

Targeting Checkpoint Kinase 1 in Cancer Therapeutics

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Abstract Progression through the cell cycle is monitored by surveillance mechanisms known as cell cycle checkpoints. Our knowledge of the biochemical nature of checkpoint regulation during an unperturbed cell cycle and following DNA damage has expanded tremendously over the past decade. We now know that dysfunction in cell cycle checkpoints leads to genomic instability and contributes to tumor progression, and most agents used for cancer therapy, such as cytotoxic chemotherapy and ionizing radiation, also activate cell cycle checkpoints. Understanding how checkpoints are regulated is therefore important from the points of view of both tumorigenesis and cancer treatment. In this review, we present an overview of the molecular hierarchy of the checkpoint signaling network and the emerging role of checkpoint targets, especially checkpoint kinase 1, in cancer therapy. Further, we discuss the results of recent clinical trials involving the nonspecific checkpoint kinase 1 inhibitor, UCN-01, and the challenges we face with this new therapeutic approach.

Background

Introduction

The cell cycle progresses in an orderly fashion and is monitored by safety mechanisms known as cell cycle checkpoints, which, on activation, function to halt cell division. Checkpoint dysfunction is common in human cancers and is considered a pathologic hallmark of neoplastic transformation. Conversely, agents used for cancer treatment, such as cytotoxic chemotherapy and ionizing radiation (IR), also activate cell cycle checkpoints. This review provides some general insight into the molecular organization of the checkpoint signaling network and how pharmacologic manipulation of checkpoints, particularly the function of checkpoint kinase (Chk) 1, can be exploited in cancer therapy.

Molecular framework of the checkpoint signaling cascade

Functional components of the checkpoint signaling network are highly conserved from yeast to human and can be categorized as sensors, proximal transducer kinases, mediators, distal transducer kinases, and effectors (Fig. 1; see refs 1–5 for review).

Although a great deal has been learned about the biochemistry/genetics of the checkpoint cascade, exactly how DNA damage is sensed at the molecular level remains elusive.

Damage sensors are operationally defined as molecules that can recognize the structural abnormality of damaged DNA or chromatin without requiring activation (3). The proximal transducer kinases ataxia-telangiectasia mutated (ATM) and ATM and Rad3 related (ATR) both possess functional properties of a sensor. However, their optimal activation requires additional sensor proteins. One example is the Mre11-Rad50-NBS1 complex, which can bind independently to DNA double-stranded breaks and facilitate ATM signaling (Fig. 1; ref. 3). On this basis, the Rad17 clamp loading complex and the proliferating cell nuclear antigen (PCNA)-like sliding clamp, Rad9, Rad1, and Hus1 complex (commonly referred to as the "9-1-1" complex) have also been assigned sensing roles in ATR-mediated signaling (Fig. 1).

The major output following damage recognition is the activation of the proximal transducer phosphatidylinositol-3-kinases, ATM and ATR. ATM seems to be the dominant kinase reactive to DNA DSBs, whereas ATR is activated by stalling of replication forks (replicative stress) induced by UV, nucleotide imbalance, and DNA cross-linking (Fig. 1). IR induces autophosphorylation of ATM at Ser¹⁹⁸¹, resulting in dissociation from inactive dimer into active monomeric forms (6). In contrast, ATR is not activated by higher kinase activity but rather by recruitment of the kinase to its substrates via its associated regulatory subunit ATR-interacting protein (Fig. 1; ref. 4).

Recent data have challenged the classic model of ATM and ATR being activated by distinct lesions and triggering independent downstream pathways (7). Coordinated cross-talk definitely exists between the two pathways. Thus, it has been shown that ATM and the nuclease activity of MRE11 are necessary for the processing of DSBs to generate the recombinant protein A-coated ssDNA that are required for ATR recruitment and Chk1 phosphorylation in response to IR (Fig. 1; ref. 7).

