

# Chapter 14

## Exploiting Cell Cycle Pathways in Cancer Therapy: New (and Old) Targets and Potential Strategies

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**Abstract** There is a now a large body of evidence supporting the notion that cancer cells have vastly altered cell cycle networks that serve to maintain their high rate of proliferation. Consequently, targeting these pathways pharmacologically has been long studied, but only recently have some promising compounds progressed into the clinic. In this chapter, we review cell cycle function in both normal cells and describe how cancer cells deregulate this fundamental process. Next we describe in detail the development of different classes of CDK inhibitors and review the failures and successes so far, and provide insight into some future directions for research and clinical trials in order to exploit the ever-expanding molecular characterization of tumors with the drugs available and in the pipelines. In addition, we present a short overview of using differential cell cycle characteristics of normal and tumor cells as a way of protecting normal cells from cytotoxic chemotherapies. Finally we describe other potential targets such as regulating p27, inhibiting PIM and MELK kinases as well as some of the mitotic kinases.

**Keywords** Cell cycle • Cyclins • CDKs • Synthetic lethality • Mitosis • Combination therapy

### 14.1 Introduction

Hanahan and Weinberg recognized the importance of cell cycle and checkpoints in their original and updated “hallmarks of cancer” papers, which describe the key features that normal cells must acquire during transformation into a tumor [1, 2].

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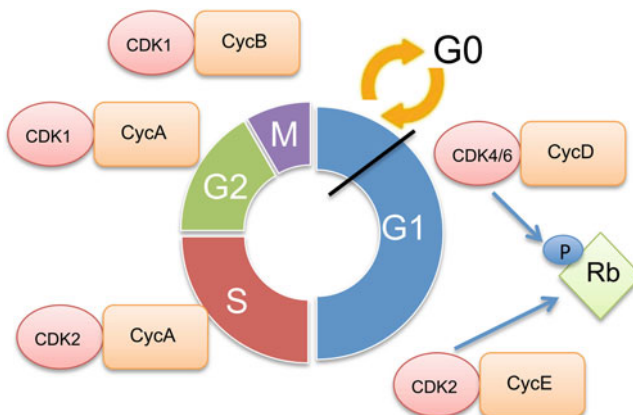
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Cell cycle deregulation has long been appreciated as a fundamental early event during tumorigenesis, which contributes to several of these hallmarks, namely self-sufficiency in growth signals” and “insensitivity to anti-growth signals”, and results in genomic instability, one of the newly added hallmarks. Since these alterations are almost universal among different tumor types, cancer biologists have expended considerable effort in interrogating these pathways as therapeutic targets for 20 years. In spite of the substantial body of literature focused on identifying the biological roles of many cell cycle pathway proteins in both normal development and in describing tumor-associated defects, the progress in the clinic has not been as rapid as desired. With this in mind, we felt that this chapter would be an ideal opportunity for us to review what is known about cell cycle deregulation in cancer, with a focus on personalized treatment strategies. Ultimately, we hope to suggest future directions for research and clinical trials to utilize the wealth of genomic knowledge we now have about cancer, and design more rational strategies likely to be effective in defined genetic contexts, as well as using the cell cycle as a means of protecting normal tissues from the chemotherapeutic insults. We will utilize a particularly promising strategy from our work as an example in a subsequent section.

## 14.2 Core Cell Cycle Proteins as Targets for Therapy

### 14.2.1 Cell Cycle Regulation

Some of the best characterized cell cycle targets are key proteins that have been highly conserved throughout evolution in all eucaryotes. These include cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKi). Cyclins are master regulators of the cell cycle, via activating CDKs which in turn stimulate downstream signaling (Fig. 14.1).



**Fig. 14.1** Cell cycle regulation by cyclins and CDKs

**Table 14.1** CDK knockout mouse phenotypes

Gene	Phenotype	References
CDK1	Not viable	[7]
CDK2	Sterile but viable	[3, 4]
CDK3	N/A – not active in most mouse strains	[210]
CDK4	Impaired proliferation of pancreatic $\beta$ cells, leading to diabetes	[5, 211]
CDK6	Hematopoietic deficiency – anemia, thymic development delay	[6]
CDK4 and CDK6	Embryonic lethal between E14.5 and E18.5, severe anemia	[6]
CDK2 and CDK6	Viable but sterile and females are small	[6]

Cell cycle progression is regulated by 4 major families of cyclins, cyclin D, E, A and B, and 4 respective CDKs (CDK4, 6, 2, or 1). Cyclin D is the first cyclin that is involved in the entry of cells from G<sub>0</sub> into G<sub>1</sub>, in response to ample growth factors and other mitogens. Cyclin D exerts its activity via its catalytic partners CDK4 and CDK6, (and can also bind to CDK2 and CDK3) which phosphorylate many substrates. One of the most studied substrates of CDK4/6 is the retinoblastoma protein (Rb), which is a negative regulator of E2F transcriptional activity. Prior to phosphorylation by CDK4/6, Rb is in a hypophosphorylated state, and is bound to E2F and DP proteins, keeping E2F inactive. However CDK4/6 phosphorylation induces conformational change in Rb, releasing E2F to bind DNA and facilitate transcription. The next cyclins that are transcriptionally regulated are cyclin E and A. Cyclin E bound to CDK2 helps drive cells through G<sub>1</sub>/S transition by further phosphorylating Rb and other substrates involved in DNA replication such as cdc6. Later during S phase, CDK2 is also regulated by cyclin A levels. After DNA replication is complete, cells enter G<sub>2</sub> phase where they prepare to enter mitosis by upregulating microtubule formation and other biosynthetic pathways necessary for chromosome segregation. Towards the end of G<sub>2</sub> phase, CDK1 takes over as the predominant kinase, since cyclin B levels begin to rise and translocate to the nucleus to bind CDK1 to initiate the G<sub>2</sub>/M transition. This complex was first identified as the M-phase promoting factor since its main function is to break down the nuclear envelope and initiate prophase. Once mitosis is almost complete, CDK1 is deactivated via dephosphorylation, and a negative feedback loop is engaged via the anaphase-promoting complex which degrades cyclin B, allowing cells to exit mitosis.

The requirement for all of the cyclins and CDKs to control the cell cycle in normal cells as described in the previous paragraph has been recently challenged based on the findings from genetic studies in knockout mice. Tables 14.1 and 14.2 summarize the phenotypes observed in the knockout models. Each interphase CDK has been knocked out individually, and except for CDK1, all of the mice are viable. However, each mouse model has cell-type specific defects, which reveal tissue-specific roles for individual CDKs. For example, the CDK2-deficient model is viable (although born at slightly lower than predicted Mendelian ratio), but sterile due to an absolute requirement for CDK2 during meiosis in both male and female germ cells [3, 4]. Cell cycle analysis and proliferation rate of mouse embryonic fibroblasts from both CDK2 wild-type and knockout embryos showed no significant difference in cell cycle distribution, and a similar rate of proliferation for the first 4 days in culture, after which the knockout cells reached a plateau phase. Similarly

**Table 14.2** Cyclin knockout mouse phenotypes

Gene	Phenotype	References
<i>D-type cyclins</i>		
Cyclin D1	Viable, but mice have neurological abnormalities, retinal hypoplasia, decreased body size, and during pregnancy only, impaired mammary epithelial cell proliferation	[212–215]
Cyclin D2	Viable, but females are sterile, and males have decreased sperm counts and hypoplastic testes. Diabetes due to impaired pancreatic beta cell proliferation	[216, 217]
Cyclin D3	Viable, but hypoplastic thymus	[218]
Cyclin D1 and Cyclin D2	Viable until 3 weeks, but decreased body size and hypoplastic cerebellum	[219]
Cyclin D1 and Cyclin D3	Some loss of viability by 4 weeks, most do not survive past 2 months due to respiratory failure and neurological defects	[219]
Cyclin D2 and Cyclin D3	Embryonic lethal at E17.5-E18.5 due to megaloblastic anemia	[219]
Cyclin D1, Cyclin D2 and Cyclin D3	Embryonic lethal at E16.5 due to megaloblastic anemia and defective fetal hematopoiesis	[220]
<i>E-type cyclins</i>		
Cyclin E1	Viable, no detected phenotype	[221, 222]
Cyclin E2	Viable, males are infertile	[221, 222]
Cyclin E1 and Cyclin E2	Embryonic lethal at E11.5. Placental failure due to lack of trophoblast-derived polyploid giant cells in the placenta	[221, 222]
<i>A-type cyclins</i>		
Cyclin A1	Viable, males are infertile	[223, 224]
Cyclin A2	Embryonic lethal at implantation	[223]
<i>B-type cyclins</i>		
Cyclin B1	Embryonic lethal at E10.5, unknown reason	[225]
Cyclin B2	Viable, no detected phenotype	[225]

