Molecular Pathways

WEE1 kinase targeting combined with DNA-damaging cancer therapy catalyzes mitotic catastrophe

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Abstract

WEE1 kinase is a key molecule in maintaining G2 cell cycle checkpoint arrest for premitotic DNA repair. Whereas normal cells repair damaged DNA during G1 arrest, cancer cells often have a deficient G1 arrest and largely depend on G2 arrest. The molecular switch for the G2/M transition is held by WEE1 and is pushed forward by CDC25. WEE1 is overexpressed in various cancer types, including glioblastoma and breast cancer. Preclinical studies with cancer cell lines and animal models demonstrated decreased cancer cell viability, reduced tumor burden and improved survival after WEE1 inhibition by siRNA or small molecule inhibitors that is enhanced by combination with conventional DNA-damaging therapy, such as radiotherapy and/or cytostatics. Mitotic catastrophe results from premature entry into mitosis with unrepaired lethal DNA damage. As such, cancer cells become sensitized to conventional therapy by WEE1 inhibition in particular those with insufficient G1 arrest due to deficient p53 signaling like glioblastoma cells. One WEE1 inhibitor has now reached clinical phase I studies. Dose-limiting toxicity consisted of hematological events, nausea/vomiting and fatigue. The combination of DNA-damaging cancer therapy with WEE1 inhibition seems a rational approach to push cancer cells in mitotic catastrophe. Its safety and efficacy are being evaluated in clinical studies.
Background

Cell cycle control by WEE1

Phosphorylation is critical for regulation of the cell cycle. Progress through the cell cycle is coordinated by cyclin-dependent kinases. In normal cells several checkpoints ensure genomic integrity by repairing damaged DNA before cell cycle progression, such as at the G1/S transition, S phase and G2/M transition. Entry into mitosis is triggered by the cyclin-dependent kinase CDK1, also known as CDC2, bound to cyclin B. The CDK1/cyclin B complex is also known as the maturation-promoting factor. CDK1 has a pivotal role in the human cell cycle, it is the only mammalian cyclin-dependent kinase out of 14 that is indispensable and that alone can drive the cell cycle (1, 2). CDK1 activity is balanced on the one hand by inactivating phosphorylation due to the protein kinases WEE1 and MYT1 at Y15 and T14, respectively, and on the other hand by activating dephosphorylation due to the protein phosphatase CDC25 (3-6).

WEE1 is a key player that serves as a mitotic inhibitor in the intricate network of kinases and phosphatases that regulate the G2 switchboard (Figure 1) (7-14). In essence, mitotic regulators can be distinguished, that either push the cell forward into mitosis (depicted in orange as mitotic activators) or hold the cell into G2 arrest (depicted in blue as mitotic inhibitors). WEE1 and its complementary counterpart CDC25 constitute the main switch for mitosis that appears to be largely regulated by post-translational modification to ensure swift switching (15). This hot handle consists of double activating feedback loops so that activated
CDK1 activates its activators (CDC25 and MastL) and inactivates its inactivators (WEE1 and MYT1) to push the cell cycle forward. The handle is withheld by three parallel CDK1 inactivating pathways: CHK1/WEE1/CDC25/CDK1, MYT1/CDK1 and PP2A/WEE1/CDC25.

WEE1 was identified through genetic studies of cell size control and cell cycle progression in Saccharomyces pombe (16). This work founded Paul Nurse’s Nobel prize in 2001. Subsequent work established WEE1 as an atypical tyrosine kinase that is considered part of the serine/threonine-specific family of protein kinases based on its structure (5, 17). The human WEE1 gene is located on 11p15.3-p15.1 (18), and the 646 amino acid protein contains three domains: an N-terminal regulatory domain, a central kinase domain, and a short C-terminal regulatory domain (19). The kinase domain appears to be strictly specific for phosphorylation of Y15 of CDK1 in vivo (3, 5, 8). In vitro kinase activity assays have demonstrated that WEE1 is able to phosphorylate CDK1 and CDK2 complexed with cyclin A, B1 or E, but not with cyclin D1 (7). These experiments also showed that WEE1 cannot phosphorylate monomeric CDK1. Alternative substrates for WEE1 kinase have however not been systematically screened. Furthermore, WEE1 has a critical developmental role in mammals because WEE1-/- knockout mice embryos die before embryonic day 4 and conditional WEE1 deletion results in growth defects and cell death due to DNA damage and chromosomal aneuploidy (20).