Chk1 and Chk2 are two structurally unrelated serine/threonine kinases that share overlapping functions. Chk1 is an essential kinase (molecular weight, 54,000) that regulates both the S and G₂-M checkpoints. Its expression is largely restricted to the S and G₂ phases of proliferating cells (8). Chk1

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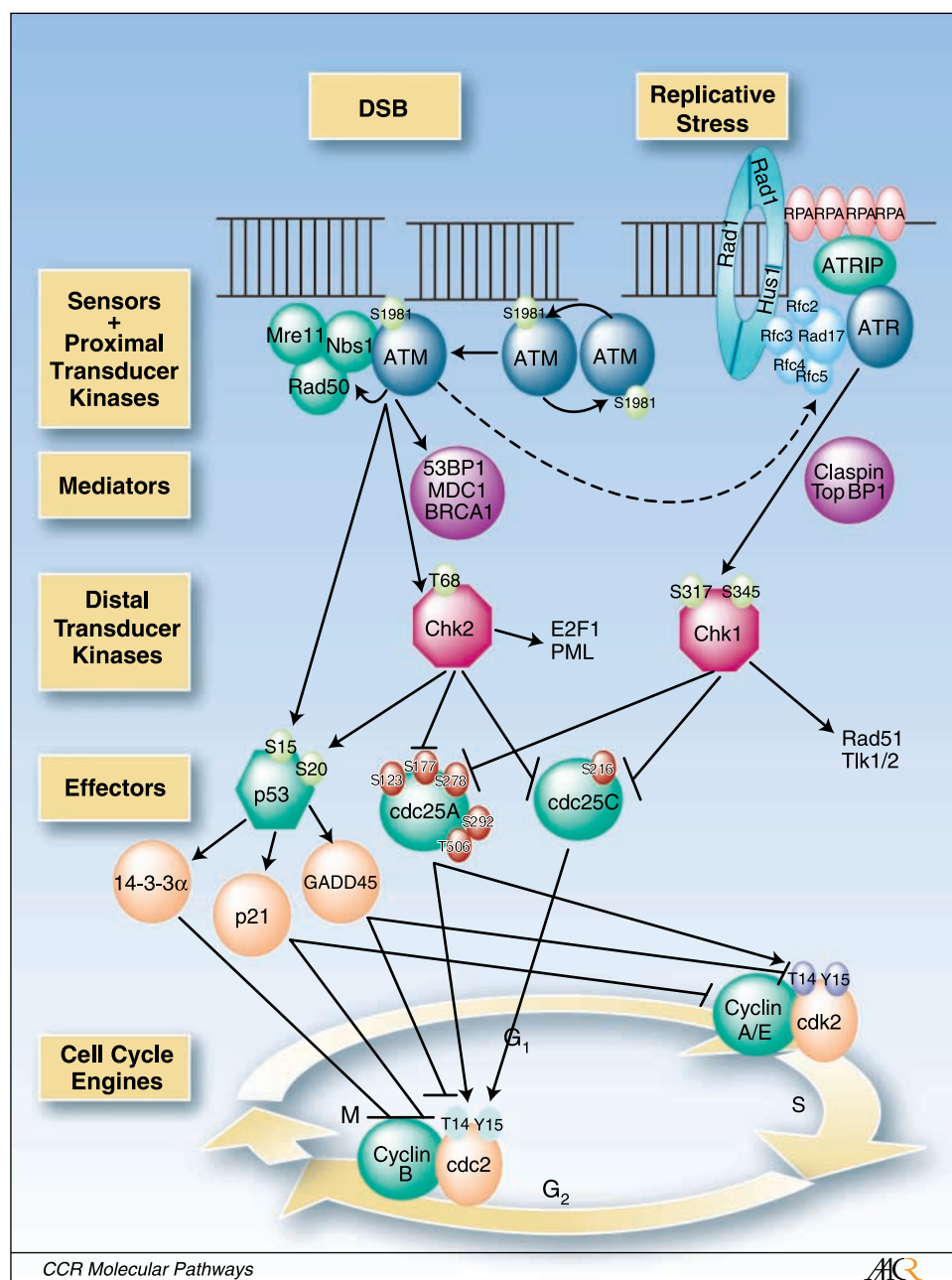