CDK4 and CDK6 are not necessary for cell cycle progression in most cells, although CDK4 is required for proliferation of pancreatic  $\beta$ -cells, leading to a diabetic phenotype in the knockout mouse [5]. Additionally, CDK6 is important in the hematopoietic system, both in the lymphocytes and erythrocytes. CDK6-deficient mice have small, less cellular thymi, since CDK6 is downstream of Notch and AKT signaling which is critical in early thymic T cell commitment to the T-cell lineage, and also have smaller spleens with less erythroid cells [6]. Double knockout of CDK4 and CDK6 induces late embryonic lethality, primarily due to the hematopoietic defects in erythroid cell production, however MEF cells from these embryos do proliferate and can become immortalized by continuous passage. CDK2 can partially compensate to phosphorylate Rb in these double-knockout cells by binding cyclin D, and therefore promote cell cycle progression, however this is not thought to fully explain the lack of cell cycle defects systemically. In stark contrast to the phenotypes seen in CDK2/4/6 knockout models, CDK1 deficiency causes cell cycle arrest and prevents embryos from developing beyond the 2-cell stage, demonstrating a lack of compensation between the mitotic CDK and the interphase CDKs [7].

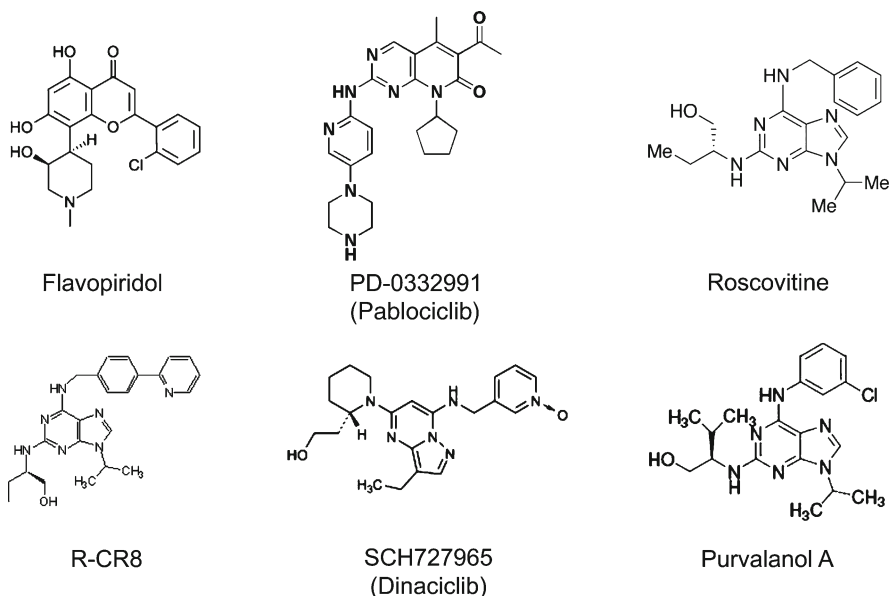
Unlike normal cells that do not depend on any single cyclin or CDK for growth, there is beginning to be some evidence that in tumor cells, altered cellular wiring can lead to oncogenic addiction to CDK signaling. For example, in a mouse model of triple negative breast cancer driven by low-molecular weight cyclin E, tumors are highly dependent upon CDK2 signaling, even though CDK2 is dispensable in normal cells [8]. This type of variation on synthetic lethality involving CDK signaling is not limited to breast cancer but can be observed in a K-Ras mutant lung cancer model. In a K-Ras-driven mouse model of lung cancer, CDK2 and CDK6 knockout only partially inhibited tumor initiation, whereas CDK4 knockout significantly decreased tumorigenesis due to an immediate induction of senescence [9]. Even though the K-Ras-mutant transgene is expressed in several other epithelial tissues in this mouse model, none of these undergo hyperplasia or tumorigenesis, and senescence is not observed in these normal tissues. CDK4 was also shown to be essential for progression of established K-Ras driven NSCLC lesions, and pharmacological CDK4 inhibition significantly inhibited tumorigenesis. The reasons for lack of immediate compensation mechanisms involving other CDKs in tumors are not clear, but this phenomenon may allow us to turn this frequent observation into an Achilles heel in cancer cells if we carefully dissect true dependencies in well-planned genetic experiments.

## 14.2.2 CDK Inhibitors

### 14.2.2.1 Pan-CDK Inhibitors

Since CDKs are the catalytically active drivers of cell cycle progression, targeting them pharmacologically has been a major effort. The early generation inhibitors, developed more than 15 years ago were pan-inhibitors targeting a large spectrum of CDKs. These drugs were somewhat promising based upon cell line and xenograft work, but when moved into early stage clinical studies failed to show considerable net benefit. The reasons for failure are likely multi-factorial, and include both biological issues as well clinical trial design flaws. In the forthcoming section we will describe the development of several generations of inhibitors and their related trials, and provide insights into future development of these classes of compounds. Figure 14.2 shows the structures of all the CDK inhibitors discussed in this section.

The most extensively tested pan-CDK inhibitor is flavopiridol, which inhibits all four of the interphase CDKs as well as CDK7 and is also the most potent known CDK9 inhibitor [10, 11]. CDK7 is both a cell cycle and a transcriptional CDK, since it is a part of the transcription factor IIH (TFIIH) complex with cyclin H and MAT [12, 13]. CDK7 promotes transcription elongation by phosphorylating the C-terminus of RNA polymerase II. CDK9 is also thought to be involved in transcriptional regulation, in complex with cyclin T, via phosphorylating different sites in the C-terminus of RNA polymerase II [14]. There is a profound response to flavopiridol in cells, that encompasses both cell cycle arrest in G1 and G2, but also transcriptional changes especially in mRNAs with short half lives such as early

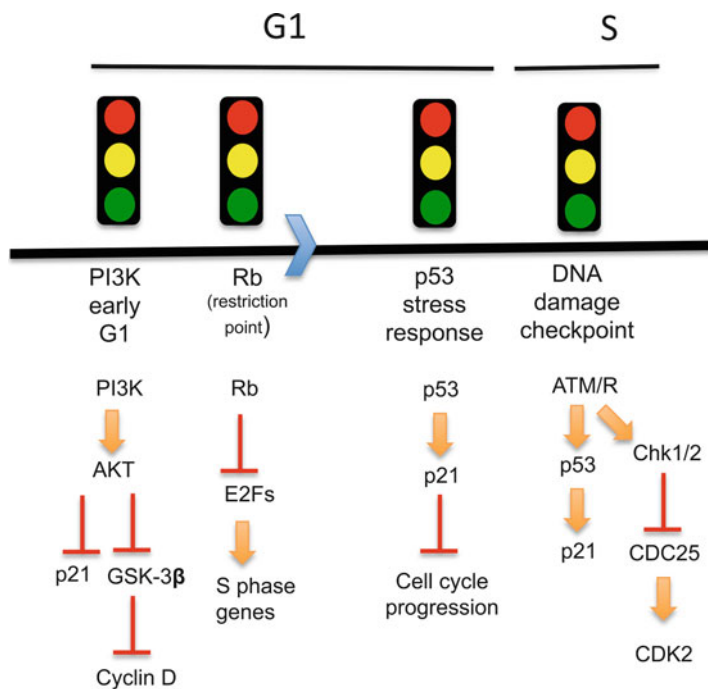


**Fig. 14.2** Structures of CDK inhibitors

response transcription factors, apoptosis regulating genes (like Mcl1) and NF $\kappa$ B responsive genes [11, 15, 16]. Whether these responses truly translated when this agent was tested in the clinic was not well studied.

