of patients with ovarian cancer was obtained, with more than 70% of the treated group showing stable disease after treatment.

The ATP-competitive PLK1 inhibitor GSK461364A, mentioned above, has been evaluated in 40 patients with advanced solid tumors [42]. The phase I study enabled the determination of MTDs for two dosing schedules. These included a weekly IV administration for 3 weeks of a 4-week cycle and twice a week IV administration for 3 weeks out of a 4-week cycle; this resulted in observed MTDs of 225 mg and 75 mg, respectively. The observed DLTs for GSK461364A were neutropenia, thrombocytopenia and bone marrow suppression, with some thrombotic events occurring in a few treated patients. As evidence for the PD effects of GSK461364A, an increase in phosphohistone H3 (a marker of mitosis) was observed in circulating tumor cells. Approximately 15% of patients showed minor antitumor responses to treatment with GSK461364A, indicated by prolonged stable disease lasting at least 3 months.

Overall, the trials performed so far suggest that GSK461364A and BI2536, the two ATP-competitive inhibitors clinically evaluated, are well tolerated in humans and that hematological effects, such as neutropenia and thrombocytopenia, are the major DLTs. Although it is early days yet, the antitumor responses observed for BI2536 as a monotherapy in advanced cancers seem to be modest at best and not consistent with potency observed in preclinical studies. Further identification of biomarkers indicative of a PLK1-specific antitumor response would be useful to guide optimization of dosing schedules, and combination treatments have yet to be clinically explored. In addition, a second-generation compound, BI6727, has similar preclinical activity to BI2536 but with considerably improved PK properties, enabling IV and oral formulations to be evaluated, thereby adding flexibility to dosing schedules [51]. Clinical studies with this compound are underway and will provide useful information as to the further utility of ATP-competitive PLK1 inhibitors as antitumor therapeutics.

Promising preclinical ATP-competitive inhibitors of PLK1

Similar to most protein kinases under investigation as antitumor drug targets, the focus of drug discovery efforts continues to be the development of direct inhibitors of catalytic activity through binding to the ATP cleft. Despite lack of progress in the years following characterization of PLK1 as a fundamental regulator of mitotic progression and driver of oncogenesis, several preclinical compounds have emerged with potential for therapeutic development. Recently, the structure–activity relationship of a series of quinazoline derivatives was presented and demonstrated unique features of this common kinase core structure. Compounds containing the 4,5-dihydro-1H-pyrazolo[4,3-h]quinazoline substructure were identified through high-throughput screening and reported to act potently against the kinase activity of PLK1 [52]. The structural basis for potency of the most promising derivative from this series (compound **1**; IC_{50} = 0.007 μM; Fig. 1) was provided through a co-crystal structure complex with a truncated Plk1 (36–345). The development potential of **1** was suggested by its submicromolar antiproliferative activity towards a panel of tumor cell lines and *in vivo* validation evidenced by tumor growth inhibition (>80%) against an HCT116 xenograft upon administration of multiple oral doses.

A structure-based approach was also used to identify and optimize the first reported case of a type II PLK1 inhibitor [53,54]. Type II compounds are inhibitors that stabilize an inactive conformation of the ATP binding site. In this approach, a comparative protein (homology) model combined with pharmacophore-based screening and high-throughput ligand-docking methods resulted in the discovery of the vanillin-derived analog, SBE13 (Fig. 1), which had subnanomolar (0.2 nM) inhibitory activity against PLK1. In addition to its lack of activity on other mitotic kinases, including Aurora A, SBE13 was found to be selective for PLK1 over other PLK isoforms tested, including PLK2 (IC_{50} > 66 μM) or PLK3 (IC_{50} = 875 nM). Treatment of tumor cells with SBE13 and analysis of phenotypic endpoints indicated decreased cellular proliferation, a G2/M arrest followed by apoptosis, and abnormal mitotic figures, therefore justifying further preclinical investigation of this promising compound.

A further ATP-competitive compound, LFM-A13 [α-cyano-β-hydroxy-β-methyl-*N*-(2,5-dibromophenyl)propenamide; Fig. 1] was identified through molecular modeling studies and *in vitro* testing against PLX1 (10 μM IC_{50}) [55]. Further screening of LFM-A13 against a panel of seven serine/threonine kinases, ten tyrosine kinases, and the lipid kinase PI3Kc established its selectivity for the PLK family. The compound is moderately selective for PLK3, with

an IC_{50} value of 61 μM; however, no PLK2 activity was reported and, therefore, a complete picture of its PLK selectivity is currently unknown. LFM-A13 demonstrated a phenotype consistent with PLK1 inhibition in that it prevented bipolar mitotic spindle assembly in breast cancer cells (BT20) and induced complete mitotic arrest in prometaphase upon treatment of epithelial cells. The *in vivo* antitumor effects of this compound were evidenced through decreased tumor progression in the MMTV/neu transgenic mouse model of human epidermal growth factor receptor 2 (HER2)-positive breast cancer. A favorable toxicity profile in mice and rats, with little evidence of hematologic toxicity, helped to establish LFM-A13 as having significant potential for further development as an antitumor therapeutic.