Fig. 1. Molecular organization of the checkpoint signaling network in response to DNA DSBs and replicative stress. Functional components of the checkpoint signaling network are hierarchically categorized as sensors, proximal transducer kinases, mediators, distal transducer kinases, and effectors. ATM is believed to be the primary proximal transducer kinase reactive to DNA DSBs. ATM activation via the formation of autophosphorylated monomers may be caused by changes in higher chromatin structure associated with DSBs. Current data suggest that recruitment of activated ATM to the site of damage requires the Mre11-Rad50-NBS1 complex. Phosphorylation of ATM substrates is facilitated by mediator molecules, such as 53BP1, Brca1, and MDC1, which themselves are often modified by ATM. In response to replicative stress, the ATR – ATR-interacting protein (ATRIP) complex is recruited to the region of ssDNA coated by recombinant protein A (RPA). Activation of Chk1 by ATR also requires the mediators Claspin and TopBP1 and loading of the sliding clamp Rad9-Rad1-Hus1 (“9-1-1”) complex onto DNA by the RFC complex. Signals originating from the sites of damage are amplified through the mobile distal transducer kinases Chk1/Chk2, which can disperse the damage alert to the rest of the cell. Both Chk1 and Chk2 can phosphorylate a variety of effectors (e.g., cdc25 phosphatases and p53), which ultimately halt cell cycle progression by inhibiting the cell cycle engines [cyclin-dependent kinases (cdk)]. Phosphorylation of cdc25A and cdc25C by Chk1/Chk2 results in enhanced degradation and cytoplasmic sequestration of the phosphatase, respectively. In addition to causing cell cycle delay, Chk1/Chk2 also mediates other aspects of the DNA damage response, including DNA repair, apoptosis, and chromatin remodeling by phosphorylating various downstream substrates (see text). A newly reported route of signaling (broken line) involves an ATM-dependent activation of the ATR-Chk1 pathway in response specifically to DSBs induced by IR. Key activating and inhibitory phosphorylation sites (green and red, respectively).

is subjected to multiple facets of regulation, including transcriptional suppression by p53 (9), ubiquitin-dependent degradation (10), and the most well-characterized activation by its upstream proximal transducer kinase ATR (11). ATR, when combined with its binding partner ATR-interacting protein, activates Chk1 by phosphorylating serine residues 317 and 345 that are present on the COOH end of Chk1, thereby removing the inhibition imposed by the COOH terminus (11). In addition, Chk1 activation also requires loading of the “9-1-1” complex by the Rad17/RFC clamp loader complex onto chromatin, as well as two recently identified mediator proteins, Claspin and TopBP1 (12, 13).

Chk2 is activated primarily by ATM-mediated phosphorylation at Thr⁶⁸. This phosphorylation event results in Chk2 dimerization and subsequent autophosphorylations leading to

its full activation (2). Like Chk1, activation of Chk2 also requires other proteins, including the Mre11-Rad50-NBS1 complex and Brca1 (2). In light of the many substrates shared between Chk1 and Chk2, it is somewhat surprising to note that Chk2 is totally dispensable for organismal development, whereas Chk1 loss results in embryonic lethality (14, 15).

When activated, Chk1 and Chk2 phosphorylate a plethora of effector molecules involved in DNA damage response, including (a) cell cycle delay (cdc25A, cdc25C, and p53; refs. 11, 14, 16, 17), (b) DNA repair (Brca1, p53, and Rad51; refs. 18, 19), (c) apoptosis (PML, p53, and E2F1; ref. 2), and (d) chromatin remodeling (Tousled like kinase 1/2; ref. 20).

