

DNA Damage Detection and Repair Pathways—Recent Advances with Inhibitors of Checkpoint Kinases in Cancer Therapy

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Abstract Insights from cell cycle research have led to the hypothesis that tumors may be selectively sensitized to DNA-damaging agents, resulting in improved antitumor activity and a wider therapeutic margin. The theory relies primarily on the observation that the majority of tumors are deficient in the G₁-DNA damage checkpoint pathway, resulting in reliance on S and G₂ phase checkpoints for DNA repair and cell survival. The S and G₂ phase checkpoints are predominantly regulated by checkpoint kinase 1; thus, inhibition of checkpoint kinase 1 signaling impairs DNA repair and increases tumor cell death. Normal tissues, however, have a functioning G₁ checkpoint signaling pathway that allows for DNA repair and cell survival. There is now a large body of preclinical evidence showing that checkpoint kinase inhibitors do indeed enhance the efficacy of both conventional chemotherapy and radiotherapy, and several agents have recently entered clinical trials. Excitingly, additional therapeutic opportunities for checkpoint kinase inhibitors continue to emerge as biology outside their pivotal role in cell cycle arrest is further elucidated.

Background

DNA damage, if unrepaired, may result in mutation or cell death. Therefore, cells have evolved complex signaling networks to carefully monitor the integrity of the genome during DNA replication, and to initiate cell cycle arrest, repair, or apoptotic responses if errors are detected. Cancer cells, on the other hand, undergo an array of genetic changes including mutations in the DNA repair pathways that impair these controls and barriers. Over the last decade, there has been a tremendous increase in the understanding of the mechanisms of DNA damage detection, signaling, and repair, and these findings have suggested a variety of therapeutic opportunities for agents that modulate these pathways (1, 2). As members of this network, checkpoint kinases have emerged as exciting targets, and inhibitors from AstraZeneca, Exelixis, Lilly, and Pfizer have recently entered early clinical development. In addition, there are many other checkpoint kinase (Chk)1 inhibitors in the preclinical phase, including compounds from Millenium, Merck, Abbott, Vernalis, and Chiron, so it is highly likely that in the near future, additional compounds will join those already in clinical trials.

Although it is only part of the cellular machinery that recognizes and responds to DNA damage, the signaling cascade that specifically regulates cell cycle arrest after DNA

damage can itself be thought of as a complex network of interconnected pathways consisting of three main components—sensors, signal transducers, and effectors (3–5) as shown in Fig. 1.

DNA damage triggers recruitment of multiprotein complexes (sensors) that then activate the transducers ataxia telangiectasia mutated (ATM) and ATR (ATM and Rad3 related), which belong to the phosphoinositide 3-kinase-like kinase family. It is generally accepted that ATR activation is driven by single strand breaks formed as a result of stalled replication forks, whereas ATM is the main initiator of response to double strand breaks resulting from ionizing radiation and other types of DNA damage (6).

Once activated, ATM and ATR phosphorylate a host of substrates, initiating a cascade that results in cell cycle arrest and DNA repair (7, 8).

Chk1 and Chk2 are checkpoint kinases downstream of ATM and ATR and play a critical role in determining cellular responses to DNA damage. Chk1 is a serine/threonine kinase and is primarily responsible for initiating cell cycle arrest, allowing time for DNA repair and cell survival (9, 10).

After its activation, Chk1 phosphorylates many serine residues on the protein phosphatase Cdc25a, facilitating recognition by ubiquitin ligases. Ubiquitination leads to its proteolysis and, thus, limits its ability to drive progression through the S phase (11–14). Chk1 also phosphorylates Cdc25c, preventing dephosphorylation and activation of CDK1, resulting in cell cycle arrest in the G₂ phase (15–17). Further support for the pivotal role of Chk1 in cell cycle checkpoint control has come from siRNA studies that have shown the importance of Chk1 in regulation of the S, intra-S, and the G₂-M phase checkpoints (11, 18–20).

Activation of Chk2 is initiated by factors that induce single strand breaks, such as replication stress or chemotherapeutic agents. Once activated, the effects of Chk2 on the effector

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proteins Cdc25a, Cdc25c, and p53 are similar to those mediated by Chk 1 (12, 21–23).

Although their effects on downstream pathways share some similarities, inhibition of Chk1 or Chk2 may have profoundly different outcomes. For example, knockout animal studies revealed drastic differences in phenotype between Chk1 and Chk2 null mice. *Chk1* (-/-) mice are embryonic lethal, whereas *Chk2* (-/-) mice are viable and seem normal. However, tissues from *Chk2* -/- mice do show significant defects in G₁-S checkpoint and IR-induced apoptosis (24).