Preclinical data had suggested that prolonged exposure to flavopiridol was necessary for maximal anti-tumor effect, so the two phase 1 trials that opened in 1994 used long infusions (72 h). The dose-limiting toxicities seen were primarily diarrhea, and at higher doses hypotension, anorexia and muscle weakness, and 21 % of patients had venous thromboses. Pharmacokinetic analysis of steady state plasma concentration revealed that 200–400nM was the range reached at the maximum tolerated dose [17]. The prior preclinical studies had found that for maximum activity, a higher concentration in the micromolar range would be desirable, so future studies attempted to reach these levels via bolus dosing on a 1 h per day for five consecutive days schedule. In these later trials, low micromolar peak concentrations were observed, and similar toxicities were observed [18]. However, when several phase 2 studies in solid tumors were analyzed, the enthusiasm for this agent waned, since no objective responses were seen in tumors ranging from melanoma to endometrial carcinoma [19–21]. In contrast, the results seen in hematopoietic malignancies appeared more promising [17]. For example, in chronic lymphocytic leukemia, 40 % of patients had partial responses, and the dose-limiting toxicity observed was tumor-lysis syndrome, indicative of strong anti-tumor activity of this agent [22].

In addition to studies using flavopiridol as a single agent, combination studies were pursued based on the hypothesis that flavopiridol may have benefit as a chemosensitizer. This hypothesis was generated based on the pre-clinical observation that



**Fig. 14.3** Key regulators of G1 to S phase transition

synchronizing the cells into S phase sensitized them to flavopiridol-induced cytotoxicity, resulting in E2F dependent cell death that is selective to transformed cells [23]. These studies used a variety of classes of cytotoxic drugs including platinum agents, anthracyclines, taxanes and 5-fluorouracil. These studies had more promising results, including a 30–40 % rate of partial responses in some studies [24–31].

In spite of some of these promising activities in both solid tumors and leukemias, recently, there has not been significant progress with this agent. The chemistry of the agent does have some challenges, since it binds to plasma proteins and also is poorly water soluble [32, 33]. There has been a novel liposomal formulation reported a few years ago, which aimed to improve the therapeutic index by slowly releasing the drug to effectively synchronize a large portion of the tumor cell population, while not being bound up in the circulation by plasma proteins [34]. As of writing, there have not been any clinical studies presented or registered using this formulation.

### 14.2.2.2 Selective CDK4/6 Inhibitors

The G1-S checkpoint (see Fig. 14.3) is altered in close to 90 % of human tumors, by various mechanisms, indicating that this phenotype provides a selective advantage for proliferation and/or survival. With this in mind, selectively targeting CDK4 and

CDK6 has been considered as an alternative strategy in several diseases including breast cancer due to the prevalence of amplification/overexpression of cyclin D1 observed (15–20 % amplification/50–70 % overexpression overall) [35, 36]. One drug candidate, PD-0332991 (see Fig. 14.2 for structure), has quickly moved to the top of its class and has rapidly moved into clinical studies [37]. This compound was selected from a high-throughput screen of pyridopyrimidines, in which both potent anti-proliferative and selective inhibition of CDK4 were used as criteria [38]. When tested against a large panel of other kinases, PD-0332291 (Pablociclib) had a highly selectivity index towards CDK4 and CDK6 (IC<sub>50</sub>'s 11/16nM, versus >8–10 μM for 36 other kinases tested). When tested in MDA-MB435 breast carcinoma cells, PD-0332991 induced a robust G1 arrest, and concomitant reduction of phosphorylation of Rb at the CDK4/6 phosphorylation sites (Ser 780–795). As expected, in Rb-negative cell lines, this compound has no activity, further demonstrating that its mechanism of action includes inhibition of CDK4/6 phosphorylation of Rb [39]. Another marker of resistance that has been identified is elevated p16 expression, since CDK4/6 is physically bound and unable to be inhibited [40].

Breast cancer is the model system which has been best studied so far in terms of understanding mechanism of action, potential synergistic combinations and predictors of resistance. At the molecular level, breast cancers can be divided into luminal or basal based on gene expression signatures. Most of the sensitive cell lines are luminal in nature, and all have intact Rb signaling, whereas the resistant cells tend to be basal-like and lack Rb activity [41, 42]. Intriguingly, basal cell lines, which retain Rb activity are still unresponsive to PD-0332991, and have hyperphosphorylated Rb. It is unclear at present what the precise mechanism is that drives hyperphosphorylation of Rb in these cell lines. It is possible that there is a greater dependence upon CDK2/CDK1, in which case these cells might respond to a combination of CDK2/1 and CDK4/6 inhibitors. Luminal tumors encompass both estrogen-receptor (ER) positive and many HER2-amplified tumors, so naturally combinations of ER antagonists or HER2 inhibiting drugs with CDK4/6 inhibitors were tested. In ER-positive cell lines, treatment with tamoxifen and PD-0332991 resulted in synergism and G1 arrest, and similarly trastuzumab and PD-0332991 are synergistic in HER2-amplified cell lines [41].

Apart from breast cancer cell lines, PD-0332991 has now been evaluated in a variety of other solid tumor types, including pancreatic neuroendocrine tumors, glioblastoma multiforme, rhabdomyosarcoma and mantle cell lymphoma with similar results [43–47]. In xenograft experiments, this drug is mostly cytostatic, with a few examples of cytotoxicity. In addition, PD-0332991 has been explored as a radiosensitizer in glioblastoma, due to its high penetrance of the blood-brain barrier, and preclinically appears to be useful in this scenario [47].

However not all tumor contexts are ideal candidates for such a strategy even if the underlying genetic changes would predict sensitivity. A recent paper described an unanticipated effect observed in pancreatic adenocarcinoma (PDAC). In PDAC cell lines examined, PD-0332991 had anti-proliferative activity, induced robust G1 arrest and hypophosphorylation of Rb [48]. However, gene expression analysis revealed that PD-0332991 upregulated genes involved in pro-angiogenic signaling,



cell adhesion, cell migration/ECM remodeling, and inflammatory pathways. In addition, EMT was induced correlating with increased invasion via TGF $\beta$ -SMAD4 signaling, suggesting perhaps combinations of TGF $\beta$  inhibitors with CDK4/6 inhibitors might be a way forward in PDAC tumors expressing wild-type SMAD4. Genetic manipulation of CDK4/6 recapitulated this phenotype, ruling out a drug-mediated off-target kinase inhibition.

The first phase I study performed using PD-0332991 was recently published, and examined patients with Rb-positive advanced cancers [49]. This study showed that the drug was generally well tolerated, with the main toxicity being myelosuppression, consistent with other cell cycle targeted therapies. Pharmacokinetic analysis suggested favorable properties including slow absorption and elimination. The response rate was moderate (~27%), however given all the usual caveats of generalizing based on phase I studies, the patients who derived some benefit (i.e. stable disease) could tolerate the drug well enough to remain on study for 10+ cycles. In breast cancer patients, a randomized phase I-II study utilizing PD-0332991 in combination with letrozole in ER-positive, HER2-normal post-menopausal patients has been completed (personal communication). In the phase I portion, there has been no biomarker selection, but in the phase II portion, the trial is specifically focused on patients with cyclin D1 amplification and/or loss of p16, since these are the patients predicted to respond best. So far, the clinical benefit rate in this combination trial was 70%, which resulted in statistically significant increase in progression-free survival and the adverse event profiles are very similar to what was reported in the single-agent phase I studies trials previously discussed i.e. this combination is generally well tolerated. The few patients so far in the study have been safely treated with some patients having partial responses. These well-designed trials with integrated biomarkers built in, are likely to provide more useful information not only about safety and pharmacokinetics but also pharmacogenomics information about responders and the biology behind responses. Caution must be taken though in considering combinations with chemotherapies that depend on actively cycling cells, based on a recent study that showed that PD-0332991 actually protected RB-positive MDA-MB-231 cells from paclitaxel-induced mitotic catastrophe [50]. Pre-treatment with PD-0332991 also resulted in a switch from homologous recombination (HR) DNA repair mechanism to non-homologous end-joining (NHEJ), which is an error prone pathway that could potentially induce further genomic instability in cancer cells. While this finding is intriguing and worthy of further mechanistic study in a broader panel of cell lines and normal mammary epithelial cells, it still remains to be determined how this result will be translated into ER+/HER2+ cell lines, which have been the focus of the majority of the breast cancer studies using this agent.