An additional class of molecules termed mediators helps to coordinate the temporal-spatial progression of the DNA damage response. Mediators involved in ATM-related signaling

comprise proteins that contain two tandem COOH-terminal BRCT (Brca1 carboxyl-terminal) domains, including 53BP1, Brca1, and MDC1/NFBD1 (21, 22). These factors are recruited to the damage site possibly by direct interaction between their BRCT domains and the phosphorylated form of the histone variant, γ -H2AX. The loss of these mediator proteins results in S and G₂-M checkpoint defects as well as hypersensitivity to IR. Analogous to Brca1 COOH terminal-containing adaptor proteins, which facilitate ATM signaling, Claspin and TopBP1 seem to be critical for ATR-mediated functions, including Chk1 activation discussed above.

Activation of the interphase checkpoints following genotoxic stress

The G₁ checkpoint. The G₁ checkpoint is governed primarily by the ATM-Chk2-p53-p21 pathway as the expression level of ATR and Chk1 remains low until late G₁ (8). Transcriptional activation of the cyclin-dependent kinase inhibitor p21 by p53 results in neutralization of the G₁-S-promoting activity of cyclin E/cyclin-dependent kinase 2, leading to a G₁ delay. As the cell traverses the G₁ restriction point, the expression of ATR, Chk1, and its downstream effector cdc25A phosphatase begins to increase. This dual-specificity phosphatase promotes the activity of cyclin E(A)/cyclin-dependent kinase 2 by removing the inhibitory phosphates at Thr¹⁴ and Tyr¹⁵ on cyclin-dependent kinase 2. Chk1 phosphorylates cdc25A at several NH₂-terminal serine residues, resulting in enhanced proteosomal degradation of the phosphatase (23, 24). Even during an unperturbed cell cycle, some constitutive activity of Chk1 is responsible for the basal turnover of cdc25A (25). With genotoxic stress, Chk1/Chk2 are activated and cdc25A is markedly destabilized, resulting in a swift p53-independent mechanism of G₁-S delay following DNA damage (1).

The intra-S checkpoint. In addition to structural DNA damage, the S-phase checkpoint is activated in response to stalled replication forks induced by deoxyribonucleotide pool perturbation, UV irradiation, and inhibition of DNA polymerases. A hallmark for a deficient S-phase checkpoint is the radioresistant DNA synthesis phenotype, in which cells continue to incorporate radiolabeled nucleotide precursors following IR exposure. The S-phase checkpoint responsible for radioresistant DNA synthesis suppression is replication independent and largely reflects its ability to act in trans to inhibit the initiation of unfired replicons. On activation of the intra-S-phase checkpoint, Chk1/Chk2 phosphorylates cdc25A on several NH₂-terminal residues, resulting in enhanced proteolysis of the phosphatase (23, 24) and inhibition of its function through 14-3-3 binding (26). In addition to the replication-independent checkpoint induced by DSBs, the S-phase checkpoint is also triggered by blockade of the replication fork. This replication-dependent S-phase checkpoint serves two functions: (1) to silence the initiation of unfired origins of replication and (2) to stabilize stalled forks and promote fork recovery. The former requires activation of the ATR-Chk1 pathway, whereas the latter function is not well understood, although both the ATR-Chk1 pathway and the Brca2 protein have been implicated (27).

The G₂-M transition checkpoint. The G₂-M checkpoint prevents mitotic entry in the presence of DNA lesions introduced during G₂ or acquired in the preceding S phase and carried through G₂. The key downstream target of the G₂-M checkpoint is the prometotic cyclin B/cdc2 kinase. During

interphase, the complex is inactivated through phosphorylation on Thr¹⁴ and Tyr¹⁵ by Myt1 and Tyr¹⁵ by Wee1. Activation of cyclin B/cdc2 requires dephosphorylation at these residues mediated by the dual-specificity cdc25 phosphatases (cdc25A, cdc25B, and cdc25C). The current model suggests that the initiation of the G₂-M checkpoint is mediated by the ATM/ATR-Chk1/Chk2-cdc25A/cdc25C pathway, whereas maintenance of this checkpoint requires p53 and its downstream effectors p21, 14-3-3 σ , and GADD45 (Fig. 1; ref. 28). A less well-characterized signaling pathway responsive primarily to UV light involves the activation of p38, resulting in phosphorylation of cdc25B and a G₂ delay (29).

