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Comparison of the different mechanisms of cytotoxicity induced by Checkpoint Kinase I inhibitors when used as single agents or in combination with DNA damage

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Abstract

Inhibition of the DNA damage response is an emerging strategy to treat cancer. Understanding how DNA damage response inhibitors cause cytotoxicity in cancer cells is crucial to their further clinical development. This review focuses on three different mechanisms of cell killing by Checkpoint Kinase I inhibitors (CHK1i). DNA damage induced by chemotherapy drugs such as topoisomerase I inhibitors results in S and G2 phase arrest. Addition of CHK1i promotes cell cycle progression before repair is completed resulting in mitotic catastrophe. Ribonucleotide reductase inhibitors such as gemcitabine also arrest cells in S phase by preventing dNTP synthesis. Addition of CHK1i reactivates the DNA helicase to unwind DNA, but in the absence of dNTPs, this leads to excessive single-strand DNA that exceeds the protective capacity of the single-strand binding protein RPA. Unprotected DNA is subject to nuclease cleavage resulting in replication catastrophe. CHK1i alone also kills a subset of cell lines through MRE11 and MUS81-mediated DNA cleavage in S phase cells. The choice of mechanism depends on the activation state of CDK2. Low level activation of CDK2 mediates helicase activation, cell cycle progression and both replication and mitotic catastrophe. In contrast, high CDK2 activity is required for sensitivity to CHK1i as monotherapy. This high CDK2 activity threshold usually occurs late in the cell cycle to prepare for mitosis, but in CHK1i-sensitive cells, high activity can be attained in early S phase, resulting in DNA cleavage and cell death. This sensitivity to CHK1i has previously been associated with endogenous replication stress, but the dependence on high CDK2 activity, as well as MRE11, contradicts this hypothesis. The major unresolved question is why some cell lines fail to restrain their high CDK2 activity and hence succumb to CHK1i in S phase. Resolving this question will facilitate stratification of patients for treatment with CHK1i as monotherapy.

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Introduction

DNA damaging chemotherapy agents have been used as standard-of-care to treat cancer patients for more than 50 years. Many types of DNA damage directly impede DNA synthesis, activate the DNA damage response, and halt cell cycle progression. A therapeutic window may be provided by the higher rate of replication in cancer cells compared to healthy tissue, albeit this is compromised by the high proliferation rate in some normal tissues. A better therapeutic window may occur for tumors that exhibit defects in DNA damage response and repair pathways. An emerging strategy to improve the efficacy of DNA damaging agents is to combine them with inhibitors of the DNA damage response [1,2]. The general rationale for improved efficacy is simple: inhibiting the DNA damage response re-activates the cell cycle before damage can be repaired, thus posing additional cytotoxic insults during replication or cell division. However, the precise molecular mechanisms by which inhibition of the DNA damage response enhances cytotoxicity of DNA damaging agents have not been fully elucidated. Additionally, inhibitors of the DNA damage response have shown efficacy as single agents in some cell lines, but the underlying causes of single agent sensitivity remain elusive.

A major component of the DNA damage response is checkpoint kinase 1 (CHK1), and numerous CHK1 inhibitors (CHK1i) have entered clinical trials (Table 1) [2]. The earliest CHK1i exhibited poor selectivity and bioavailability. The development of many subsequent inhibitors was terminated for business reasons or due to toxicity, yet whether the toxicity was due to an on-target or off-target effect has yet to be resolved. In April 2019, development of LY2606368 (prexasertib) was terminated, likely due to a high rate of observed toxicity (>90% grade 3/4 neutropenia). The only CHK1i currently undergoing further clinical development is SRA737. It has just completed two phase I trials, one as monotherapy [3], the other in combination with gemcitabine [4] and has the advantage of being orally bioavailable. SRA737's observed toxicities also differ from prexasertib in type and severity suggesting prexasertib's toxicities may have been due to off-target effects. Several inhibitors of ATR, the kinase upstream of CHK1, are also in clinical trials, including 22 trials of AZD6738 either as a single agent or in drug combination [5].

Elucidating the molecular mechanisms by which DNA damaging agents interact with the DNA damage response pathway may help provide rationale for optimal administration schedules of these combination therapies. Identification of patients whose tumors might be uniquely sensitive would be of particular importance. For example, one patient had a complete and durable response to administration of a topoisomerase I inhibitor plus a CHK1i that was attributed to a mutation in a damage response gene, RAD50 [6]. Similarly, defining the underlying mechanisms for hypersensitivity of a subset of cell lines to CHK1i as a single agent, assuming this extrapolates to tumors in patients, would facilitate administering drug to the patients likely to have the greatest benefit.

As will be highlighted in this review, there is a different mechanism of action for CHK1i as a single agent as opposed to its mechanisms in combination with DNA damaging agents. Consequently, a confounding factor in many prior studies is their reliance on cancer cell lines sensitive to checkpoint inhibitors as single agents when investigating mechanistic

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effects of combination therapies. In particular, the osteosarcoma cell line, U2OS, is hypersensitive to CHK1i as a single agent [7,8], yet is frequently used as the primary or sole model in studies investigating DNA damage or replication [8–21]. The use of cell lines hypersensitive to monotherapy CHK1i may not accurately represent how the majority of cell lines respond to combination therapies. In addition, it was reported that U2OS cells exhibit far more γH2AX when incubated with CHK1i than ATRi [22], and several subsequent studies were predicated on this observation [8]. However, the original study lacked a rationale for the concentration of drugs that were compared. We find that U2OS cells are about 4-fold more sensitive to CHK1i (MK-8776) than ATRi (AZD6738), and exhibit γH2AX at a similar 4-fold different concentration (unpublished observations). Furthermore, we find that far more cell lines are sensitive to ATRi than CHK1i, and conclude, in contrast to the above publications, that ATRi is deleterious to many more cell lines than CHK1i. Investigations into the different mechanisms of action of CHK1i as monotherapy, in combination with drugs that directly damage DNA, and in combinations with ribonucleotide reductase inhibitors that prevent dNTP synthesis are discussed in this review.

The DNA damage response arrests cell cycle progression

DNA damaging agents activate a protective DNA damage response, which is controlled through three major regulatory kinases: ATM, ATR, and DNA-PK (Fig. 1) [2,23]. The MRE11-RAD50-NBS1 (MRN) and KU70/80 complexes both sense double-strand DNA breaks (DSB). The MRN complex activates ATM in S and G2 phase leading to DNA repair by homologous recombination, while KU70/80 activates DNA-PK in G1 phase resulting in non-homologous end joining. ATR is primarily activated by single-strand DNA (ssDNA) caused by replication stress. Replication protein A (RPA) rapidly binds ssDNA which serves as a platform for TopBP1 and ATRIP to activate ATR. In addition, resection of DNA at DSB leads to ssDNA that activates ATR.