Going forward in hormone-receptor positive breast cancer, targeting CDK4/6 should eventually become a first line therapy in combination with endocrine therapy for a number of reasons. Certainly, as described above, the preclinical data regarding this combination is compelling, and this includes cell lines that acquired tamoxifen resistance being re-sensitized by PD-0332991. Secondly, genomic data have confirmed the relevance of this pathway in resistance to endocrine therapy alone, such as the fact that cyclin D1 is overexpressed/amplified in endocrine-therapy resistant tumors [51, 52]. In addition to deregulating the cell cycle, amplified cyclin

D can directly activate ER in a hormonally-independent manner that does not require CDK/Rb activity [53]. Thirdly, the fact that cyclin D1-CDK4 is downstream of multiple pathways that mediate resistance to anti-estrogens (e.g. EGFR/HER2, ERK, AKT, NF $\kappa$ B) may make this strategy useful regardless of which pathways are upregulated in any particular patient [54]. Despite being a good target however, resistance to CDK4/6 inhibition is likely to occur, since resistance arises to every targeted therapy tested so far. Indeed, there is evidence currently for activation of CDK2 due to p27 down regulation as a mechanism of resistance to these agents, which could potentially be targeted via CDK2 inhibitors as will be discussed in the next section [55].

Further clinical studies in other diseases are also underway, for example in mantle cell lymphoma. The single agent study showed some evidence of benefit, and now a subsequent study has been designed using PD-0332991 in combination with bortezomib in this patient subset.

#### 14.2.2.3 Selective CDK1/2 Inhibitors

The other subclass of CDK inhibitors that have been developed are more specific for CDK1 and CDK2 (versus CDK4/6), such as R-roscovitine (also known as CYC202 or seliciclib), SNS-032 and the newer agent SCH727965 (see Fig. 14.2 for the structures). Roscovitine, a 2,6,9-trisubstituted purine was generated in a screen of olomoucine-related analogues for CDK1/cyclin B inhibition, and found to potently inhibit CDK1 kinase activity (IC<sub>50</sub> of 0.45  $\mu$ M) [56]. Once olomoucine was shown to co-crystallize with CDK2, roscovitine was also confirmed to bind directly to CDK2 [57]. Several years later, roscovitine became the first orally bioavailable drug from this class to go into clinical trials based on the preclinical data showing that CDK1/2 inhibition causes S and G2 arrest followed by apoptosis in tumor cells [58–60]. Apart from the effects on these cell cycle CDKs, roscovitine also inhibits CDK7 and CDK9, thereby inhibiting transcription as well, via reducing key anti-apoptotic proteins such as Mcl1 [61–64].

Several studies have shown that apoptosis is further induced when CDK1/2 inhibitors are combined with most cytotoxic therapies including taxanes, anthracyclines as well as radiation. For example, a combination of purvalanol A and taxol caused profound apoptosis in Hela cells, when taxol was used first to stabilize microtubules then purvalanol A was added [60]. Intriguingly when the drugs were used in the reverse order, the response was decreased, demonstrating that synchronization of cells in mitosis (i.e. the end results of taxol) is important for the mechanism of CDK inhibitor-induced cell death. A similar synergistic combination strategy was demonstrated in MCF7 breast cancer xenografts using roscovitine and doxorubicin, however in this context, cell cycle synchronization in G2/M phase with roscovitine was used to prime the cells to respond to doxorubicin (versus the taxol $\rightarrow$ CDKi strategy in Hela cells) [65, 66]. Similar to the Hela cell study described above, the taxol-purvalanol A combination was found to be similarly effective in MCF7 xenografts [67]. These dichotomous results in two different systems just

illustrate one of the challenges we have moving forward with sequential combination therapies that exploit mechanism of action of drugs. Likely a number of factors could contribute to which direction of treatment is likely to be best, including genomic factors (such as Rb status, p53 pathway status), timing of exposure to agents as well as which specific drugs under investigation. Clearly, further mechanistic work is still needed to dissect out these details in order to rationally match treatments to individual patients.

In spite of this incomplete understanding of mechanism of action of these agents in both solid tumors and hematopoietic malignancies, roscovitine was moved into clinical trials in the early 2000s [68]. The phase 1 studies demonstrated that this agent could be administered both in an intravenous formulation as well as orally [69, 70]. It has reasonable pharmacokinetic properties including high bioavailability, slow GI absorption, however it is rapidly metabolized to an inactive metabolite, making its half-life fairly short (~1 h). However, there were a number of dose-limiting side effects observed including liver and kidney toxicity, electrolyte disturbances, rashes and fatigue that accumulated over time, making repeated administration daily for more than 5 days too challenging for patients. Responses were unimpressive over a few phase 1 studies, with primarily stable disease and very few partial responses seen as monotherapy. Two phase 2 studies were undertaken in non-small cell lung cancer and nasopharyngeal carcinoma which essentially replicated the results in the phase 1 studies [71]. Looking at the pharmacological and response data together, the researchers concluded that one of the major challenges was maintaining a plasma dose that is high enough for anti-tumor activity based on the preclinical studies, and even when white blood cells were used as a surrogate for tumor cells, Rb phosphorylation was not decreased, further supporting the claim of insufficient dose reaching tumor cells. One way of potentially overcoming this problem might be to use a more frequent dosing schedule such as 2–3 times a day, since the preclinical studies showed that 8–16 h of continued exposure is needed to effectively inhibit tumor growth. Whether this would actually work might not be known since the excitement regarding this agent has waned, in light of newer compounds that have been developed. One such potential compound is CR8, which is a N6-biaryl-substituted derivative of roscovitine that is 2–4 fold more potent at inhibiting CDK1, CDK2 and CDK7, which translated to 40–70 fold higher potency in cellular activity measures such as PARP cleavage and caspase activation [72–74]. Since discovery of this compound a few years ago, animal studies have not been published in cancer models, although a very recent paper utilizing CR8 in a mouse model of traumatic brain injury demonstrated that this drug could be delivered safely *in vivo*.

The other CDK inhibitor that targets CDK1 and CDK2 (as well as CDK5 and CDK9) that appears promising is SCH727965 (Dinaciclib) (see Fig. 14.2 for structure), a compound developed to address some of the issues with previous generation inhibitors with respect to therapeutic index [75]. Indeed, in direct comparison to flavopiridol this agent had > tenfold greater therapeutic index in the A2780 ovarian cancer xenograft model (defined as the ratio between MTD defined as 20 % body weight loss and minimal effective dose to cause 50 % inhibition of tumor growth when given i.p. once daily for 7 days). In addition, cell line studies showed that even

a brief 2 h exposure to SCH727965 was sufficient to inhibit progression of cells into S phase. *In vivo*, this drug was at least as effective as paclitaxel in A2780 xenografts, and well tolerated with the main toxicity being reversible myelosuppression.

In addition to ovarian cancer, SCH727965 has been tested and found to be potentially effective in pancreatic cancer, melanoma and various forms of sarcoma including osteosarcoma [76–79]. The pancreatic study was particularly exciting, as it was performed using low-passage patient-derived xenografts (PDX) as opposed to cell line xenografts. These PDX models are thought to more faithfully recapitulate human tumorigenesis for multiple reasons including the fact that they maintain human stroma for multiple passages [80]. This intense desmoplastic stroma and hypovascular microenvironment which characterizes pancreatic cancer, is known to be a major barrier to chemotherapy drug access. Therefore the data showing efficacy in multiple mouse models with these characteristics bodes well for subsequent trials in humans. In addition to using these better disease models, the authors performed gene set enrichment analysis on the tumors to interrogate potential mechanisms of resistance, an area of research that is very undeveloped in the cell cycle field. They compared sensitive and resistant tumors, and found that in the most resistant tumors, the Notch and TGF $\beta$  pathways were upregulated, suggesting that perhaps combinations of these inhibitors may be future directions for research.