**Mitotic catastrophe as anti-cancer strategy**
Cancer cells often have a deficient G1 checkpoint, for instance due to deficient p53 signaling, which can result in increased DNA damage at the G2 checkpoint compared to normal cells (21). Abrogation of the G2 arrest releases cells with unrepaired DNA damage into premature mitosis. This G2 abrogation can be brought about by pharmacodynamic manipulation resulting in mitotic catastrophe and subsequent cell death through the apoptotic program when the extent of unrepaired DNA damage is sufficient (22-26). This appears to be a viable anti-cancer strategy in combination with DNA-damaging therapy. As WEE1 kinase gatekeeps the G2 arrest, the pharmacotherapeutic inhibition of WEE1 seems rational, but other explorable targets for G2 abrogation and mitotic catastrophe are ATR, MYT1, CHK1, Hsp90 and PP2A.

**WEE1 INHIBITION**

**WEE1 as a target for anti-cancer therapy**

Kinases that have been proven as useful targets in cancer therapy exert their driving action either by mutation, protein fusion or overexpression (27). Therefore arguments for WEE1 as a candidate kinase cancer target can be derived from gene and protein expression in human cancers.

Several observations with increased WEE1 expression in human cancers have been reported. First, activity of WEE1 was found to be increased in samples of 20 patients with advanced hepatocellular carcinoma compared with non-cancerous cirrhotic tissue (28). Second, WEE1 protein was overexpressed in
35% of breast cancers specifically in the luminal and HER2-positive subtypes as determined by immunohistochemistry in 208 patient samples (29). Third, WEE1 expression correlated with patient survival in mantle cell lymphoma (30). Fourth, to establish expression profiles of the entire kinase gene family for a spectrum of human cancers, we performed an in silico analysis of gene expression microarray data based on 1451 patient tumor samples and 479 normal tissue samples (31). WEE1 was on top of the list of kinases with substantial and frequent overexpression in glioblastoma. Other G2/M transition-related kinases that were overexpressed in glioblastoma include CDK1 and AURKA. WEE1 was also substantially overexpressed in other human cancers, such as colon and lung carcinoma, and seminoma.

In contrast, a number of others observations reported a lack of WEE1 expression in human cancer and normal tissues. First, the WEE1 gene was differentially underexpressed in colon cancer cell lines and tumor samples of 12 patients with non-mucinous and mucinous colon carcinoma compared with non-dysplastic colonic mucosa (32). Second, normal human prostate epithelium cells and prostate epithelium have low protein expression of WEE1 with increased susceptibility to unrepaired DNA damage in mitosis (33). Third, WEE1 protein was not detectable in 66% of tumor samples of 79 patients with NSCLC (34). The WEE1 gene expression in tumor tissue was similar to normal tissue. Patients with WEE1-negative NSCLC had a higher recurrence rate and poorer survival.

Another approach to identify kinase cancer targets is to perform RNAi screening. A screening with silencing of 89 tyrosine kinases in breast cancer cells,
representing estrogen, progesteron, and HER2-negative subtypes, identified WEE1 as a potential target for breast cancer therapy (35). In another RNAi screening, cell viability was determined after targeting nearly all kinases in breast, lung and cervical cancer cells and WEE1 was identified as potential therapeutic target (29).

Taken together, WEE1 can be considered to be a tumor suppressor, loss of which emanates normal prostate epithelium and possibly colon and lung epithelium cells susceptible to genetic aberrations and cancerous transformation. On the other hand, cancer cells with genomic instability, typically those with deficient p53 signalling, depend on WEE1 for survival of mitosis. Under these circumstances, WEE1 can be considered a cancer-conserving oncogene, inhibition of which holds potential as an effective sensitizer in combination with DNA-damaging therapy.