Regardless of the source of DNA damage, CHK1 is a critical effector of the intra S and G2/M checkpoints (Fig. 1). CHK1 is activated by ATR-mediated phosphorylation on serines 317 and 345 [24]. Active CHK1 inhibits the CDC25 family of phosphatases to prevent activation of cyclin-dependent kinase 1 and 2 (CDK1 and CDK2). CDK1 and CDK2 are highly conserved master regulators of cell cycle progression in eukaryotes: CDK2 promotes S phase entry and progression, while CDK1 promotes mitotic entry (Fig. 1) [25,26]. The inhibitory kinase, WEE1, phosphorylates tyrosine 15 (Y15) on both CDK1 and CDK2 to prevent their activity at an inappropriate phase of the cell cycle. CDC25 phosphatases counteract Y15 phosphorylation to promote CDK activity and cell cycle progression. Thus, DNA-damage induced signaling through ATR-CHK1 prevents cells from replicating on damaged DNA, or undergoing mitosis before repair is complete.

Inhibition of the DNA damage response elicited by direct DNA damaging agents

CHK1i have been demonstrated to abrogate S and G2 phase arrest from many sources of DNA damage including, alkylating agents, interstrand cross-links, ionizing radiation and topoisomerase poisons [27]. As a representative of this class of damaging agents, we and

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others have recently dissected the mechanism of CHK1i-mediated sensitization to the topoisomerase I inhibitor, SN38 [6,28–31]. Topoisomerase I relieves torsional strain in the DNA back bone during normal replication by creating a nick that allows DNA to unwind [32]. Small molecule inhibitors trap topoisomerase I on DNA, replication forks collide with the trapped protein, creating a DSB (Fig. 1). These DSB activate the DNA damage response thus accumulating cells in S or G2 phase depending on the concentration of inhibitor used [28,29,31,33].

To dissect the impact of CHK1i, we arrested cells in S phase with SN38. Addition of CHK1i reinitiated DNA synthesis in a CDK2- and CDC7-dependent manner and forced cells to progress to G2 and M prior to repairing damage (Fig. 2) [28,29,31,33,34]. Interestingly, CHK1i did not activate CDK1 until the cells reached G2, at which time they rapidly rounded up and entered mitosis. However, the majority of cells replated after a few hours with fragmented nuclei, suggesting failed cytokinesis [31]. These multinucleated cells persisted for several days suggesting this is a survival mechanism, at least transiently, but eventually most of these cells died.

Inhibition of WEE1 also caused S phase progression and aberrant mitosis almost immediately after the cells reached G2, but few cells replated thereafter, and they died more rapidly than the combination with CHK1i [31]. Interestingly, completion of S phase is not required for WEE1i to induce premature mitosis. Concurrent inhibition of CDC7 and WEE1 prevented abrogation of S phase arrest, but the cells still underwent aberrant mitosis within 6 h. This is consistent with more direct activation of CDK1 by WEE1i compared to CHK1i. Aberrant CDK1 activation in S phase could further enhance cytotoxicity by recruiting MUS81-SLX4 to under replicated sections of chromatin [35]; following WEE1i, MUS81-SLX4 association promoted premature mitotic entry as well as increased DSB during mitosis.

The order of administering inhibitors of topoisomerase I and CHK1 impacts efficacy. Either concurrent inhibition for 24 h or staggering topotecan then PF477736 both synergistically inhibited ovarian cancer cell growth *in vitro* whereas PF477736 prior to topotecan did not [29]. This is consistent with the role of checkpoint inhibition causing premature S phase progression and mitotic entry before repair of DNA damage. Similarly, in the clinical trial of topotecan plus the CHK1i AZD7762 mentioned above, in which there was one long term survivor, both drugs were administered on day 1 and 8 [6].

Inhibition of the DNA damage response elicited by ribonucleotide reductase inhibitors

Many pre-clinical studies have focused on CHK1i combinations with the ribonucleotide reductase inhibitors hydroxyurea and gemcitabine [1,2,36], while the majority of clinical combinations have involved CHK1i plus gemcitabine (Table 1). Gemcitabine is incorporated into DNA where it terminates replication, but pre-clinical and clinical evidence suggests that inhibition of ribonucleotide reductase is the primary cytotoxic mechanism of gemcitabine [37,38]. For example, overexpression of ribonucleotide reductase subunit 1 conferred resistance in cell lines, xenografts, and human patients [39–41]. Additionally, CHK1i does

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not abrogate S phase arrest caused by gemcitabine, as would be expected if the primary mechanism was a simple replication block as observed with SN38 [42]. Hence, we conclude the critical mechanism of gemcitabine action is to inhibit ribonucleotide reductase.

Regardless of the mechanism of stalled replication, antimetabolites uncouple the DNA polymerase from its helicase complex which can then continue unwinding DNA to generate regions of ssDNA (Fig. 1). Recruitment of RPA and TopBP1 to ssDNA activates ATR that, through activation of CHK1, prevents firing of dormant origins. Another consequence of ATR activation is fork regression in a FBH1- and SMARCAL1-dependent manner [10,11]. By 16 h following addition of hydroxyurea, the replication machinery dissociates from stalled replication forks [43]. Similarly, RAD51 foci are induced 16 h following gemcitabine suggesting initiation of homologous recombination [44]. However, if cells lack dNTPs, the attempt to undergo homologous recombination is futile. By 36 h, MUS81 cleaves unresolved holiday junctions, forming DSBs [11,12]. Cells are able to avoid DSBs and restart replication if dNTP levels recover [13]. Inhibiting CHK1 18 h after gemcitabine, but not concurrently, re-activates the DNA helicase in a CDC7- and CDK2- dependent manner; this presumably occurs at dormant origins (Fig 2.) [42]. However, this increased helicase activity occurs in the continued absence of dNTPs and results in excessive ssDNA to which RPA binds. When the level of ssDNA exceeds the capacity of RPA to protect it, nucleases then degrade the ssDNA in a process that has been termed replication catastrophe [9].