Recently, further insight was gained into the inter-relationship of Chk1 and Chk2 by the generation of conditional mutant mice in which *Chk1* was only deleted in the T lineage (25). It was found that in the absence of Chk1, the transition of CD4⁺CD8⁻ double-negative thymocytes to CD4⁺CD8⁺ double-positive cells was blocked by an increase in apoptosis. The loss of Chk1 resulted in the activation of checkpoint kinase Chk2 in

these thymocytes and conversely the loss of Chk2 resulted in the activation of Chk1. Interestingly, the double knockout mitigated some of the effects of Chk1 deletion. These data suggest that with Chk1 deletion, crosstalk between the pathways leads to Chk2 induced apoptosis (25). Therefore, a dual inhibitor of both Chk1 and Chk2 might provide some protective effects on normal tissues such as thymocytes.

Chk2 has also been shown to be modified in *Chk1lox/-ES* cells expressing an inactive form of Chk1, again supporting crosstalk between the pathways (26). Based on the crosstalk between these pathways, it could be hypothesized that it may be beneficial to target both Chk1 and Chk2 simultaneously to avoid compensatory mechanisms. Any such benefit, however, is likely to be dependent on the genetic background of the tumor because there are conflicting studies demonstrating inhibition of Chk1 and Chk2 has no benefit over inhibition of Chk1 alone (27, 28).