**Selectivity of WEE1 inhibition**

A number of small molecule compounds can inhibit WEE1. These compounds are based on pyrimidine and pyrrolo-carbazole derivatives and their working mechanism aims to abolish CDK1 phosphorylation at Y15 (5, 36-39). PD0166285 is a pyrido-pyrimidine derivative that is a potent but non-selective inhibitor of WEE1 (IC50 of 24 nM). Other kinase targets of PD0166285 are c-Src, MYT1, EGFR, FGFR1, CHK1 and PDGFRβ (IC50 values of 8, 34, 35, 39, 72 and 85 nM, respectively) (40-42). PD0407824 is a pyrrolo-carbazole derivative that is a less potent but more selective inhibitor of WEE1 (IC50 of 97 nM), but also of CHK1
(IC50 value of 47 nM) (5, 37). Other kinase targets, such as AKT, CDK4, FGFR, PDGFR and c-Src, are inhibited only at much higher concentrations (IC50 values >3000 nM). Other potent pyrrolo-carbazole derivatives that inhibit WEE1 include WEE1 inhibitor II and PHCD (29, 35, 37). MK1775 is a pyrazolo-pyrimidine derivative that is a potent and more selective inhibitor of WEE1 (IC50 of 5 nM). Other kinase targets of MK1775 consist of YES (IC50 of 14 nM) and 7 unspecified others out of 223 kinases tested with an IC50 of more than 500 nM (43, 44).

**Preclinical observations**

In vitro, several cancer cell lines have been exposed to WEE1 inhibition which resulted in inhibition of outgrowth of cancer cells. First, inhibition of WEE1 by PD0166285 at nanomolar concentrations demonstrated effective G2 abrogation when combined with irradiation in various cancer cell lines including ovarian, colon, cervical, lung and hepatocellular carcinoma (42, 45, 46). The efficiency of G2 abrogation by PD0166285 appeared to correlate with the functional status of p53. Second, in the wild type-p53 B16 melanoma cell line PD0166285 monotherapy demonstrated an antiproliferative effect at micromolar concentration with an early G1 arrest and an inhibition of microtubule stabilization but no cell death (47). Third, knockdown of WEE1 in cervical cancer cells, but not in normal human epithelial cells, in combination with adriamycin treatment induced G2 abrogation and resulted in apoptosis (48). Fourth, WEE1 inhibition by
MK1775 at nanomolar concentrations in combination with gemcitabine induced premature mitotic entry and cell death in colon and lung carcinoma cells, both were p53-deficient. MK1775-treated ovarian carcinoma cells expressing shRNA against p53 were much more sensitive to gemcitabine, carboplatin, or cisplatin than the wild-type p53 cells (44). Fifth, the same group reported that MK1775 also enhanced the cytotoxic effects of 5-FU at nanomolar concentrations in p53-deficient colon cancer cells and pancreatic cancer cells but not in wild type-p53 colon cancer cells (49). Furthermore, combination therapy with other cytostatics, such as pemetrexed, doxorubicin, camptothecin and mitomycin C, provided similar cytotoxic effects. Sixth, knockdown of WEE1 by siRNA reduced viability of breast cancer cells but not of normal mammary epithelial cells (35). WEE1 inhibition by WEE1 inhibitor II at micromolar concentrations as monotherapy reduced cell viability, increased DNA damage and induced apoptosis in various breast cancer cells, that represent estrogen receptor positive, HER amplified and triple negative subtypes, but not in normal mammary epithelial cells and fibroblasts (35). Remarkably, WEE1 expression levels in these cell lines did not correlate with sensitivity to inhibitor. Seventh, WEE1 kinase inhibitor PHCD reduced viability and induced apoptosis in breast and cervical cancer cells with high WEE1 gene expression but not in prostate carcinoma cells and normal mammary epithelium with low levels of WEE1 gene expression (29). Eight, silencing of WEE1 by siRNA sensitized stathmin-transfected breast cancer cells to paclitaxel and vinblastine (50). Finally, we have demonstrated profound cell death in several glioblastoma cell lines and primary glioblastoma cell cultures.
after PD0166285 exposure at micromolar concentrations most notably in combination with DNA-damaging therapy such as irradiation or the alkylating cytostatic temozolomide but not in normal human fibroblasts and astrocytes (31). These effects in glioblastoma cells were paralleled by siRNA WEE1 silencing and did not correlate with p53 status. We observed similar cell death in glioblastoma stem-like cell populations both as CD133-positive fraction and when grown as neurospheres.