Clinical-Translational Advances

Inhibitors of cell cycle checkpoint: UCN-01 as a prototypical Chk1 inhibitor

If checkpoint dysfunction represents a common molecular defect acquired during tumorigenesis, an important question is whether additional checkpoint disruption by pharmacologic means will render tumors more sensitive to cytotoxic agents. In 1982, Lau and Pardee (30) showed that the methylxanthine derivative, caffeine, could potentiate the cytotoxicity of DNA-damaging agents by abrogating the G₂-M checkpoint and promoting premature mitosis. It was subsequently shown that caffeine is a nonspecific inhibitor of ATM and ATR with IC₅₀ values of 0.2 to 1 mmol/L (31).

A second wave of enthusiasm came along when the staurosporine analogue, UCN-01 (7-hydroxystaurosporine), was shown to be a potent but nonselective inhibitor of Chk1 (32). We and others have shown that UCN-01 sensitizes tumor cells to a wide variety of genotoxic agents (Table 1). Chk1 as a target for chemosensitization has also been validated by nonpharmacologic approaches using dominant negative, antisense, and siRNA to down-regulate Chk1 activity and sensitize cells to DNA damage (1).

In the single-agent phase I study led by the National Cancer Institute, pharmacokinetic data from the first few patients revealed that UCN-01 has a very small volume of distribution, low systemic clearance, and a prolonged half-life of elimination (>200 h) as a result of avid binding of the drug to serum α 1 acidic glycoprotein (33). Three phase I combination studies in patients with solid tumors have been published recently, in which UCN-01 was combined with cisplatin (34), 5-fluorouracil (35), and topotecan (36), respectively. Two partial tumor responses were observed in the cisplatin and topotecan trials. A fourth study evaluated UCN-01 in combination with the antimetabolite Ara-C in patients with acute myelogenous leukemia (37). Only the correlative science portion of the trial has been reported to date. Circulating leukemic blasts were collected from patients for pharmacodynamic evaluation. However, inhibition of Chk1-mediated signaling by UCN-01 has not been convincingly shown in leukemic samples presumably because only a small proportion of the circulating blasts were cycling (37).

Novel Chk1 inhibitors

The clinical development of UCN-01 has been hampered by its unfavorable pharmacokinetic properties and untoward toxicities (e.g., hyperglycemia; ref. 33). Thus, identification of alternative Chk1 inhibitors represents a high priority. A partial list of the newer Chk1 inhibitors is shown in Table 1. One

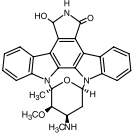
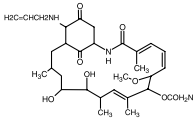
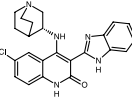
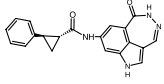
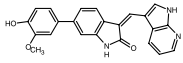
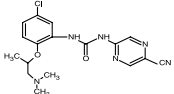
candidate is CHIR-124, a quinolone-based compound that potently and selectively inhibits Chk1 *in vitro* (IC₅₀, 0.0003 μmol/L; ref. 38). We have shown that CHIR-124 interacts synergistically with topoisomerase poisons in reducing proliferation in several p53-mutant cell lines (38). CHIR-124 abrogates the SN-38–induced S and G₂-M checkpoints and enhances apoptosis in MDA-MD-435 breast cancer cells. In an orthotopic xenograft model, CHIR-124 potentiates the growth-inhibitory effects of CPT-11 by abrogating the G₂-M checkpoint and increasing tumor apoptosis (38).