Preclinical studies in adult and pediatric leukemia are also underway, and a report of efficacy in CLL cells showed promise independent of high-risk genomic features (del 17p13.1 and gVHI unmutated). Short-term exposure of CLL cells directly taken from patients was sufficient to induce apoptosis [81]. Moreover, SCH727965 was shown to abrogate microenvironment-derived cytokine-induced survival signaling, in a PI3K-dependent mechanism, suggesting that a logical combination to explore might be SCH727965 in combination with PI3K inhibitors.

These pre-clinical studies provided strong rationale for moving this agent into clinical studies. A phase 1 study using SCH727965 dosed once every 3 weeks as a single agent in unselected adult patients has been performed which showed moderate responses (mainly stable disease) and similar to the preclinical studies, myelosuppression was the DLT [82]. Notably, unlike flavopiridol, there was no diarrhea, and much less fatigue, making this agent better tolerated. Since nausea and vomiting were common side effects as well, a subset of patients was given the anti-emetic drug aprepitant [83]. Aprepitant is known to weakly inhibit CYP3A4 which is one of the enzymes involved in metabolizing SCH727965, so the pharmacokinetic parameters were compared in patients treated with aprepitant with those not given it. These results showed no interaction between aprepitant and SCH727965, suggesting that use of this agent prophylactically in the clinic is a feasible and safe strategy moving forward to maximize use of SCH727965 in different patient populations.

Several phase 2 studies with SCH727965 as a single agent have also begun in both solid tumors and hematopoietic diseases, and some have been presented as abstracts at meetings. The first and most promising was performed in adult acute myeloid or lymphocytic leukemia patients and reported at ASH in 2010 [84]. The response rate was 60 %, and many patients had rapid decrease in their blast counts. Correlative studies including pharmacodynamics analyses showed that CDK activity

(i.e. Rb phosphorylation and McI1 decreased expression) was effectively inhibited in the samples taken at 4 h post infusion, however these biomarkers returned almost to baseline by 24 h, suggesting a need for frequent dosing. A similar study is also underway in multiple myeloma, but no results are currently available [85].

With respect to solid tumors being examined in single-agent phase 2 trials, the progress has been slower. One single arm study has been reported in melanoma, which had ~72 patients enrolled [86]. The response profiles were very modest, with 22 % of patients with stable disease and no partial or complete responses, and toxicities were common. Another phase 1–2 study in unresectable melanoma is now approved and about to open. The only other trial that has been started is a multi-arm randomized phase 2 in breast cancer and NSCLC, in which SCH727965 is being compared to active treatments for each respective disease (oral capecitabine for breast cancer, and erlotinib for NSCLC). Importantly crossover from the control arm was allowed after disease progression, which is likely to make detection of a significant difference in overall survival extremely challenging. The study has been completed but no data has been presented as of writing (Nov 2012) [87].

Similar to the other CDK inhibitors, combinations with chemotherapy/other targeted agents are ultimately going to be necessary for optimal activity of SCH727965, and already a number of combination studies have been started. These trials include combinations with the PARP inhibitor Veliparib with or without carboplatin, rituximab in CLL, bortezomib and dexamethasone in myeloma, and our own trial with epirubicin in triple negative breast cancer [88–91]. Our trial differs from the others in that by limiting our patients to a specific subtype of breast cancer in which we have preclinical data showing dependence on CDK2 signaling because of LMW-E expression [92]. In addition, another group has shown elevated c-Myc expression is synthetically lethal with CDK inhibition in triple negative breast cancer [93]. By pre-selecting patients with a high likelihood of being oncogenically addicted to CDKs either via the LMW-E pathway or via amplification of c-Myc, we believe we will enrich for potential responders. Further biomarkers of response or markers predicting resistance will be needed in order to make this drug clinically useful. For example, basal like breast cancers or serous ovarian cancers with defects in DNA repair such as BRCA1/2 or ATM mutations may be more sensitive to CDK2 inhibition (in combination with chemotherapy), analogous to their propensity for sensitivity to PARP inhibition. BRCA1 or ATM knockdown sensitized various cell lines to CDK2 inhibitors, since CDK2 can regulate DNA repair independently of its effect on the cell cycle [94].

### **14.2.3 Targeting p27**

Moving away from directly targeting components of the cell cycle that promote growth, the other strategy that has been proposed is targeting negative regulators such as p27. p27 is a small nuclear protein involved in negatively regulating G1 to S phase transition via inhibiting cyclin E-CDK2, cyclin A-CDK2 and cyclin D-CDK4/6

activity [95]. However, in addition, it has novel functions when mislocalized in the cytoplasm that contribute to tumor cell survival and cancer progression [95]. For example, in response to stress, p27 is driven into the cytoplasm via AMPK-mediated phosphorylation at Thr198 which blocks apoptosis and induces a cytoprotective autophagy response [96, 97]. Other cytoplasmic functions also include increased invasion and metastatic potential, perhaps via binding to RhoA and stimulating changes in actin cytoskeleton formation [98]. A p27 knock-in mouse model where p27 has been mutated to be unable to bind cyclins and CDK2, was generated to determine whether there are cell-cycle independent roles for p27 [99]. One of the major findings from this model includes a role for cytoplasmic p27 in stem/progenitor cell enrichment, leading to lung tumorigenesis [100]. Taken together these results clearly show that p27 has pleiotropic functions and therefore merely upregulating it without understanding cellular context may not be very effective.

In many cancers p27 levels are decreased as a result of deregulation of transcriptional, translational or post-translational pathways. Much effort has been expended into understanding these mechanisms with the end goal being to determine ways of upregulating nuclear p27 to prevent cell cycle progression. To briefly summarize these studies, which have been reviewed extensively elsewhere, p27 transcriptional regulation is complex, and involves both repression via oncogenic transcription factors such as c-Myc and Id3, as well as activation via FOXO transcription factors and E2F1 [101]. In addition to transcriptional regulation, p27 has been shown to be regulated via miRNAs such as miR-221/222, which are overexpressed in some tumors [102]. p27 is also extensively regulated at a post-translational level, including multiple phosphorylation sites that dictate localization, as well as that phosphorylation sites that stabilize the protein via inhibiting ubiquitination. Some of these most important sites include Ser10, Tyr74/88/89, Thr157, Thr187, and Thr198.

Beyond phosphorylation, p27 localization can also be regulated via protein-protein interactions, such as Jab1, which binds to p27 and induces its nuclear export and degradation in the cytoplasm [103]. Jab1 is an interesting potential target for a number of reasons. Jab1 expression in tumors is inversely correlated with p27 levels and overexpression is correlated with poor prognosis [104–107]. This proto-oncogene has also been linked to radiation resistance and cisplatin-resistance due to inhibiting several apoptotic and DNA repair pathways [108]. Apart from p27, Jab1 can induce degradation of other tumor suppressors such as Smad4 and p53 as well as inhibit DNA repair via HR pathways that involve Rad51 [109–111].

p27 proteolysis via the proteasome is regulated by Skp2 [112]. Skp2 is part of a larger SCF complex, comprised of cullin1, Skp1, ROC1/Rbx1 and requires an adaptor protein called Cks1. Together, this complex functions as an E3-ligase that regulates a number of substrates including p27, p21, p57, FOXO1 and c-Myc [113–116]. Towards the end of G1 phase, Skp2 recognizes p27 phosphorylated at Thr187, which serves as a degradation signal, allowing cells to enter S phase. However, in cancer cells, Skp2 can be amplified and/or overexpressed, leading to decreased p27 levels constitutively [117–119]. As a result of this reciprocal relationship, the concept of targeting Skp2 has been proposed, however these studies have not progressed well. Genetic studies using a Skp2 deficient mouse model have demonstrated that acute inactivation of Skp2 in the context of Pten or Arf heterozygosity induced

senescence but not in the Skp2-deficient mice without other oncogenic signals [120]. Senescence induction correlated with decreased tumorigenesis and p27 induction in the preneoplastic lesions that could be detected in some mice, providing additional rationale for the development of Skp2 inhibitors.