Dissection of this pathway led to the realization that the schedule of drug administration is important for maximal cell killing. To approximate a schedule that is clinically relevant, we used a nominal 6 h incubation of cells with gemcitabine. This reflects the fact that gemcitabine is generally administered to patients as a short infusion (30 min). Continuous incubation with gemcitabine as often used in cell culture has little relevance as there is no potential for recovery of the cells [45]. Administering MK-8776 18 h after gemcitabine produced the most robust sensitization of cancer cells to gemcitabine *in vitro* and in xenograft studies [42,44,46]. Similar results were obtained in xenograft models treated with gemcitabine plus either LY2603618 or SRA737 administered at 24 h [47,48]. This delayed administration schedule has been adopted in many of the clinical trials highlighted in Table 1.

Two events explain why delayed, but not concurrent, CHK1i sensitizes cancer cells to gemcitabine or hydroxyurea. First, more cells are arrested in S phase at 18 h, and second, DNA replication machinery dissociates from stalled replication forks at delayed time points [42,43]. An increase in the proportion of S phase cells makes more cells reliant on CHK1 to keep replication suppressed. However, this only partially explains the delay as concurrent CHK1i and gemcitabine failed to activate DNA helicases even in the cells that were already in S phase. Dissociation of replication machinery that occurs after a prolonged arrest permanently stops replication at stalled forks, because new origins are only licensed during G1 phase. After the replication machinery dissociates at stalled forks, the only way a cell can finish replication is to fire dormant origins. This is consistent with findings that cells restart replication at stalled forks when released into fresh media after 1–2 h of hydroxyurea treatment (because ribonucleotide reductase inhibition by hydroxyurea is rapidly reversible), but at dormant origins during recovery from 24 h of treatment with hydroxyurea [13].

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Furthermore, the helicase co-factor CDC45 is limiting for origin firing [49,50]. Therefore, we conclude that dissociation of CDC45 or other factors from stalled forks is required before dormant origins can fire.

Several reports suggest that the mechanism of CHK1i-mediated sensitization to gemcitabine is due to premature mitotic entry [51–54]. However, these studies used low concentrations of gemcitabine, which failed to durably arrest cells in S phase; CHK1i caused gemcitabine-treated cells to enter mitosis only after reaching G2 phase [51,53]. The relevance of this model is questionable as gemcitabine arrests cancer cells in S phase for at least 48 h following a bolus treatment in xenograft models, and for at least 24 h in bladder cancer patients at the standard-of-care dose [46]. Furthermore, while CHK1i can force gemcitabine-treated cells to prematurely enter mitosis from S phase, this is highly cell line dependent [42] and is not required for cytotoxicity; neither CDK1 inhibition nor knock down of cyclin B reduced CHK1i-mediated DNA damage following gemcitabine [42,55]. We conclude that the clinically relevant mechanism of sensitization to this drug combination involves CHK1i-mediated firing of dormant origins of replication in the absence of dNTPs, induction of excess ssDNA, exhaustion of protective RPA, nuclease degradation and replication catastrophe.

Mechanism of action of CHK1 inhibitors as monotherapy

Several reports have demonstrated that a few cancer cell lines are very sensitive to CHK1i as a single agent. Most of these studies used prexasertib but reported a different percent of cell lines that are sensitive. For example, 20% of head and neck tumors were deemed sensitive to prexasertib, based on a threshold of 10 nM [56]. Alternately, the majority of small cell lung cancer lines were sensitive to prexasertib based on a threshold of 300 nM [57]. In colorectal cells, 33% were deemed sensitive to 100 nM prexasertib [58]. Interestingly, all 25 pediatric cancer cell lines tested responded to prexasertib with an EC50 in the range of 0.9–22 nM [59]. While MK-8776 is less potent in cells, we reported that 15% of cell lines from multiple tumors types are sensitive at <1 μM and after only a 24 h incubation [7]. MK-8776 still inhibited CHK1 in the resistant cells thereby ruling out uptake or metabolism as a mechanism of resistance. Hence, despite the difference between these studies, they all agree that a subset of cancer cell lines exhibit hypersensitivity to CHK1i as a single agent, and treatment of various xenografts has established this efficacy can be translated to *in vivo* models. In addition, when assessed, non-transformed cells were deemed resistant to CHK1i [56,60]. This is important because it suggests a large therapeutic window might be achieved in appropriately selected patients.

Cytotoxicity required a longer exposure time of cells to CHK1i, as well as a higher concentration, than when combined with gemcitabine [46]. The cytotoxic activity of CHK1i as a single agent occurs in S phase cells, so a longer incubation is required to impact those cells that have to progress to S phase before succumbing. Consequently, animal and clinical studies have used repeated administration over 3 days or continuous daily treatment.

The generally accepted explanation for sensitivity to CHK1i as a single agent is that the cells are under endogenous replication stress, and a similar argument has been made regarding

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sensitivity to ATRi [27,61,62]. Replication stress has been defined as slowing or stalling of replication fork progression usually resulting in stretches of ssDNA [63]. Endogenous replication stress is often observed as high constitutive activation of the DNA damage response pathway or as elevated phosphorylation of RPA and H2AX. Replication stress is often equated to oncogene-induced stress, with tumors over-expressing MYC or cyclin E, possessing oncogenic RAS activity, or deficient in ATM or p53, reported to be sensitive to CHK1i or ATRi [22,64–67], but as discussed below, these dysfunctions do not correlate with sensitivity to CHK1i as a single agent. In the case of cyclin E-induced replication stress, evidence was obtained after transfection of an exogenous cyclin E gene into U2OS cells [68]. This suggests that U2OS cells do not have constitutive endogenous replication stress, yet they are already one of the cell lines most sensitive to CHK1i. Hence, this experiment appears to contradict the requirement for endogenous replication stress in sensitivity to CHK1i.

A key function of both ATR and CHK1 is to suppress origin firing in the face of replication stress [18,20,69]. In addition, ATR-CHK1 promote transcription of the ribonucleotide reductase subunit M2 (RRM2) by phosphorylating the transcription factors E2F1 or E2F3 [8,70,71]. The addition of ATRi or CHK1i results in firing of excess origins of replication leading to rapid utilization of dNTPs with concomitant slowing of each replication fork [8,70]. In addition, ATRi and CHK1i decrease the transcription of RRM2. Finally, several reports have demonstrated rapid degradation of RRM2 protein upon incubation with ATRi and CHK1i (and WEE1i) as a result of CDK2/cyclin A- or cyclin B/CDK1-mediated phosphorylation of thre-33 on RRM2 [72,73]. Hence, decreased transcription and increased degradation of RRM2, together with firing of dormant origins, conspire to deplete dNTPs leading to excess ssDNA and replication catastrophe.