In addition to developing small molecular kinase inhibitors, alternate approaches to down-regulating Chk1 function have been studied. It has been shown that Chk1 is a client protein of the molecular chaperone heat shock protein 90 and treatment of

tumor cells with 17-allylamino-17-demethoxygeldanamycin, a heat shock protein 90 inhibitor, results in depletion of Chk1 protein and sensitization to gemcitabine (Table 1; ref. 39). We have found that in addition to Chk1, exposure of tumor cells to 17-allylamino-17-demethoxygeldanamycin also depletes another critical Chk, Wee1 (40). Decreased expression of these kinases was associated with abrogation of the G₂-M checkpoint and enhancement of apoptosis following treatment with SN-38– in p53-deficient tumors (40). A phase I clinical trial combining irinotecan and 17-allylamino-17-demethoxygeldanamycin in solid tumor patients is now under way and the relationship between tumor response and p53 status will be explored in the trial.

PD-321852 inhibits Chk1 with an *in vitro* IC₅₀ of 5 nmol/L against the kinase (Table 1; ref. 41). At nontoxic concen-

Table 1. Chk1 inhibitors in development

Agent and chemical structure if available	Drug company	DNA-damaging agent(s) studied in combination	Clinical study stage
UCN-01 	Kyowa	Gemcitabine (50), Ara-C (51), topoisomerase I poisons (52), cisplatin (45), mitomycin (53), temozolomide (47), IR (45)	Phase I/II
17AAG 	Kosan	Gemcitabine (54), Ara-C (55), topoisomerase I poisons (40, 56)	Phase I (combination studies)
XL844	Exelixis	Undisclosed	Phase I
CHIR-124 	Chiron	Topoisomerase I poisons (38)	Preclinical
PF-00394691 	Pfizer	Gemcitabine, topoisomerase I poison, cisplatin (42)	Preclinical
PD-321852	Pfizer	Gemcitabine (41)	Preclinical
CEP-3891	Cephalon	IR (57)	Preclinical
N-aryl-N'-pyrazinylurea 	Abbott	Doxorubicin (58, 59)	Preclinical
and 3-ethylidene-1,3-dihydro-indol-2-one analogs 			

trations, it potentiates clonogenic inhibition induced by gemcitabine in colorectal SW620 and pancreatic BxPC3 but not in pancreatic PANC-1 cancer cell lines (41). Interestingly, in cell lines of which clonal growth was synergistically inhibited by the combination, PD-321852 (but not UCN-01) caused a dramatic decrease of Chk1 protein (41). It will be of interest to determine whether PD-321852 affects Chk1 stability by inhibiting heat shock protein 90 like 17-allylamino-17-demethoxygeldanamycin (39).

PF-00394691 is a diazepinoindolone-based selective inhibitor of Chk1 with a IC_{50} of 0.75 nmol/L (Table 1; ref. 42). It potentiates the antitumor activity of gemcitabine, irinotecan, and cisplatin without increasing host toxicity in human tumor xenograft models (42). Of note, PF-00394691 inhibits Chk1 phosphorylation on Ser³¹⁷ induced by irinotecan in tumor xenografts via an unknown mechanism (42). One possible explanation is that the interaction between Chk1 and its upstream phosphatidylinositol-3-kinase ATR is somehow affected by PF-00394691.

Several undisclosed Chk1 inhibitors found in the patent literature have been described in a recent review article on the subject (43).