A high-throughput screen for inhibitors of Skp2 was performed using purified components of the complex and p27 as a substrate [121]. A compound designated CpdA was discovered to induce cell cycle arrest at low micromolar doses in multiple myeloma cells, and this led to caspase-independent cell death via autophagy. In addition, because low levels of p27 is associated with resistance to therapy in myeloma cells, CpdA was examined as a chemosensitizer, and was found to synergize with bortezomib, and overcome resistance to doxorubicin and melphalan. Despite this useful spectrum of activity however, further progress has not been made using this agent since it is difficult to make, and was not potent enough to use *in vivo* (required 5–10  $\mu$ M dose in cell lines). More recently, another screen was performed using a chemical-genetics approach using automated microscopy to identify compounds that upregulate p27 in prostate cancer cell lines [122]. After several rounds of stringent validation, two compounds were identified – SMIP001 and SMIP004. These compounds are not broad proteasome inhibitors, but specifically target Skp2, resulting in elevated p27 and decreased CDK2 activity at low micromolar doses. At this point it is not known whether either of these compounds will be effective *in vivo*.

Targeting p27 presents a considerable challenge, despite the large body of knowledge regarding the mechanisms of its regulation and their redundancy. Some open questions include which of the upstream regulatory enzymes would be the best target to induce a sustained increase in p27 cells in tumor cells specifically. In some ways, the lack of complete specificity of substrates presents the largest conundrum. Assuming it would be possible to move one or more of the identified compounds (or a derivative) into the clinic, it is possible that some of the off-target effects may be undesirable e.g. in some contexts the upregulation of cyclin E or c-Myc may drive additional genomic instability. In fact there is data suggesting this might be the case using siRNA in A549 lung cancer cells targeting p27, Skp2 or the combination of both mRNAs [123]. In the dual-siRNA treated cells, there was an increase in centrosome number, abnormal mitoses/nuclear atypia, which could be attributed to an increase in both full length and low-molecular weight forms of cyclin E. In tumors, such as triple negative breast cancers, which already have LMW-E expression we propose that a Skp2-targeting strategy could be detrimental because of this off-target effect, unless combined with CDK inhibitors.

The other concern with Skp2 targeting relates to the specific genetic background of p27. As mentioned previously, p27 transcriptional silencing such as by miRNAs or promoter methylation are not uncommon events, and could co-exist with Skp2 overexpression. In this scenario even a potent Skp2 inhibitor would be ineffective at restoring p27 expression, with similar potential consequences as described above. In addition, because cyclin D-CDK complexes also bind p27, the level of p27 induction that may be necessary to slow down the cell cycle might be fairly high. Clearly, much work lies ahead in developing more potent inhibitors and understanding in greater depth the cellular contexts in which this strategy could be beneficial and which contexts to avoid.

### 14.3 Exploiting Normal and Cancer Cell Differences for Protection of Normal Tissue

One of the most challenging problems in cancer therapy involving cytotoxic chemotherapy is how to selectively kill tumor cells while sparing normal dividing cells. Previous work from our group has demonstrated two potential strategies that utilize cell cycle synchronization as a mechanism of selectively arresting normal cells. We proposed that using UCN-01, a staurosporine analog which was developed as a PKC inhibitor, but was later shown to inhibit other kinases including CDK1, CDK2 and CDK4 at low nanomolar concentrations could be used in this manner. UCN-01 has been found to induce a reversible G1 arrest in normal cells, while Rb-deficient tumor cells arrest in S phase instead [124]. Cytostatic doses of staurosporine (0.5–10nM) can also be used to arrest normal cells in G1 without any detectable effect on tumor cells [125]. Importantly, staurosporine priming does not compromise the ability for tumor cells to respond to cytotoxic therapies, while normal cells are arrested in G1 and therefore not responsive to chemotherapy that targets cycling cells.

More recently with the availability of specific CDK4/6 inhibitors that are more clinically relevant than staurosporine, this hypothesis has been revived and tested in mouse models using PD-0332991 [126]. Myelosuppression induced by platinum drugs and anthracyclines are one of the most life-threatening toxicities seen in cancer patients, due to both heightened risk of infection while immunosuppressed and also due to the subsequent chemotherapy delays or dose reduction, which can compromise treatment efficacy. A study was performed comparing two different mouse models of breast cancer, one with intact Rb signaling (the MMTV-neu model) and the other with inactive Rb (C3-Tag), to examine whether CDK4/6 inhibition could protect the hematopoietic progenitor cells from carboplatin-induced quiescence. The C3-Tag model which best resembles basal-like breast cancers, is Rb-deficient and, unsurprisingly, does not respond to CDK4/6 inhibition as a single agent. Carboplatin is highly active in basal-like breast cancer, though, and co-treatment with PD-0332991 did not protect tumor cells from death. However, these mice had reduced thrombocytopenia. In contrast, MMTV-neu mice, which have previously been shown to be dependent upon CDK4 and cyclin D, were sensitive to PD-0332991 as a single agent. In addition, when carboplatin was combined with PD-0332991, tumors grew back faster, indicating the CDK4/6 inhibition in this context protects tumor cells as well normal cells from toxicity, therefore not gaining any significant therapeutic index. These results suggest that CDK4/6 inhibitors may have a new utility – in tumors that are CDK4/6 sensitive, these drugs can be used for anti-tumor effect (and should be used separately from other cytotoxic therapies), and in other tumors that are insensitive (e.g. Rb deficient, p16 overexpressed), these drugs can be normal tissue protectors from other cytotoxic therapies. As a practical consideration as a result of these discoveries, we advocate for Rb mutation status to become one of the biomarkers tested routinely in the clinic.



## 14.4 Newer Cell Cycle-Related Targets for Therapy

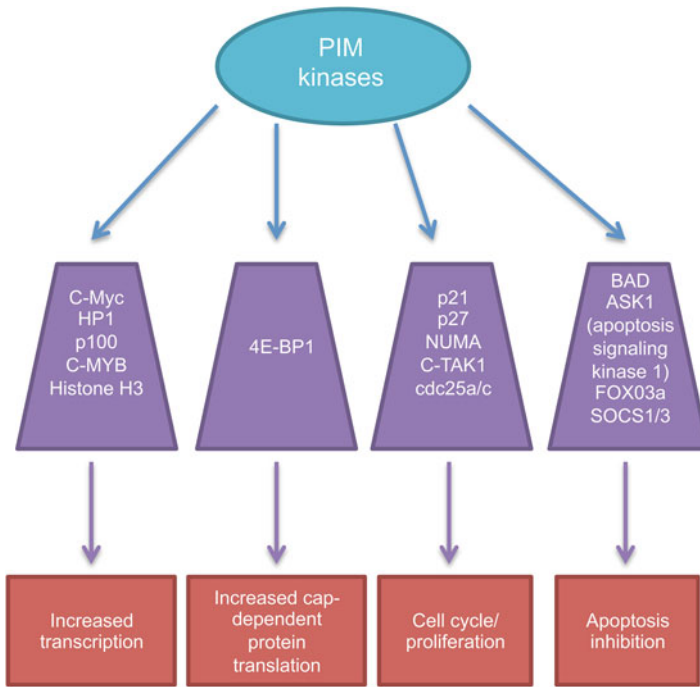
In this last section, we briefly outline rationale for targeting other cell cycle-related proteins and review the state of drug development for each class of agent. Some of these proteins are intimately involved in mitosis regulation, and will be discussed separately.

### 14.4.1 *PIM Family Kinases*

One of the most interesting emerging targets in the PIM family of kinases, which regulate multiple pathways including the cell cycle. The PIM family of serine/threonine kinases consists of three isoforms that have a significant degree of sequence homology, but differ in their tissue distribution [127]. Overall these proteins are expressed throughout in hematopoietic progenitors as well as liver, spleen and other epithelial and mesenchymal tissues, and have considerable functional redundancy. PIM1 is the best studied member of this family, and is thought to be the most widely relevant gene (of the 3 PIM isoforms) in cancer.