How might endogenous replication stress enhance sensitivity to CHK1i and ATRi? It is generally believed that oncogenes drive cells into S phase before they have adequate RRM2 or dNTP pools, and that they have a decreased ability to limit the number of replicons that fire. Presumably, this decrease in RRM2 and dNTPs means the cells are closer to a critical threshold for replication catastrophe. However, it has been reported that sensitivity to CHK1i is not predicated on the existence of endogenous replication stress, although sensitivity to ATRi may be [8]. Below, we will discuss other reasons why the replication stress hypothesis for sensitivity to CHK1i has to be questioned.

The critical role of CDK2 activity thresholds in sensitivity to CHK1 inhibitors

If sensitivity to both CHK1i and ATRi is due to exacerbation of endogenous replication stress, then it would suggest that sensitivity to these agents should occur in the same cell lines, yet we find a very poor correlation. While cells sensitive to CHK1i are also sensitive to ATRi, there are many additional cell lines sensitive to ATRi [7] [and unpublished observations]. Oncogene-associated replication stress [22,66] and loss of DNA damage response proteins [64,65,74,75] have been reported to sensitize cells to ATRi, but again these defects do not correlate with sensitivity to CHK1i. Another concern for the replication stress

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hypothesis is that cells would be expected to respond to endogenous stress in a manner similar to exogenous stress such as gemcitabine treatment, yet there are major differences in the reliance of monotherapy on CDK2 and MRE11 as discussed here. While replication stress might enhance sensitivity to CHK1i, we propose that there is another mechanism that results in much greater sensitivity to CHK1i due to aberrant hyper-activation of CDK2.

In CHK1i-sensitive cells, an increase in phospho-RPA32, γH2AX and DSB is observed in S phase cells within 6 h of addition of CHK1i [7,18,76]. These events correlate with CHK1i-mediated accumulation of CDC25A that in turn activates both CDK2/cyclin E and CDK2/cyclin A, the latter of which promotes MRE11-dependent ssDNA and MUS81-dependent DSB (Fig. 2) [7,17,76]. While MRE11-mediated resection of DNA usually occurs following formation of DSB, recent experiments support the hypothesis that MRE11-mediated degradation of nascent DNA strands can precede MUS81-induced DSB [77].

A discernible difference between sensitive and resistant cell lines is the robust increase in CDC25A protein following CHK1i which leads to the increase in CDK2 activity [7]. Consequently, we and others conclude that differential activation of CDC25A and CDK2 is a critical determinant of sensitivity to CHK1i as a single agent [7,21,78]. This observation led to a conundrum in that only a few cell lines are sensitive to CHK1i as a single agent, but all cell lines appear sensitive to CHK1i when used in combination with SN38 or gemcitabine. How can both mechanisms of cytotoxicity require CHK1i-mediated activation of CDK2? The answer is that CDK2 exhibits different activity thresholds for different substrates that are required for single agent activity versus S phase progression. It appears that a high CDK2 activity threshold can be achieved prematurely in S phase only in the cells sensitive to CHK1i.

To begin to explain this concept, we need to consider prior difficulties in resolving CDK2 from CDK1 [25]. One of the most commonly used markers of activation of CDK1 or 2 is disappearance of the inhibitory phospho-Y15. However, this tyrosine residue resides within a highly conserved amino acid sequence in CDK1 and CDK2 that antibodies can not distinguish. Thus, a decrease in pY15 is not informative for either CDK1 or CDK2 specific activity. Unfortunately, many reports have used these antibodies as if they are selective for one or other phospho-CDK.

Advances in fluorescent western blot strategies have enabled separation and quantification of this phospho site [31,42]. An important issue is that CDK2 is approximately one-tenth the level of CDK1 and may therefore be missed [79]. Fluorescent detection provides much more focused bands and greater dynamic range and readily detects the lower signal of CDK2 and its phosphorylated form. Using this strategy, we demonstrated that CHK1i was much more effective at decreasing pY15-CDK2- than pY15-CDK1 [31,42]. In contrast, WEE1i caused rapid dephosphorylation of both kinases [31].

Many substrates of CDK2 have also been implicated as CDK1 substrates (and vice versa) so may not provide a clear resolution of the specific CDK activity. One robust reporter of CDK2-specific activity is the expression level of its binding partner, cyclin E [25]. Following DNA damage, CHK1/CDC25A suppress CDK2 activity resulting in accumulation

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of cyclin E protein [31,42,80]. Upon addition of CHK1i, CDK2 is activated and, in concert with GSK3, leads to phosphorylation and proteasomal degradation of cyclin E [14]. We have observed that cyclin E is preferentially degraded in cells sensitive to CHK1i consistent with activation of CDK2 [7].

Unfortunately, there are no small molecule inhibitors specific to CDK2. CVT-313 appears to be the most selective for CDK2, but also inhibits CDK5 at roughly 2-fold higher concentrations, and CDK1 at 10-fold higher concentrations in cell-free extracts [81,82]. RO-3306 is commonly used as a selective inhibitor of CDK1, with a 10-fold greater affinity for CDK1 than CDK2 in cell free assays [82,83], although it has been reported that RO-3306 inhibits both CDK1-dependent mitotic entry and CDK2-dependent cytotoxicity from CHK1i monotherapy with similar potency in cells [7]. Many other less selective CDK inhibitors have also been used to draw conclusions about CDK-mediated biology [25,82,84].

A major advance in understanding CDK2 activity occurred as a result of titrating CVT-313 over a large range of concentrations. At low concentrations (<2.5 μM), CVT-313 effectively protected against CHK1i-mediated γH2AX as a single agent, and rescued sensitive cells from cytotoxicity [7,31]. At 10 μM, CVT-313 induced G2/M arrest consistent with its inhibition of CDK1. Initially, it was surprising that CVT-313 did not prevent CHK1i-mediated abrogation of S phase arrest induced by SN38, nor did it effectively prevent the degradation of cyclin E, both events believed to depend on CDK2 activity. This was resolved using significantly higher concentrations of CVT-313 (40 μM) which prevented recruitment of the helicase co-factor, CDC45, to DNA following SN38 or gemcitabine treatment [31,42]. Degradation of cyclin E protein was also fully inhibited by 80 μM CVT-313 [31,42].

These novel observations in human cancer cells exhibit a striking resemblance to experiments using a monomolecular CDK/cyclin module in *S. pombe* [85,86]. As the fission yeast cells pass through S and G2, different CDK substrates are phosphorylated as a result of the steadily increasing CDK activity. It was proposed that differential phosphorylation of CDK substrates temporally controls S phase progression and mitotic entry. These phosphorylation events could also be discriminated using a CDK inhibitor whereby low concentrations were sufficient to reduce CDK activity below the threshold required for late phosphorylations, whereas up to 1000-fold higher concentrations were required to suppress CDK activity below the threshold for low activity substrates [85]. Hence, the term "high activity threshold" was coined for CDK activity levels that are required to phosphorylate substrates late in the cell cycle, and "low activity threshold" for levels required early in S phase.