The role of p53 in determining tumor sensitivity to cytotoxic agents and Chk1 inhibitor combination

Because functional loss of p53 is frequent in human cancers, pharmacologic disruption of additional checkpoints may create "synthetic lethality" in tumors lacking p53 following DNA damage. Theoretically, this can be achieved by targeting a different checkpoint of the cell cycle (e.g., inhibiting the S or G₂-M checkpoint in tumors that lack a normal G₁ checkpoint due to p53 loss) or by blocking the complementary pathway of the same checkpoint (44). For the G₂-M checkpoint, p53 and Chk1 seem to function in two parallel pathways in regulating mitotic transition (Fig. 1; ref. 44). However, there has been conflicting data as to whether Chk1 inhibitor selectively enhances the cytotoxicity of DNA-damaging agents in cells with nonfunctional p53 (45–48). It seems that such discrepancies in part relate to the different treatment schedules used and the *in vitro* assays used to evaluate cytotoxicity. Thus, we showed that HCT116 colon cancer cells treated with SN-38, the active metabolite of irinotecan, undergo a senescence-like G₂ arrest (44). When these cells were treated sequentially with SN-38 first for 24 h and then followed by UCN-01, the G₂-arrested cells undergo checkpoint abrogation, enter an aberrant mitosis (mitotic catastrophe), and subsequently develop micronucleation or apoptosis (44). Isogenic p53-null cells, by virtue of an intrinsic G₂ checkpoint defect, are clearly more susceptible to undergoing mitotic catastrophe and apoptosis induced by UCN-01. However, this did not translate into a further reduction in clonogenicity as determined by a colony formation assay (44). Therefore, it seems that sequential treatment with SN-38 followed by UCN-01 converts a senescent, yet metabolically active, cell into a dead cell, and this process is enhanced by p53 loss. At this juncture, it

remains to be determined as to which mode of "reproductive death" in tumors (i.e., cellular senescence, apoptosis, and mitotic catastrophe) is therapeutically desirable clinically.

Potential challenges for clinical development of Chk1 inhibitors

One potential shortcoming associated with checkpoint inhibitors is that they may enhance the toxicity of DNA-damaging agents. Although administration of full-dose chemotherapy in combination with Chk1 inhibitor seemed feasible in xenograft-bearing mice (38, 41, 42), increased myelosuppression was seen in the phase I combination trial of UCN-01 with topotecan at doses of topotecan lower than the ones when the drug is used as a single agent, suggesting some synergistic effect on normal proliferating cells. Another concern with Chk1 inhibitors is whether secondary cancers will arise as a result of genetic instability induced by checkpoint inhibition. The initial testing of this therapeutic approach should therefore be limited to patients with advanced disease for which no curable options exist. One way to optimize its therapeutic index is to combine checkpoint inhibitors with a treatment modality that targets directly at the tumor, such as radiotherapy, regional infusion of chemotherapy, and radiolabeled antibodies.

Validation of target inhibition in clinical studies involving Chk1 inhibitors presents a unique challenge as it requires demonstration of successful suppression of an activated Chk1-mediated signaling event by the inhibitor. Although it is frequently stated that Chk1 phosphorylates Ser²¹⁶ of cdc25C following DNA damage, this site is constitutively phosphorylated by a yet unidentified kinase in undamaged cells, making it an unreliable marker for Chk1 inhibition (49). Conversely, reversal of DNA-damaging agent-induced suppression of cdc25A protein level by Chk1 inhibitors as analyzed by Western blots can be used as a pharmacodynamic readout for Chk1 inhibition in tumors (38). An indirect but simpler way to evaluate for target inhibition is to measure the extent of G₂-M checkpoint abrogation using the mitosis-specific antibody against phosphorylated histone H3, which can be assayed by immunohistochemistry (38). In addition, further activation of the DNA damage response as assayed by increased H2AX phosphorylation at Ser¹³⁹ (γ -H2AX) over that from chemotherapy alone can also be used as a downstream pharmacodynamic marker for Chk1 inhibition.⁴

Conclusions

Over the past several years, an enormous effort has increased our knowledge about how cell cycle checkpoint functions. Chk1 has now emerged as a novel target for combination anticancer therapy. The challenge ahead is to identify genetic determinants in tumors that would predict response to such an approach and to define the optimal clinical settings where such a therapeutic strategy can be applied.

⁴ A.N. Tse and G.K. Schwartz, unpublished data.

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