The PIM1 gene was identified in the 1980s as a frequent proviral integration site for Moloney murine-leukemia virus (MuLV) which induced T-cell lymphomas in transgenic mice [128]. Subsequently PIM1 was shown to cooperate with c-Myc in inducing lymphomas in utero or around birth, whereas E $\mu$ -Myc transgenic mouse crossed onto a PIM1 and PIM2-deficient background had delayed lymphomagenesis [129–131]. More recently, PIM family kinases have been discovered to be over-expressed or mutated in other solid tumors such as prostate, pancreatic, ER-negative breast cancer and head and neck squamous carcinomas as well as many leukemias and lymphomas (AML and CLL), leading to the question of whether they could be targeted [132–137].

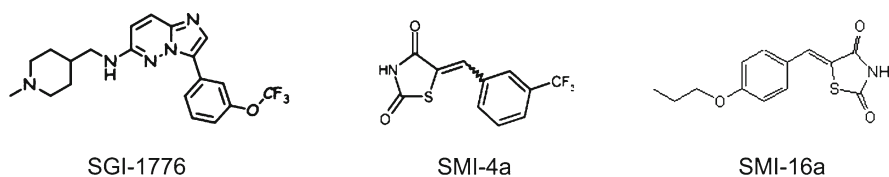
PIM kinases are unusual in that they are constitutively active, but are regulated largely at the transcriptional and translational level [127–138]. A wide range of cytokines and growth factors can activate PIM kinases, mainly via the JAK-STAT pathway and NF $\kappa$ B pathways [139, 140]. Since the mRNA has a short half-life, inhibitors of JAK-STAT could potentially be used to inhibit PIM signaling as well. An emerging paradigm places PIM1 at the center of a cellular stress response, since PIM1 can be induced by hypoxia and DNA damage via various mechanisms. For example hypoxia can induce PIM1 expression rapidly in a HIF1 $\alpha$  independent mechanism, as well as induce nuclear translocation [141, 142]. PIM1 induction in response to hypoxia promotes cell survival via inhibition of apoptosis and is linked to chemoresistance under these conditions. PIM1 can also be induced in response to DNA damage by Kruppel-like factor 5 [143]. In a study of head and neck squamous carcinoma patients, upregulation of PIM1 in response to irradiation was shown to be associated with a poor response [144]. Since EGFR expression is also correlated



**Fig. 14.4** PIM1 kinase substrates and cellular functions

with radiation resistance and EGFR is autophosphorylated and nuclear localized after IR, the authors asked with EGFR can regulate PIM1 levels/activity. In cell lines, EGF-ligands induced PIM1 nuclear-translocation, and this effect could be blocked by the EGFR antibody cetuximab or tyrosine kinase inhibitor, gefitinib. Similarly, HNSCC cells that were irradiated had more nuclear PIM1, and PIM1 knockdown demonstrated the pro-survival role that PIM1 plays in this context. Taken together, these studies show that PIM1 may be a good target in a number of different cancer systems.

PIM1 plays a number of cellular functions that all contribute to tumorigenesis, as depicted in Fig. 14.4. One of these is regulation of the cell cycle via phosphorylating several substrates such as p21, p27, cdc25A, cdc25C and HP1 [145–151]. One of the most robust readouts of PIM1 activity is phosphorylation of the pro-apoptotic protein BAD at Ser 112, which inactivates it, therefore enhancing anti-apoptotic activity of Bcl2 [152]. As mentioned previously, PIM1/PIM2 cooperates with c-Myc to regulate lymphomagenesis, and one of the mechanisms it does so is via phosphorylation of c-Myc which stabilizes the protein [153]. Overexpression of PIM1 also induces genomic instability via deregulating the mitotic spindle checkpoint, which causes abnormal mitoses, centrosome amplification and aneuploidy [154]. In hematopoietic malignancies in which PIM2 is highly expressed, 4EBP1 is also a target that is involved in promoting cap-dependent translation initiation of



**Fig. 14.5** Structure of PIM1 kinase inhibitors

proteins that have growth promoting roles such as c-Myc and cyclin D1 [136]. In prostate cancer specifically, the androgen receptor is also a substrate of PIM1, and this phosphorylated form is transcriptionally inactivated and degraded [155, 156].

Structurally PIM kinases are distinct from other kinases in terms of how ATP binds to them, which has allowed chemists to design highly selective inhibitors. One of the most attractive features of PIM1 as a drug target is the lack of obvious phenotype in the knockout mouse, which is viable and fertile [157]. Compensation by other PIM family members is unlikely since compound knockout mice are also viable and fertile. The only phenotype that was observed in the *Pim1*<sup>-/-</sup> mouse is a subtle hematopoietic effect, such that the red blood cells are abnormally small but this did not lead to any physiological effects. When other potential hematopoietic functions were examined closely, it was found that bone-marrow-derived cells in culture had a significant impairment in IL-3 and IL-7 growth factor response [131].

The first compound that has been developed that has moved into cellular and *in vivo* studies is SGI-1776, which is an imidazo [1, 2-b] pyridazine compound that inhibits all three PIM kinases with IC<sub>50</sub>s of 7, 363 and 69nM (PIM1, PIM2 and PIM3 respectively), and has some activity against FLT3, another target in AML (see Fig. 14.5 for structure of this and other PIM inhibitors) [158]. In xenograft models of AML cells, this drug was highly active as an oral agent, inducing complete regression of blasts [159]. In addition, SGI-1776 can re-sensitize chemoresistant prostate cells to taxanes due to inhibiting multidrug resistance proteins including MDR1 [160]. Unfortunately when moved into phase 1 trials in humans (one trial was focused on prostate cancer and the other was non-Hodgkin's lymphoma) this drug was found to cause dose-limiting cardiac toxicity for reasons that are not clear, and the studies were stopped [161]. Another two structurally-related PIM1 inhibitors were identified in a chemical library screen called SMI-4a and SMI-16a which are benzylidene-thiazolidine-2, 4-diones [162]. When tested *in vitro*, these agents both had growth inhibitory activity in leukemia and prostate cancer cell lines, induced G1 arrest and induced p27 nuclear translocation. In addition, PIM1 inhibitors including SMI-4a synergize with both rapamycin in prostate cancer cells, and more recently with the Bcl2 inhibitor ABT-737 [163]. In our opinion despite these compounds having promising pre-clinical activity, the drug discovery market relating to PIM1 kinase is wide open right now. Special attention should be focused on the potential cardiac toxicity profile of future inhibitors (compared to SGI-1776), to try to understand what the off-target mechanisms are that underlie the Qt prolongation seen with SGI-1776 in patients.

### ***14.4.2 Mitotic Kinases: Aurora Kinase Family***

In much of this chapter so far we have discussed targets that function early in the cell cycle in regulating G1 and S phases. However, G2 and M phases are also very kinase-rich and have tremendous potential as drug targets. Several classes of chemotherapies already target these processes, such as taxanes, which bind to tubulin and disrupts the assembly of the spindle. In order to design better therapies against proteins that act in G2 and M phase, we must understand their functions at a mechanistic level and how they contribute to the events that are necessary for progression through these stages. During G2 phase when cells are preparing for mitosis, the cell is very active in ensuring the DNA was replicated correctly, and dividing the other organelles. In addition, microtubule proteins are being synthesized in order to form the mitotic spindle along which the chromosomes will segregate during mitosis. G2-M phase targets include proteins that are involved in entering mitosis (such as Aurora kinase A), the spindle assembly checkpoint (such as BUB1), and mitotic exit (such as APC). Each of these proteins and processes could be the focus of entire chapters, so we will provide a high-level overview of each here, and point the readers to recent reviews on these proteins.

Aurora kinases, of which there are three highly related isoforms (A, B and C) in mammalian cells, are key regulators of mitosis that have been well conserved throughout eukaryotic organisms. All three isoforms have been the focus of drug development over the past several years, and most of the inhibitors target two or three of them due to the highly conserved catalytic domain [164]. Aurora kinase A (AURKA) and Aurora kinase B (AURKB) have the strongest evidence for a role in tumor cell growth, whereas Aurora kinase C has scant evidence. This may be because for many years Aurora kinase C was thought to be primarily expressed in the testes where it plays a role in spermatogenesis, by playing similar roles to AURKB [165]. However more recently it has also been found to be expressed at high levels in some cancer lines, and several point mutations have been found in lung tumors but relatively little is known about its function [166, 167].