In a similar manner to yeast, CDK2 activity increases as human cells progress through S phase [87]. The CHK1i-mediated abrogation of S phase arrest is achieved at a low activity threshold of CDK2 (inhibited at a high concentration of CDK2i), while the monotherapy action of CHK1i requires a high activity threshold (inhibited at a low concentration of CDK2i). Consequently, the mechanism underlying the CHK1i monotherapy action can be distinguished from its mechanism in drug combination, providing evidence that these are two distinct mechanisms to induce cytotoxicity.

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To our knowledge, this is the first evidence of differential phosphorylation of CDK substrates at different kinase activity levels in human cells. However, the specific substrates responsible for CDK2-mediated effects following CHK1i remain unknown. Candidate proteins can be detected using an antibody directed to the consensus sequence of CDK1/2 substrates (e.g., pTPXK). Incubating cells with CHK1i following gemcitabine significantly increased phosphorylation of numerous CDK2 substrates [42]. Inhibiting CHK1 concurrently with a titration of CVT-313 demonstrated that different substrates exhibited drastically different IC50 values for CVT-313. Potential candidates include Treslin, responsible for loading CDC45 on to DNA [15,16], and hence requiring only low CDK2 activity. In contrast, phosphorylation of CtIP, an activator of MRE11 [88], and RRM2, that is degraded upon phosphorylation thereby limiting dNTP production, would require high CDK2 activity levels if they are involved in sensitivity to CHK1i monotherapy.

The underlying reason why only a small percentage of cancer cell lines hyperactivate CDK2 in response to CHK1i remains unresolved. While CHK1-mediated inhibition of CDC25A is generally considered critical for inhibiting CDK2, the literature provides several alternate mechanisms that might contribute to the differential regulation of CDK2 (Fig. 3). For example, there are alternative kinases that may suppress CDC25A including CHK2 [19] and BRSK (SAD) kinases [89]. Alternately, MK2 may regulate CDC25B in a manner that complements inhibition of CDC25A [90]. Inhibition of CDK2/cyclin binding has also been associated with components of the Hippo pathway [91]. Direct inhibition of CDK2 activity by CDKN2A/B may also be involved [56]. Several of these pathways are activated by ATR, hence may explain why many more cell lines are sensitive to ATRi.

The affinity of counteracting phosphatases acting on CDK substrates is another factor that may impact the phosphorylation level of different substrate. $PP2A^{Cdc55}$ preferentially dephosphorylates threonine substrates of CDK in yeast during early S phase [92]; hence it may require higher CDK activity to counteract the phosphatase activity on these substrates, thus contributing to the temporal regulation of cell cycle (the human homolog is $PP2A^{B55}$). Conversely, the CDC14 phosphatases specifically counteract CDK-mediated phosphorylation of a limited number of substrates in budding yeast, but may only be transiently activated during early anaphase [93,94]. The role of these phosphatases in CDK2 activity following CHK1i remains unclear. Better understanding of the regulation of CDK2 activity and the phosphorylation of its substrates in cancer cells is critical to identifying patients that may respond to single-agent treatment of CHK1i.

From CDK2 activity to cytotoxicity

While aberrant activation of CDK2 in S phase may be necessary for sensitivity to CHK1i, it may not be sufficient as steps downstream of CDK2 may also determine the outcome of drug treatment. The events downstream of active CDK2 are very reminiscent of the replication catastrophe induced by the combination of gemcitabine plus CHK1i, for example extensive phosphorylation of RPA and H2AX, but the origin of these events appear different. First, as discussed above, phosphorylation of RPA is inhibited by low concentrations of CVT-313 following CHK1i as a single agent, but it requires much higher concentrations of CVT-313 to inhibit the replication catastrophe induced by gemcitabine. This suggests that

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the single agent activity of CHK1i is not dependent on firing of dormant origins of replication. Second, replication catastrophe in CHK1i-sensitive cells requires MRE11, but it is not required when CHK1i is combined with gemcitabine [42]. Similarly, cells lacking MRE11 protein failed to create ssDNA in response to CHK1i, but exogenous re-expression of MRE11 reverted the phenotype [76]. Hence, events that modify the activity of MRE11 may also impact sensitivity to CHK1i.

There are several links between CHK1 and MRE11. In particular, CHK1-mediated phosphorylation of BRCA2 and RAD51 recruits RAD51 to DNA, and this protects DNA from resection by MRE11 [95,96]. In contrast, CDK2-mediated phosphorylation of BRCA2 dissociates RAD51 from DNA, although this normally occurs late in the cell cycle to terminate homologous recombination and permit mitosis [97]. If CHK1 is inhibited in early S phase, the protection afforded by BRCA2 and RAD51 is lost, and MRE11 can degrade the DNA. This predisposes that replication fork reversal or homologous recombination occurs continuously during normal replication, thereby requiring the protection afforded by BRCA2/RAD51, or that CHK1i-sensitive cells have a much higher level of constitutive fork reversal. While MRE11 is not a direct target of CDK2, its binding partner CtIP is [88], and this could contribute to the mechanism by which aberrant CDK2 activity leads to MRE11-dependent DNA degradation.

Defects in MRE11 are fairly common, often due to mRNA missplicing [98,99]. In our cell panel, we note that ATLD cells (defective for MRE11) are somewhat more sensitive to CHK1i and ATRi when complemented with MRE11 [76] and unpublished observations]. Other proteins may also regulate MRE11 (Fig. 3); for example, both Fanconi anemia group J helicase and DYNLL1 can suppresses MRE11 activity [100,101]. An alternate means to induce hyper-resection of DNA is through activation of EXO1 which can also be activated by CDK2; ATR suppresses this hyper-resection of DNA by degrading EXO1 [102]. Finally, differences in repairability of DNA breaks might influence the outcome of incubation with CHK1i.