PLK family inhibition: selective or not?

The ATP-competitive PLK inhibitors reported to date, although highly selective for the PLK family, in general do not discriminate between the individual isoforms. Indeed, similarities in their ATP binding sites suggest that obtaining selective PLK inhibitory drugs based on the ATP binding site will be challenging. This is an important consideration because the roles of PLK2, 3 and 4 are incompletely understood, expressed at different cell cycle stages and potentially have opposing functions to PLK1. In particular, there is mounting evidence that PLK3 acts directly or indirectly as a tumor suppressor. It is induced by DNA damage and its knockdown leads to increased proliferation [56–58]. Conclusions that PLK1 and PLK3 activities might be in opposition stem from observations associated with the ataxia telangiectasia mutated (ATM) DNA damage response network. Specifically, PLK1 is inhibited as a consequence of DNA damage-induced ATM signaling [59–62], whereas PLK3 is activated by ATM signaling [56,63]. The suppression by PLK1 of the transcriptional and pro-apoptotic activity of wild-type p53s was also attenuated by ATM [31]. PLK3-deficient mice develop tumors that appear to have increased levels of angiogenesis [64]. Recently, it has been demonstrated that the phosphatase and tensin homolog (PTEN) tumor suppressor is a substrate of PLK3 and that phosphorylation leads to increased stability of PTEN [65]. Collectively, these studies suggest that inhibition of PLK3 is an undesirable feature of drugs targeting the PLK family and would potentially oppose the effects of blocking PLK1, while contributing to tumorigenesis in normal cells. In addition, the opposing effects of PLK1 and PLK3 on proliferation might be the cause of the lack of *in vivo* antitumor efficacy for BI2536 discussed above.

Although limited success has been achieved in selective PLK1 inhibition through binding to an inactivated state of PLK1, a more general approach has been demonstrated in targeting the polo-box domain (PBD) of PLK1. The PBD contains a specific binding domain involved in substrate interactions and in subcellular localization of the kinase [66]. A phosphorylated peptide motif has been identified in known PLK1 substrates, such as CDC25c, which regulates the activation of CDK1/cyclin B, and crystal structures of peptides based on this motif and in complex with the PBD have been solved (Fig. 2) [66–68]. When ectopically expressed, this motif was shown to interfere with PBD-substrate binding and