In vivo, WEE1 inhibition has resulted in tumor growth reduction in several studies utilizing xenograft animal models. First, nude rats subcutaneously bearing colon carcinoma WiDr xenografts were administered MK1775 orally at a dose of 5-20 mg/kg in combination with gemcitabine showed reduced tumor growth without animal toxicity (44). Similar results were obtained when combining MK1775 with carboplatin for cervical cancer HeLa-luc xenografts and with cisplatin for ovarian carcinoma TOV21G-shp53 xenografts. In this study, inhibition of CDK1 phosphorylation at Y15 as downstream substrate and pHH2 to monitor mitotic entry were established as potential surrogate markers in skin hair follicles for monitoring pharmacodynamic effects in tumor tissue. In another study by the same group, a gene expression profile was obtained from skin samples of rats with xenografts of WiDR cells after combination therapy of gemcitabine and MK1775, that was consistent with WEE1 inhibitor sensitivity (43). Effects of MK1775 monotherapy were only moderate. Second, the same group demonstrated similar tumor growth reduction for MK1775 in combination with 5-FU or capecitabine in nude rats with colon carcinoma WiDr xenografts or breast
cancer MX1 xenografts, again without toxicity (49). Third, the same group also observed marked tumor regression in p53-deficient primary pancreatic cancer subcutaneous xenografts in mice after combination therapy with MK1775 and gemcitabine compared to monotherapy and control, but not in p53-intact xenografts (51). Fourth, in mice harbouring intracranial xenografts of glioblastoma U251 cells, which were transduced with shRNA against WEE1, we observed a reduced tumor burden and survival advantage in combination with irradiation (31). A comparable survival advantage was demonstrated for combination therapy of PD0166285 and irradiation in the same glioblastoma model. Repeated experiments with highly invasive E98 glioblastoma xenografts again showed a similar therapeutic response for combination therapy of E98 transduced with shRNA against WEE1 or PD0166285 with irradiation in comparison with irradiation only or control shRNA. No toxicity was observed in these animals.

These in vitro and in vivo results provide solid arguments that WEE1 inhibition combined with DNA-damaging therapy, either irradiation or cytostatics, results in efficient cell death in various human cancer types by mitotic catastrophe. Cells with intact G1 checkpoint arrest, such as normal cells or cancer cells with intact p53 signaling, are less dependent on the G2 checkpoint arrest and are therefore not as sensitive to WEE1 inhibition. These effects are unlikely due to off target effects of the evaluated inhibitors, because knockdown of WEE1 yielded similar results. Furthermore, it is encouraging that adverse events were not met in rat or
mouse even at high dose, although toxicity studies in animals have not been published yet.

**Clinical-Translational Advances**

**Clinical studies with WEE1 inhibitor**

Clinical studies are currently at an early stage with preliminary phase I results for MK1775. The other inhibitors PD0166285 and PD0407824 have not been tested in patients. Preliminary results of an ongoing phase I study (NCT00648648) of oral MK1775 as monotherapy and in combination with gemcitabine, cisplatin, or carboplatin in 91 patients with advanced solid cancer, excluding central nervous system malignancies, were reported with good tolerance and strong target engagement (52). Adverse events were observed in 20 patients and mainly consisted of hematological events, nausea/vomiting and fatigue. Patients on monotherapy of oral MK1775 did not present dose-limiting toxicity up to 1300 mg. The maximum tolerated dose in combination therapy varied between 200-325 mg of MK1775. Four phase I/II studies with MK1775 are currently active: combined with carboplatin in ovarian cancer (NCT01164995, phase II), combined with gemcitabine, cisplatin or carboplatin in advanced solid tumors (NCT00648648, phase I), combined with 5-fluorouracil or 5-fluorouracil/cisplatin in advanced solid tumors (NCT01047007, phase I), and combined with topotecan/cisplatin in cervical cancer (NCT01076400, phase I/II).
Clinical studies with other therapeutic targets for G2 checkpoint abrogation

Alternative therapeutic targets for mitotic catastrophe by G2 abrogation with clinical data are CHK1 and Hsp90 (53). These are also discussed here.