Future Directions

Identifying robust predictive biomarkers is critical to the future development of CHK1i. This is exemplified by the patient whose tumor had a defect in RAD50 and was highly responsive to the combination of irinotecan plus AZD7762 [6]. This suggests that additional defects in the MRN complex, or other proteins in this pathway may confer sensitivity to this combination. Unfortunately, this is a unique case and the numerous other molecular mechanisms described here would likely require predictive biomarkers specific to each therapeutic strategy. Furthermore, it is intriguing that defects in MRE11 appear to have the opposite effect in conferring resistance to CHK1i monotherapy due to its essential function in cytotoxicity [76]. At the current time, it is unknown whether any mechanism may predominate thereby making patient stratification easier. To reiterate, the differential response does not appear to be due to differential uptake or metabolism as the CHK1i still inhibit their target in resistant cells. This is best exemplified by the fact that cells resistant to CHK1i monotherapy are still very sensitive to CHK1i when used in combination with gemcitabine. Our current results suggest that the majority of predictors of sensitivity to

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CHK1i as a single agent are likely to be upstream of CDK2, although defects in downstream proteins such as MRE11 clearly provide an important factor to be considered.

Many possible contributors to response are highlighted in Figure 4. The potential role of each factor could be established through the use of small molecule inhibitors (when available), siRNA, knock-out or knock-in strategies, or through the use of inducible constructs. An alternative approach is to treat patients with drug, hoping to identify those with an outlier response, and then perform genomic and/or proteomic analysis to identify the predictors of response (as performed for the RAD50 case discussed above). This predisposes that the administered drug schedule is optimal for response. This approach might also be limited as it requires that responders be found before the drug development is terminated for lack of efficacy.

Until determinants are found that predict sensitive tumors, an alternate approach could be to administer drug to tumor samples from each patient ex vivo for indicators of response; for example, phosphorylation of CDK2 substrates and H2AX could be assessed in tumor slices. These events could be assessed in a first cycle of drug administration, which would then be continued if predicted markers are observed. As the percentage of sensitive tumors appears to be relatively low, this approach may also require analysis of a large number of tumors and would not be applicable to routine clinical management.

Although phosphorylation and chromatin binding of RPA32 is of particular interest as a marker for characterizing the extent of replication catastrophe, it relies on depletion of soluble RPA which would be extremely difficult to assess in patient samples. One common approach in cell culture has been the incorporation of BrdU into DNA, then assessing the amount of BrdU in single-strand DNA, yet this would not be possible in clinical samples. A potential alternative may be to use a commercially available antibody to single-strand DNA but whether this has the sensitivity to detect the excess single-strand DNA associated with replication catastrophe has not been evaluated.

This review has emphasized the importance of CDK2 activity thresholds that discriminate sensitivity to CHK1i as a single agent versus in combination. Further dissection of the critical CDK2 substrates is required. The high activity threshold for CDK2 must be attained for mitosis [85], and whether the high CDK2 activity required for CHK1i monotherapy activity is simply premature phosphorylation of mitotic proteins remains to be established, albeit cells exhibit extensive γH2AX without concomitant phospho-histone H3, a common marker for mitosis. We have also reported the essential role of cyclin A in CHK1i-mediated toxicity [7], suggesting that the critical activity may be specifically attributed to cyclin A/ CDK2 complexes, and hence may relate to differential regulation of cyclin A. Ongoing studies are addressing these possibilities.

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Competing interests

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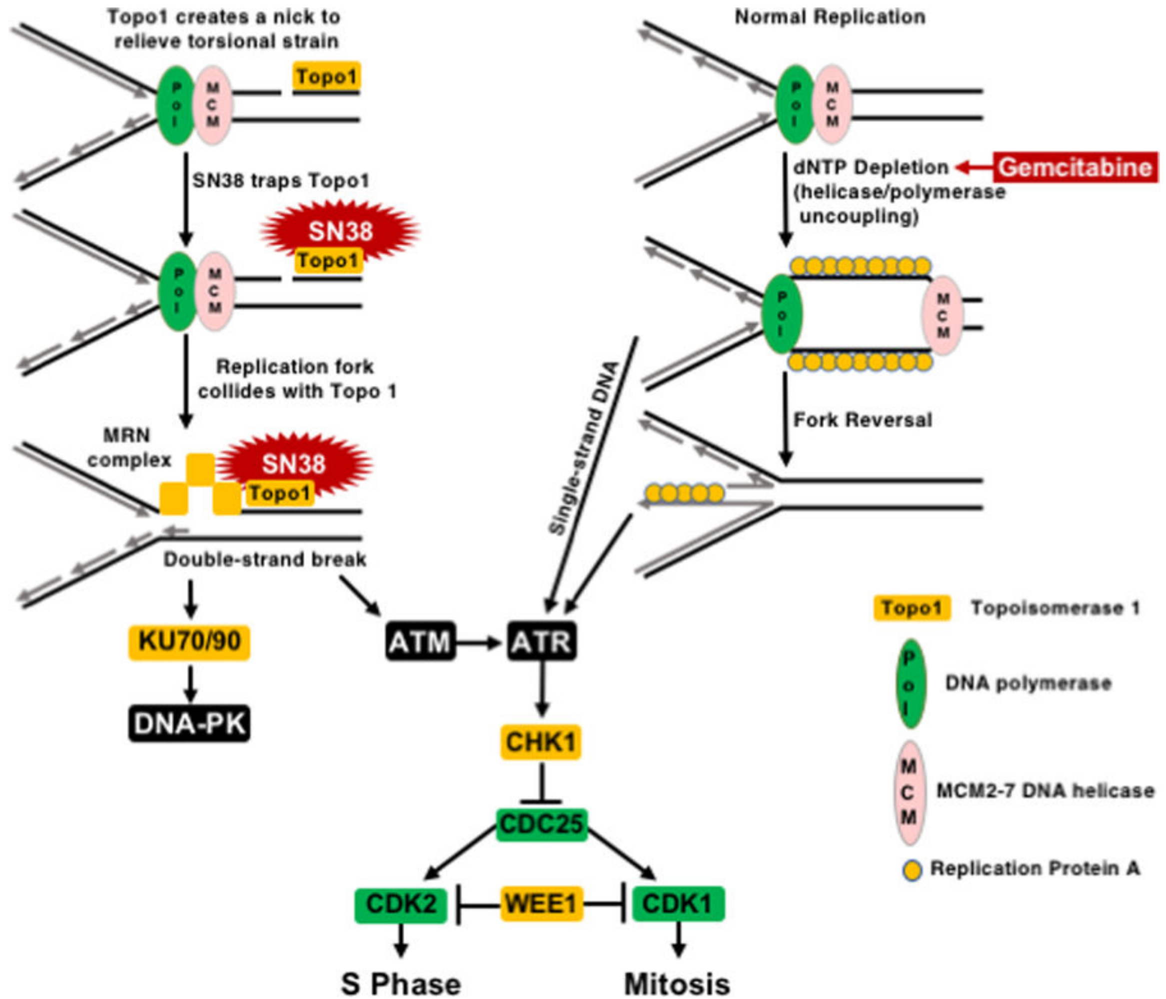

Figure 1. SN38 and gemcitabine arrest cell cycle progression by activating the DNA damage response.