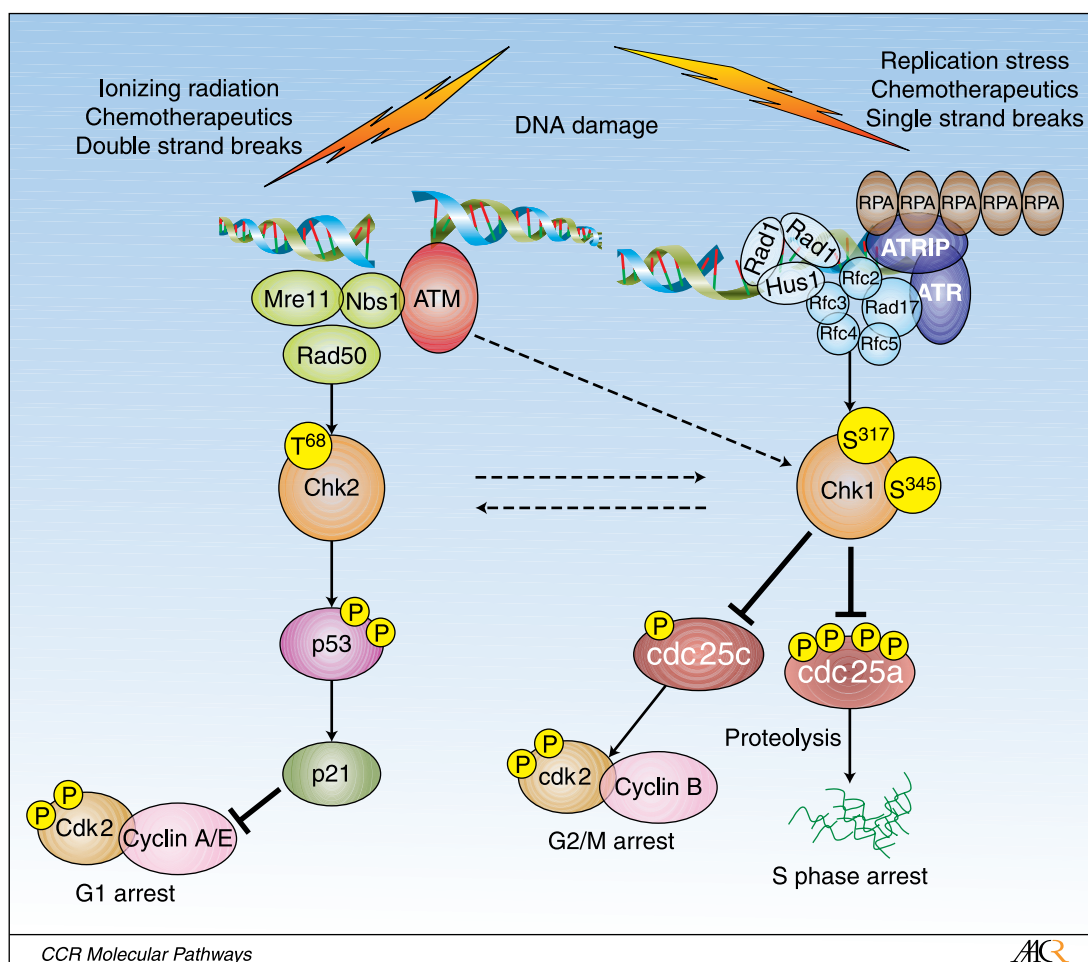


Fig. 1. Chk1 and Chk2 kinases are serine/threonine kinases that are activated by the ATM and ATR kinases in response to DNA damage. The checkpoint kinases are transducers of the DNA damage signal and both phosphorylate a number of substrates involved in the DNA damage response. Chk1 and Chk2 share a number of overlapping substrates, although it is clear that they have distinct roles in directing the response of the cell to DNA damage. Current understanding is that the checkpoint kinases are involved not only in cell cycle regulation but also in other aspects of the cellular response to DNA damage. The G₁ checkpoint is modulated primarily by the ATM-Chk2-p53 pathway, as expression of ATR, Chk1, and Cdc25a is limited until the cell passes this restriction point. At this point, levels of ATR, Chk1, and Cdc25a all increase. If DNA damage is detected, Chk1/Chk2 are activated, Cdc25a is phosphorylated, and thus, destabilized, resulting in a p53-independent S arrest. In S phase, the same cascade can result in an intra-S arrest in response to stalled replication forks. The G₂-M checkpoint prevents entry into mitosis with unrepaired DNA lesions. Initiation of this checkpoint is mediated by the ATM/ATR/Chk1/Chk2 cascades as shown, which ultimately suppresses the promotive activity of cyclin B/cdc2. Along with their pivotal roles in the modulation of the cell cycle checkpoints, Chk1 and Chk2 are also involved in other aspects of the DNA damage response, including DNA repair, induction of apoptosis, and chromatin remodeling.

The phenotypic effects of the pharmacologic modulation of checkpoint pathways were first recognized in early work with caffeine (an inhibitor of ATR and ATM), and with the staurosporine analogue, UCN-01 (KW-2401, NSC 638850). It was shown that these agents could abrogate DNA damage-induced G₂ arrest and selectively sensitize p53 mutant cells to radiation (29–34). When one of the targets of UCN-01 was identified as Chk1 (IC₅₀, 10 nmol/L), it was recognized that Chk1 could be a useful therapeutic target to induce enhanced cytotoxicity in tumor cells in response to DNA damage (35, 36).

It should be noted, however, that UCN-01 also potently inhibits a number of other kinases including Chk2 (IC₅₀, 10 nmol/L) and a number of the cyclin-dependent kinases; thus, the clinical effects of this agent cannot be presumed to predict the effects that will be seen with more specific inhibitors, although it is believed that the potentiation of DNA-damaging agents is primarily driven through the inhibition of Chk1 and, hence, abrogation of cell cycle arrest (32). A number of clinical trials with UCN-01 in combination with a variety of DNA-damaging therapies are still ongoing.

The tumor cell specificity of the sensitization of the effects of DNA damage relies on the observation that the majority of tumors are deficient in the G₁ DNA damage checkpoint pathway or other components of checkpoint signaling and response. For example, high p53 mutation rates result in reliance on S and G₂ phase checkpoints to repair DNA damage and promote cell survival. Therefore, abrogation of these remaining intact checkpoints should lead to enhanced tumor cell death compared with normal tissue. Inhibition of Chk1 signaling using small-molecule inhibitors, dominant negative enzymes, interference RNA, and ribozymes abrogated the S and G₂ checkpoints, impaired DNA repair, and selectively increased tumor cell death. This has provided strong preclinical support for this approach (2, 11, 32–39).

Recent evaluation of checkpoints activated by stalled replication forks showed that, in contrast, nontransformed cells have a more robust DNA replication checkpoint than tumor cells. In nontumor cells, both Chk1-dependent and independent pathways are activated in response to stalled replication forks. Tumor cells, on the other hand, rely entirely on Chk1 activity for a proper response. Thus, Chk1 inhibition enhanced the toxicity of hydroxyurea treatment in tumor cells but not in nontransformed cells as determined by clonogenic assays. This study therefore supports a rationale for tumor-selective effects of combined therapies such as Chk1 inhibition and agents that induce stalled replication forks such as gemcitabine, 5-fluorouracil, and hydroxyurea (40).

Clinical-Translational Advances

DNA-damaging therapies are among the most common cancer treatments and have produced significant increases in the survival of patients, particularly when used in combination with drugs with different mechanisms of action. Due to the efficacy of these anticancer treatments, DNA-damaging agents are likely to remain a standard of care for the treatment of many cancers for the foreseeable future.