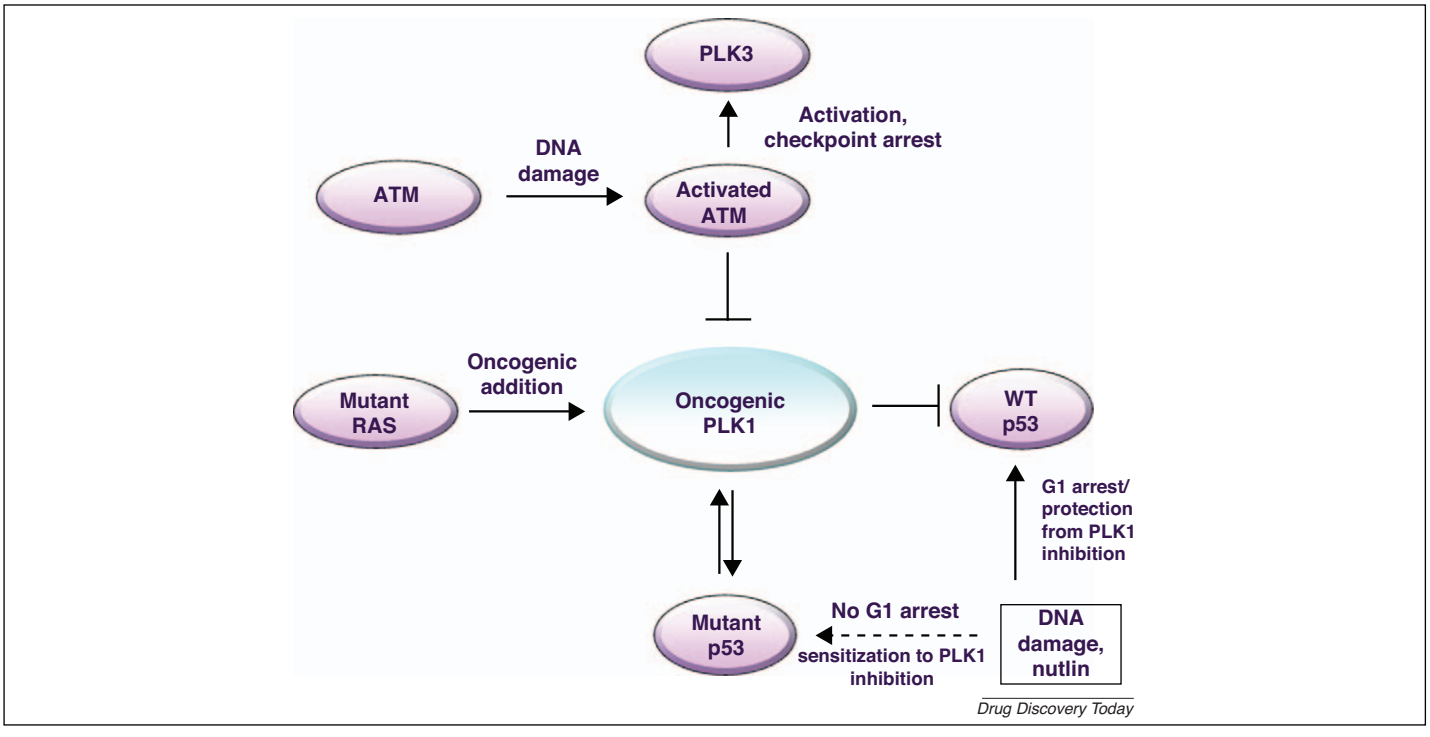

FIGURE 2

Synthetic interactions of PLK1 that are potentially exploitable with PLK1 inhibitors for cancer chemotherapy. Evidence suggests that mutant Ras tumors become dependent on PLK1 overexpression. In the presence of oncogenic PLK1, wild-type p53 functions are suppressed. Conversely, loss of wild-type p53 de-represses wild-type PLK1. Treating cells with agents that upregulate wild-type p53, such as the nutlins, and induce a G1 arrest could protect normal cells from toxicity associated with PLK1 inhibition. In p53-defective cells, the lack of G1 arrest facilitates cell cycle progression and subsequent sensitization to PLK1 inhibition. This has important implications for combination therapies. Lastly, there is evidence that ATM activation by DNA damage represses PLK1 expression while upregulating the PLK3 tumor suppressor, thus providing an important rationale for PLK1-specific inhibition.

localization of the PBD to centrosomes [69,70]. These results indicate that PLK1 can localize to specific sites within cells in response to priming phosphorylation events of PLK1 substrates, including CDC25c (phosphorylated by CDK1). It has recently been shown that minimized phosphopeptides bind potently and selectively to the PLK1 PBD in contrast to the PLK2 and 3 phosphopeptide binding site [68]. In addition to this, crystal structures of these inhibitors have been determined, therefore providing a window into the design of small molecule PBD inhibitors.

The uniqueness of the PBD and the compactness of the phosphopeptide binding site suggest that it can be exploited as a drug target to generate selective PLK inhibitors. To date, limited success in identifying small molecule hits has been achieved [71–73]. Using a fluorescence polarization-based high-throughput screening assay, one group screened 20,000 small molecules for PBD binding. Despite the diversity of this approach, only one compound was reported to inhibit peptide binding to the PBD and with a relatively low potency. Poloxin, a synthetic derivative of the natural product thymoquinone **8**, had an IC_{50} of 5 μM (compared with 30 nM for the reported optimized phosphopeptide) [72]. Thymoquinone, which inhibited the PLK1 PDB more potently than did Poloxin [72], also affected other subtypes of phosphothreonine/phosphoserine binding domains and was therefore found to be a less specific kinase inhibitor. Although Poloxin is cell permeable and demonstrated a PLK phenotype, the potential redox activity and lack of chemical stability make it unlikely that this compound can be further developed without significant modification.

Another study reported testing of a 2500-member natural product library for binding to the PBD [73]. Of this set, only one compound (purpurogallin, PPG) was shown to inhibit peptide binding by more than 30%. PPG had to be administered at high concentrations (50 μM) to observe cellular effects and inhibited several other targets at high concentrations. The modest inhibition and limited success of high-throughput screening towards the PBD suggests that other approaches are required. The authors are currently exploring the application of REPLACE methods for generating nonpeptidic inhibitors of protein–protein interactions to the PBD in an iterative fashion. This structure-based strategy involves computational prediction of the binding of small molecule fragments to replace peptidic determinants when synthetically ligated onto a truncated peptide. These methods have been successfully applied and proof-of-concept obtained in identifying non-ATP-competitive inhibitors of CDK2/cyclin A [74]. Using REPLACE, low molecular weight fragment alternatives for the N-terminal tripeptide of the CDC25c endogenous sequence (LLCSpTPNGL) have been identified using a fluorescence polarization (FP) competitive binding assay and represent hits for further optimization as potent and selective PLK1 therapeutics.

Future developments

In light of the connections to mutant Ras and p53 status, oncogenic PLK1 remains an attractive target for novel anticancer agents. Given the developing clinical experience that epidermal growth factor receptor (EGFR) inhibitors are ineffective against tumors with mutant Ras, such a target will be desirable for inhibitor development. However, the accumulating evidence of the functions of the other PLKs suggests an agent that selectively inhibits PLK1 without affecting other isoforms is needed. Such inhibitors would also be valuable chemical biology probes to further track and understand the consequences of the subcellular localization of PLK1. Work understanding the functions of the PBDs for each PLK suggests that targeting the PBD domain can achieve this specificity.

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