(Left) Topoisomerase I creates a nick in the DNA backbone to relieve torsional strain. SN38 traps topoisomerase I on the DNA. As the replication machinery collides with topoisomerase I, a double-stranded break is formed, thus activating the DNA damage response through the MRN complex and ATM. *(Right)* Gemcitabine depletes dNTPs in cells by inhibiting ribonucleotide reductase, which stalls the DNA polymerase while the helicase continues unwinding DNA. Replication protein A binds exposed ssDNA to activate ATR and stalled replication forks. ATR activates CHK1 to arrest the cell cycle by inhibiting CDC25 phosphatases and downstream CDK1 and CDK2.

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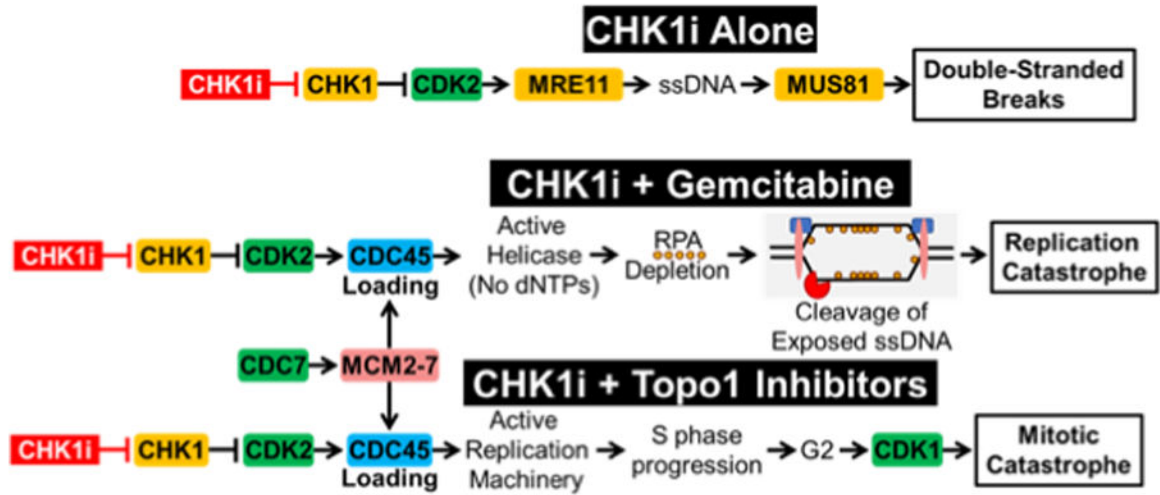

Figure 2. The molecular mechanisms of CHK1i.

In a small subset of cells, CHK1i alone stabilizes CDC25A protein to activate CDK2. High CDK2 activity activates MRE11 nuclease to create ssDNA, and subsequent MUS81-dependent double-stranded breaks. In gemcitabine-arrested cells, CHK1i re-activates the DNA helicase to unwind DNA in the absence of dNTPs. This overcomes the ability to protect ssDNA and results in DNA cleavage. In SN38-arrested cells, CHK1i restarts DNA synthesis and cells are forced into mitosis prior to repairing SN38-mediated damage.

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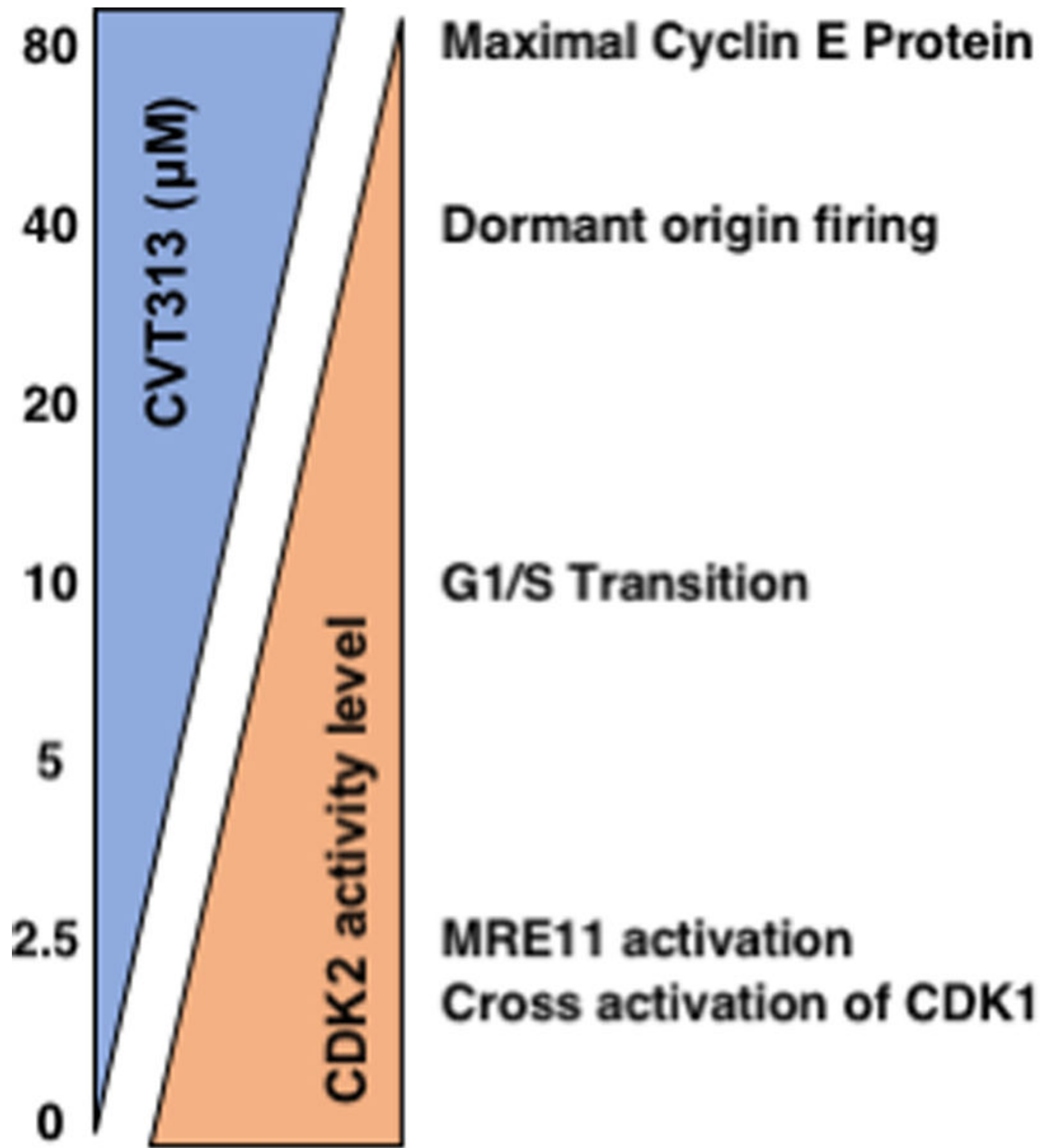

Figure 3. Effects of different levels of CDK2 activity.