Although the induction of DNA damage is an effective approach to tumor control, this mechanism also leads to significant side effects as the majority of these agents are used at the maximum tolerated dose. Toxicities to the hematologic,

gastrointestinal, and other organ systems are commonly observed and limit the dose that can be tolerated and the degree of tumor control that can be achieved. Another limitation of DNA-damaging agents is that many patients develop resistance and therefore become refractory to treatment.

The hypothesis that modulation of the DNA checkpoint pathways offers the potential to sensitize cancer cells to DNA damage induced by chemotherapeutics or radiotherapy, while sparing normal cells was first proposed in the 1980s and has continued to gather support. More specifically, abrogation of cell cycle checkpoint pathways via inhibition of Chk1 and Chk2 has become an increasingly appealing approach to broaden the therapeutic window of commonly used anticancer therapies including alkylating agents, topoisomerase inhibitors, antimetabolites, and radiotherapy.

Recent advances in checkpoint biology have also suggested the potential utility of combining Chk1 inhibitors with antimetabolic agents. Chk1 inhibition sensitized tumor cells to paclitaxel (27). Mechanistic studies followed, which showed that Chk1 plays a critical role in the spindle checkpoint. Chk1-deficient DT40 avian B-lymphoma cells or human BE colon cells were found to fail to undergo mitotic arrest in the presence of Taxol. Mechanistic studies showed Chk1 colocalization with kinetochore proteins, and in the Chk1-depleted cells, failure of critical spindle checkpoints. The spindle checkpoint failure is suggested to be due to lack of activation of aurora B kinase. Proper spindle checkpoint activation was also found to be dependent on the catalytic activity of Chk1 (41). In contrast to studies suggesting that Chk1 is a positive regulator of the spindle checkpoint, there is also a finding that it is a negative regulator of the spindle checkpoint through regulation of polo-like kinase 1 (42). It is not understood whether these differences are due to the different models or techniques to deplete Chk1 that were used, but both studies clearly indicate that Chk1 plays a role in the spindle checkpoint.

As well as a way of potentiating the efficacy of DNA-damaging therapies in sensitive tumors, several recent advances have implicated checkpoint pathway activation as a major mechanism driving both chemoresistance and radioresistance. This may further broaden the clinical utility of such agents. For example, the ATR-Chk1 pathway is strongly activated in BCR/ABL-positive cells, and this was shown to contribute to the resistance of these cells to treatment with DNA crosslinking agents (43). Increased Chk1 activity has also been found to be associated with cellular resistance to Adriamycin in K562/A02 human erythroleukemic cell lines (44). In addition, glioma stem cells, the cell population representing radioresistant tumor cells, were shown to promote radioresistance through activation of the checkpoint pathway, thus targeting the checkpoint response in this setting may overcome radioresistance and improve the therapeutic outcome in malignant brain cancer (45).

New findings have also led to the hypothesis that there may be certain tumor types that are particularly sensitive to Chk1 inhibition. These include triple negative breast cancer where Chk1 has been found to be significantly up-regulated (46), and colorectal cancer where Chk1 was identified as a protein that discriminated between normal and tumor mucosa (47).

In parallel with recent biological advances, there have been many recent developments in the medicinal chemistry of

checkpoint kinase inhibitors, and the area has been the subject of a number of excellent reviews that give a very good overview of the design and preclinical activity of these compounds (48–56). Overall, the data from this class of agents are compelling and consistently support the hypothesis that abrogation of DNA damage–induced checkpoints will potentiate the effects of radiotherapy and chemotherapy as exemplified below using the three Chk1/Chk2 inhibitors that have recently entered clinical trials (XL-844, AZD7762, and PF00477736). All three agents are in phase I trials, and pursuing combination approach (Table 1). All three agents have been extensively profiled preclinically, and the reported profiles unsurprisingly show a number of similarities. XL-844, AZD7762, and PF-00477736 represent different chemical classes from both UCN-01 and each other. They are all potent inhibitors of both Chk1 and Chk2 (Table 1). They have all been shown to abrogate DNA damage–induced cell cycle arrest and to potentiate the effects of DNA damage induced therapies, both *in vitro* and *in vivo* (54, 57–63). The degree of potentiation observed is dependent on the cell line and DNA damage agent used, but in all cases, a robust response is seen in combination with gemcitabine (cell lines and xenograft models), and this is the initial focus for testing the hypothesis clinically.