CHK1 kinase activates WEE1 and inactivates CDC25 and therefore appears to be a suitable alternative therapeutic target. The current CHK1 inhibitors are not selective and vary in potency to inhibit CHK2 activity. This is reflected by the off target effects presumably responsible for sensitization of wild type-p53 cells to CHK1 inhibition in preclinical studies. Clinical trials in patients with advanced solid tumors and lymphoma with the non-selective CHK1 inhibitor UCN-01 (also inhibiting CHK2, WEE1 and AKT) either as monotherapy or in combination with cisplatin, carboplatin, topotecan or irinotecan, demonstrated dose-limiting toxicity consisting of hyperglycemia with resultant metabolic acidosis, pulmonary dysfunction, nausea, vomiting, hypotension and arrhythmia. At maximal tolerated doses, biological responses were detected in biopsies with inhibition of CHK1 activity, but without clinical responses (54-58). The CHK1 and CHK2 inhibitor XL844 was evaluated in combination with gemcitabine in two phase I trials in patients with chronic lymphocytic leukemia, advanced solid tumor or lymphoma with unreported results (NCT00475917 and NCT00234481). The CHK1 and CHK2 inhibitor AZD7762 has also been tested in phase I trials combined with gemcitabine or irinotecan in patients with advanced solid tumors (NCT00413686 NCT00473616 NCT00937664) with unreported results. For further details, we refer to a recent review (59).
Hsp90 is a cytoplasmatic molecular chaperone that activates several client proteins including WEE1 and CHK1 (53). The Hsp90 inhibitor tanespimycin (17AAG) results in nonspecific CHK1 and WEE1 depletion inducing p53-dependent G2 checkpoint abrogation (53). Clinical trials of tanespimycin monotherapy lacked response activity in metastatic prostate cancer, renal cell cancer and melanoma and demonstrated some activity in relapsed multiple myeloma with acceptable toxicity (60-64). Combination therapy with sorafenib, paclitaxel, trastuzumab appears to be well tolerated (63, 65, 66). For further details, we refer to a recent review (67).

**Role of WEE1 in glioblastoma**

WEE1 kinase inhibition appears to be of particular interest in glioblastoma therapy on the basis of several arguments. First, the resistance of glioblastoma to radiotherapy is due to preferential checkpoint response and repair of DNA damage (68). Second, spontaneous epigenetically-silenced MGMT, a DNA-repair protein that dealkylates temozolomide-alkylated DNA, is related with a more favorable response to chemo-irradiation therapy in patients with glioblastoma (69). This argues in favor of combining DNA-damaging chemo-irradiation with WEE1 inhibition in order to prevent DNA damage repair. Third, the genomic alterations of glioblastoma are characterized by profound chromosomal instability (70), a constitutively-activated PI3K/AKT pathway in 88% and deficient p53 signaling in 87% of glioblastomas (71). These alterations bring about a profound G2 checkpoint dependency for glioblastoma. Fourth, we demonstrated that WEE1 is the top-ranking overexpressed kinase in glioblastoma, that WEE1
expression in glioblastoma correlates with patient survival inhibition and that inhibition of WEE1 by siRNA or small molecular compound results in efficient cell death and survival benefit in animal models (31).

FUTURE PERSPECTIVES
Interestingly, pushing the cell cycle forward into mitosis may be a more effective therapeutic strategy than stagnation of the cell cycle by halting mitosis. Many kinase inhibition strategies aim to interrupt the replicative cycle of cancer cells, including inhibitors of EGFR, PDGFR and AKT. These inhibitors have so far not shown relevant patient responses in glioblastoma therapy (72). Preclinical studies indicate that timely pushing of the cancer cell cycle can be more efficient than interrupting cell proliferation (31). Explorations to push cancer cells in mitotic catastrophe by optimal combination therapy in patients holds potential to turn the tide.