CDK2 activity levels are inversely correlated with the concentration of CVT-313 that is required to inhibit the observed effects.

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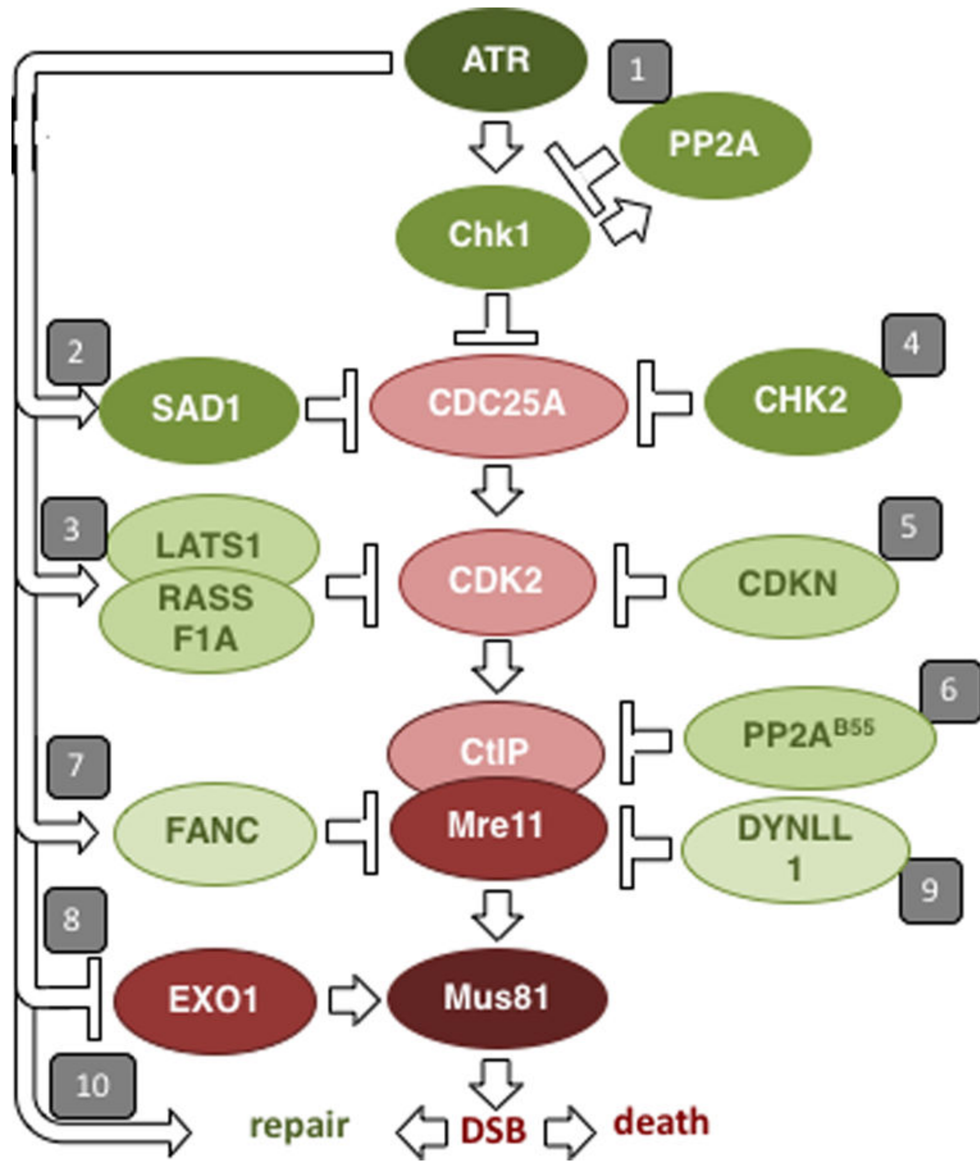

Figure 4. Potential regulatory mechanisms for sensitivity to CHK1i monotherapy.
Mechanisms #1–5 represent pathways suppressing CDK2 activity, while the phosphatase (#6) can reverse phosphorylation of CDK2 substrates. Mechanisms #7–9 impact the activity of downstream nucleases that may degrade DNA. Finally, #10 recognizes that differential DNA repair may influence the outcome of all the upstream events. Green reflects potential protective pathways. Red reflects potential cytotoxic pathways.

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Table 1.

CHK1 inhibitors that have undergone clinical development.

Name	Stage	Year of 1st Trial	Clinical Combinations	Half-Life	References
UCN-01	Phase II; discontinued-α1-acid glycoprotein binding caused poor bioavailablity	1995	Monotherapy, Carboplatin, Cisplatin, Fludarabine, Fluorouracil, Gemcitabine, Irinotecan, Perifosine,Prednisone Topotecan	250–1600 h	[103]
XL844	Phase I; discontinued-business	2005	Monotherapy, Gemcitabine	2–28 h	[104]
AZD7762	Phase I; discontinued-cardiotoxicity	2006	Irinotecan, Gemcitabine	8–18 h	[105,106]
PF-00477736	Phase I; discontinued-business	2006	Gemcitabine	8–20 h	[107]
LY2606318	Phase I; discontinued-thrombembolic toxicity, poor efficacy	2007	Pemetrexed, Cisplatin	14 h	[108,109]
MK-8776	Phase I; discontinued-Business	2008	Cytarabine, Gemcitabine	6–10 h	[110,111]
LY2606368	Phase II; discontinued-neutropenia	2010	Monotherapy, Cetuximab, Cisplatin, Cytarabine, Etoposide, Fludarabine, Gemcitabine, Mitoxantrone, Olaparib, Ralimetinib	11–27 h	[112,113]
GDC-0425	Completed Phase I, no further trials	2011	Gemcitabine	15 h	[114]
GDC-0575	Completed Phase I, no further trials	2012	Gemcitabine	23 h	[115]
SRA737	Completed Phase I; Phase II pending	2016	Monotherapy, Gemcitabine	8.6–13.8 h	[47]