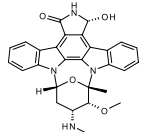
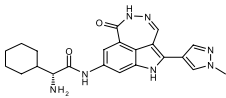
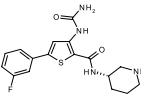
Although they all inhibit Chk1 and Chk2, AZD7762, PF-00477736, and XL844 show a range of potencies against Chk2, with PF-00477736 demonstrating the most selectivity for Chk1 (~100-fold). Interestingly, PF-00477736 has been shown to have somewhat different effects on downstream protein phosphorylations than either XL-844 or AZD7762. For example, both *in vitro* and *in vivo* PF-00477736 combination studies have shown a decrease in Chk1 phosphorylation, in sharp contrast to the increase in Chk phosphorylation observed with other checkpoint kinase inhibitors. However, in all cases, an increase in the levels of phosphohistone 2AX (p-H2AX), a marker of double-stranded DNA damage, is seen (57, 58, 64). It is not yet clear how these differences will affect the clinical

profile of these agents, but recent findings using molecular tools such as shRNA and siRNA suggest that potentiation of the effects of DNA damage is driven primarily through Chk1 inhibition rather than Chk2 inhibition. It has also been hypothesized that Chk2 inhibition may lead to the enhanced effect of sensitizing p53-null cancer cells while protecting normal cells (48). In support of this argument, it has been shown that an ATP-competitive inhibitor of Chk2 protected human CD4(+) and CD8(+) T cells from apoptosis after IR (65). More recently, the selective Chk2 inhibitor VRX0466617 (Chk1: IC₅₀, >10 000 nmol/L; Chk2: Ki, 11 nmol/L) has been described (66), and this together with Chk1-selective compounds such as EXEL-3611 (Chk1: IC₅₀, 2.4 nmol/L; Chk2: IC₅₀, 2,400 nmol/L; ref. 67) may shed further light on the most desirable balance of Chk1/Chk2 inhibition required to achieve the greatest therapeutic effect while minimizing detrimental effects to normal tissue.

Conclusions

This is a complicated but promising area of research that extends far beyond the originally described roles of Chk1 and Chk2 in cell cycle arrest. Although our understanding is expanding rapidly, there are many issues and challenges still to be overcome before the potential of such agents can be fully realized. Combination dose scheduling, biomarker, and patient selection strategies are complex. The p53 hypothesis may provide guidance, but loss of p53 is not the only mechanism by which checkpoint pathways may be compromised in tumor cells. Another area with many unanswered questions is the potential for side effects from checkpoint kinase inhibition in normal tissues, as well as the possibility of exaggerated toxicity from the combination of these agents with existing chemotherapeutics. Chk1 inhibition is known to cause some genetic instability and so, long term, the risk of secondary cancer formation remains to be determined. In addition, as mentioned briefly above, emerging checkpoint biology already suggests

Table 1. Checkpoint kinase inhibitors in clinical trials

Agent	Structure	Chk1 IC ₅₀ (nmol/L)	Chk2 IC ₅₀ (nmol/L)	Combination	Tumor setting	Phase	Status
UCN-01*		10	10	Irinotecan	Breast, gastric, head/neck, NSCL, small cell, ovarian, pancreas, prostate	I	Ongoing
				Cisplatin	Bladder, melanoma, thyroid	I	
				Topotecan	Lung	II	
XL844	Structure undisclosed	2.2	0.2	—	CLL	I	Terminated
PF-00477736		0.49 (Ki)	47 (Ki)	Gemcitabine	Aggressive or indolent NHL, solid tumors	I	Ongoing
				Gemcitabine	Solid tumors	I	Ongoing
AZD7762		5	<10	Gemcitabine	Solid tumors	I	Ongoing
				Irinotecan	Solid tumors	I	

Abbreviations: NSCL, non-small cell lung; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma.

*Nonselective kinase inhibitor with known activity against checkpoint kinases—only ongoing trials in combination with DNA-damaging agents listed.

that there may be additional opportunities for these agents in specific patient populations as in the case of drug resistance. Identification of the optimal clinical settings and those patients most likely to respond are key issues that remain to be addressed.

Overall, the discovery and development of checkpoint kinase inhibitors is an area of intense interest, and is likely to continue grow. The preclinical data thus far provide confidence that Chk inhibitors will have broad utility in combination with many established DNA-damaging therapies across a wide range of tumor types. Hopefully, clinical validation of the potentiation hypothesis will soon be achieved. Ultimately, this approach

could achieve equal or greater efficacy than is possible today, and possibly even with a lower dose of chemotherapeutic agent, thereby greatly improving patient well-being and providing a significant step forward in oncology treatment. Exciting additional opportunities, such as those outlined above in triple negative breast and colorectal cancer, also continue to emerge and offer even further opportunities for development with this class of agent.

Disclosure of Potential Conflicts of Interest

S. Ashwell and S. Zabludoff are employees of AstraZeneca.

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