The best therapeutic target to induce mitotic catastrophe by G2 abrogation remains elusive, be it ATR, MYT1, CHK1, Hsp90, PP2A, WEE1 or other unknown G2/M transition targets or combinations thereof. This is one of the focuses of future work. Another focus is to improve the selectivity of WEE1 inhibitors. Current inhibitors suffer from a lack of selectivity. As WEE1 appears to have a strictly specific function, off-target effects due to non-selective effects of the available inhibitors are not required for adequate G2 abrogation and are therefore considered undesirable. Increased WEE1 selectivity would likely account for a more beneficial toxicity profile, although MK1775 monotherapy
already showed minimal toxicity. Another focus is to verify alternative biomarkers for WEE1 engagement at the tumor site. So far, the phosphorylation status of the downstream substrate CDK1, phosphorylation of HH2 to monitor mitotic entry and a gene expression profile that correlates with WEE1 inhibitor sensitivity have been postulated (43, 44). The obvious incentive is to provide a reproducible, reliable and accurate representation of target engagement utilizing minimal sample tissue with maximal ease to acquire. Furthermore, the optimal timing of treatment with the DNA-damaging component and the G2-abrogating component is of critical importance. In experiments so far these issues have not been addressed.

Some concern can be raised when assuming that a subpopulation of cancer cells with sublethal DNA damage can survive forced mitosis, despite genomic instability and treatment-induced DNA damage, with viable daughter cancer cells with accelerated clonal evolution as a consequence. These offspring cells could theoretically be more resistant to the combination therapy by natural selection. However, more likely is DNA damage to be a stochastic process, so that these offspring cells are also led to mitotic catastrophe by acquiring additional DNA damage that becomes lethal in the following cell cycles. Another concern is that normal cells are exposed to combination therapy that may deregulate these cells by inhibiting the tumor suppressor function of WEE1. At this point no experimental suggestions exists for this phenomenon, presumably due to an adequate G1 checkpoint response.
CONCLUSION

WEE1 kinase, as gatekeeper of the G2 checkpoint arrest, holds potential as therapeutic target to manipulate entry into mitosis of cancer cells. Compelling preclinical data indicates that targeting WEE1 induces a catastrophic mitosis due to premature mitotic entry with unrepaired lethal DNA damage. This strategy selectively targets cells that depend on G2 checkpoint arrest in which cancer cells with deficient p53 signaling contrast with normal cells. The combination of DNA-damaging treatment with subsequent WEE1 inhibition-induced release into mitotic catastrophe is an attractive paradigm that justifies current evaluation of safety and efficacy in clinical studies, but is only at its infancy.
Figure. Mitotic activators (orange) and inactivators (blue) regulate the transition from G2 checkpoint arrest to mitosis. CDK1 is deactivated by WEE1 kinase phosphorylation and is activated by CDC25 phosphatase dephosphorylation. Three parallel inactivating pathways maintain G2 checkpoint arrest: CHK1/WEE1/CDC25/CDK1, MYT1/CDK1 and PP2A/WEE1/CDC25. Double positive feedback loops with activated CDK1 activating its activators and inactivating its inactivators trigger mitosis. Mitosis is positively regulated by PI3K/AKT, AURKA/PLK1, MASTL/PP2A, KLF2 and PIN1. Pharmacotherapeutic manipulation with inhibitors of AKT, CDK1, PLK1 or AURKA stagnate the cell cycle at the G2/M transition. Inhibitors of WEE1, CHK1, Hsp90 or PP2A trigger mitosis. The WEE1 kinase is controlled by several mechanisms. WEE1 activity is enhanced through phosphorylation by CHK1 and binding to 14-3-3 peptides (73-75). Other positive regulators of WEE1 are PP2A and Hsp90 (76, 77). At the onset of mitosis WEE1 must be downregulated rapidly to activate CDK1. Therefore, phosphorylation of WEE1 by CDK1 and PLK1 at S53 and S123 creates phosphodegrons that signal WEE1’s ubiquitination by CDC34 and proteasome-dependent degradation by the F-box proteins β-trcp-1 and Tome-1 (11, 78-81). Furthermore, phosphorylation of WEE1 at S642 by AKT1 creates a 14-3-3θ peptide-binding site that ports WEE1 from the nucleus and decreases its level of activity (12). WEE1 expression levels are also negatively regulated by Kruppel-like factor 2 (KLF2) (81). Moreover, the circadian clockwork controls WEE1 as well (82).
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