AURKA is ubiquitously expressed and is the first of the family members to be activated starting in late S phase and working through to completion of mitosis. Many related processes are regulated by AURKA, including maturation and separation of the centrosomes, assembling the mitotic spindle, chromosome alignment and cytokinesis [168–170]. Regulation of AURKA levels is also important for mitotic exit, as either too much or too little activity leads to failure of cytokinesis and multinucleation [171]. AURKA regulation occurs at both transcriptional and posttranslational levels, including activation by autophosphorylation at Thr288 on the activation loop, and deactivation via protein phosphatase 1 [172]. In cancer, AURKA is frequently amplified/overexpressed due to various mechanisms, especially in higher grade tumors and has been demonstrated to be a poor prognostic factor [173–176]. It was established as a bona fide oncogene when it was shown to be capable of inducing rodent fibroblast cell transformation due to formation of multipolar mitotic spindles that induce genomic instability [177]. Interestingly, these chromosomal

abnormalities that occur in AURKA overexpressing cells does not lead to cell death, because AURKA also promotes cell survival pathways including AKT-mTOR and nuclear accumulation of cyclin D1 [178]. NF $\kappa$ B is another anti-apoptotic pathway that is regulated by AURKA phosphorylation of its inhibitor, I $\kappa$ B [179]. AURKA has also been shown to interact with the p53 network, specifically via phosphorylating p53 inducing its degradation via MDM2, as well as phosphorylating p53 on Ser 215 which inhibits its DNA binding ability [180, 181]. These findings demonstrate the wide spectrum of roles that AURKA plays in cellular transformation and provide significant rationale for targeting this kinase.

Similar to Aurora kinase A, Aurora kinase B (AURKB) is also expressed in all proliferating cells, however it plays more limited roles as a chromosome passenger protein. AURKB is primarily expressed starting during prophase, where it is localized at the kinetochore to ensure correct chromosome alignment to the spindle and also helps ensure chromosomes segregate correctly [182]. In addition, AURKB phosphorylates Histone H3 at Ser10 and Ser28, which facilitates chromosome condensation [183, 184]. In cancer AURKB is not amplified, however it is still highly expressed in several tumor types [185–187]. Apart from regulating kinetochore-spindle interactions, in cancer cells, AURKB has been linked to degradation of p53 via phosphorylation at multiple sites [188], providing further rationale for targeting this kinase.

Inhibition of AURKA leads to G2 arrest, and has been shown to increase chemo- and radiosensitivity in cancer cells [189]. Quite a number of inhibitors have been generated by most of the major pharmaceutical companies and are currently being tested in early stage clinical trials. Many of these target both AURKA and AURKB, although Millennium has two AURKA specific compounds, MLN8054 and MLN8237. For a recent review with information about the clinical development of these agents, see [169]. Inhibition of AURKB are known as mitotic drivers, since they cause overriding of the mitotic checkpoints and results in aberrant mitosis and aneuploidy. This contrasts with AURKA inhibitors which block passage through mitosis. The question of whether inhibiting both AURKA and AURKB is better than either kinase alone has still not been answered. In preclinical genetic studies, the results have been equivocal. In one study in pancreatic cancer, antisense oligonucleotides to AURKA, AURKB or the combination were added to cells and responses compared [190]. The combination of both oligonucleotides was not better at inducing caspase activation, accumulating tetraploid cells or reducing formation in soft agar than either one alone. Targeting AURKA alone had a slightly better response overall versus AURKB alone, and this correlated with cells rounding up and detaching from the plate versus becoming large and multinucleated with the AURKB oligonucleotide. In contrast to this pancreatic study however, in colon cancer cells, AURKB inhibition was better than AURKA [191]. In order to move these targets forward, greater emphasis will have to be placed on understanding what contexts predict response to inhibition of each protein, and multiple readouts of each kinase inhibition should be analyzed since it is possible that each drug will have a slightly different profile. One clue that has already emerged is that p53-deficient cells more readily undergo apoptosis in response to the VX-680 inhibitor,

however since this is a pan-aurora inhibitor, it is difficult to dissect out which target is most relevant in p53-deficient tumors [192]. Further molecular and pharmacodynamic characterization of sensitive and resistant patients in the many clinical trials in progress should elucidate more such factors, as well as more detailed preclinical work with patient derived xenograft models should be the *in vivo* assay of choice in these studies.

### 14.4.3 Other Mitotic Targets of Interest

Moving forward as genomic studies are completed and more functional screens are performed it is likely more novel cell cycle targets will be found. Some examples of this nature that have been identified so far include MELK, Bub1, and Mps1. We will briefly summarize some of these proteins and how targeting them might be useful in cancer.

Maternal embryonic leucine zipper (MELK) is an atypical member of Snf1/AMPK family of kinases that has received only a little research attention so far. MELK is upregulated in several solid tumors including high-grade prostate cancer, astrocytoma, medulloblastoma and in breast cancer [193–195]. In addition, MELK is highly expressed in neural and breast cancer stem cells, making it a potentially attractive target to eradicate this population of cells thought to be the main drivers of drug resistance and eventual disease progression [196–198]. Expression of MELK is known to be increased in mitotically-arrested cells, and in prostate cancer cells is highly correlated with several other cell cycle/proliferation related genes including AURKB, cyclin B2 and DNA topoisomerase 2 alpha [193, 199, 200]. A few recent studies have suggested a role for MELK in radioresistance and chemoresistance, and have provided some *in vitro* evidence that knockdown can sensitize cancer cells to additional therapies [201, 202]. The only known pharmacological agent that targets MELK so far is the antibiotic siomycin A which reduces MELK expression and has been shown to decrease glioblastoma growth *in vivo* via targeting the neural stem cells [203].

The spindle assembly checkpoint (SAC) is a mechanism of delaying anaphase if the kinetochores are unattached to microtubules. There are at least 14 proteins involved in this process, four of which are kinases that are potentially targetable. These kinases are Bub1, BubR1, Mps1 and aurora B, although aurora B is dispensable for the checkpoint. If the SAC checkpoint is active, some of the components, such as Bub1, sequester Cdc20 which is the active part of the APC/C complex which degrades cyclin B. Bub1 may be a master regulator of the spindle assembly checkpoint by recruiting other important proteins involved such as BubR1, Mad1 and Mad2 [204]. The underlying concept behind targeting this checkpoint is that by preventing SAC activation, severe chromosome segregation occurs which causes cell death. Even partial inhibition of any of these essential mitotic checkpoint components can sensitize tumor cells to mitotic-targeting chemotherapies such as taxanes, whereas normal cells are not sensitized since normal cells can maintain a diploid population of cells [205]. Proof of this principle *in vitro* has been obtained for inhibitors of Mps1 [206, 207].

Cdc20 has also been proposed as a target in cancer due to the strong phenotypes seen in genetic studies from blocking mitotic exit. The *cdc20* homozygous knockout mouse is embryonic lethal at the two-cell stage due to a metaphase arrest [208]. When an inducible knockout model was generated, a similar phenotype could be observed upon induction, and very high levels of cyclin B was observed in the cell, consistent with a defect in APC/C function [209]. When tumors of either epithelial or mesenchymal origin were induced in this model, and then *cdc20* knockout was induced, the tumors rapidly regressed due to mitotic arrest and apoptosis.

In summary, there are various strategies that are being investigated to interfere with mitosis including delaying mitotic entry and spindle formation, preventing activation of the spindle assembly checkpoint or targeting mitotic exit via the APC/C-*cdc20* complex. Such strategies may synergize with current chemotherapies that act in mitosis such as taxanes and vinca alkaloids, allowing lower doses of these agents to be administered. The question remains however whether a sufficient therapeutic index can be reached since normal cells also require these processes to be intact to undergo normal mitosis.

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