Combination Therapy Targeting the Chk1 and Wee1 Kinases Shows Therapeutic Efficacy in Neuroblastoma

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Abstract

Neuroblastoma is uniquely sensitive to single-agent inhibition of the DNA damage checkpoint kinase Chk1, leading us to examine downstream effectors of this pathway and identify mitotic regulator Wee1 as an additional therapeutic target in this disease. Chk1 was overexpressed in both neuroblastoma cell lines and high-risk patient tumors. Genetic or pharmacologic abrogation of Wee1 signaling results in marked cytotoxicity in 10 of 11 neuroblastoma cell lines with a median IC50 of 300 nmol/L for the Wee1-selective small-molecule inhibitor MK-1775. Murine tumor lines derived from mice that were either heterozygous or homozygous for Mycn were particularly sensitive to single-agent inhibition of Wee1 (IC50s of 160 and 62 nmol/L, respectively). Simultaneous pharmacologic inhibition of Chk1 and Wee1 acted in a synergistic fashion to further impede neuroblastoma cell growth in vitro, in a manner greater than the individual inhibitors either alone or combined with chemotherapy. Combination Chk1 and Wee1 inhibition also revealed in vivo efficacy in neuroblastoma xenografts. Taken together, our results show that neuroblastoma cells depend on Wee1 activity for growth and that inhibition of this kinase may serve as a therapeutic for patients with neuroblastoma. Cancer Res; 73(2); 1–9. ©2012 AACR.

Introduction

Neuroblastoma is a common pediatric tumor derived from the cells of the sympathetic nervous system that manifests with significant clinical heterogeneity (1–3). Patients are typically stratified into risk groups on the basis of several criteria at diagnosis, including age, tumor ploidy, MYCN amplification status, and histologic features (1, 2). Although low-risk patients are successfully treated with surgery, approximately 50% of all children with neuroblastoma are diagnosed with high-risk disease, requiring myeloablative chemotherapy followed by maintenance with retinoids and anti-GD2–based immunotherapy (3). Despite this intense multimodal treatment regimen, half of these patients will eventually relapse and succumb to the disease and those who survive are typically burdened with treatment-related chronic illnesses (4). The intense chemotherapy schedule and poor survival rate characteristic of high-risk and/or relapsed neuroblastoma underscores the need for novel therapies to successfully treat children burdened with this disease. To this end, our group recently identified the DNA damage response (DDR) protein checkpoint kinase 1 (Chk1), as a bona fide molecular target through an siRNA screen of the neuroblastoma kinome (5). Our findings showed that neuroblastoma possesses disproportionate Chk1 activity, and compared with any other tumor histotype, is uniquely sensitive to single-agent Chk1 inhibition, likely due to MYC and MYCN oncogene–induced replicative stress. Because of this reliance upon Chk1 signaling, we hypothesized that neuroblastoma is particularly susceptible to DDR pathway interference, leading us to investigate potentially tractable pathway members.

The maintenance of genomic integrity through error-free DNA replication and precisely timed cellular division is essential for the accurate transfer of genetic information to daughter cells. Flawless cell-cycle progression is dependent upon the tightly regulated coordination between several cell-cycle checkpoint proteins and the cyclin-dependent kinase (CDK) family of proteins. Malignant cells frequently exploit defects in DNA repair and/or cell-cycle checkpoint pathways to stimulate aberrant cellular division, thereby gaining a distinct survival advantage (6). Key regulators of DNA damage surveillance pathways, such as the checkpoint kinases ATR, Chk1, and Wee1, recognize DNA aberrations and arrest cellular division until genomic integrity is restored (7, 8). Paradoxically, however, the aberrant activity of DDR proteins can also serve to dampen the replication stress generated by oncogenic transformation, thereby protecting cancer cells (9).

Both of the serine/threonine kinases Chk1 and Wee1 are overexpressed and/or aberrantly activated in several cancer types, suggesting that they may serve as attractive molecular targets for pharmacologic intervention (7–13). Chk1 exerts indirect checkpoint control through both S-phase and G2–M
phases of the cell cycle and is phosphorylated by ATR in response to DNA damage caused by oncogene-induced stress, stalled replication forks, ultraviolet light, or genotoxic agents (14, 15). Activation of Chk1 results in the sequestration and/or subsequent degradation of the Cdc25A and Cdc25C phosphatases, consequently causing cell-cycle arrest through accumulation of inactive cyclin-dependent kinases (CDK; ref. 14). In a parallel pathway, Wee1 signals in concert with Chk1 by directly catalyzing an inhibitory phosphorylation at the Y15 residue of Cdc2 (CDK1), abrogating its activity and inhibiting mitotic progression at the G2 checkpoint (16). Because both Chk1 and Wee1 signaling ultimately result in cell-cycle arrest, it seems counterintuitive that constitutive activity of these kinases would contribute to a malignant phenotype. However, many cancer types harbor G1 checkpoint deficiencies, such as TP53 inactivation, necessitating reliance upon G2 checkpoint signaling for continued survival. In particular, cancers with G2 checkpoint hyperactivation may be more efficient at repairing the genetic lesions generated by genotoxic insults (17, 18). Therefore, small-molecule inhibitors targeting the G2 checkpoint are intriguing as both single-agent and combination therapies aimed at enhancing conventional chemotherapy with the rationale that abrogation of Chk1/Wee1 signaling forces mitotic progression with incompletely replicated or damaged DNA, leading to mitotic catastrophe and subsequent cell death (11, 18, 19).

Therefore, with the goal of identifying additional therapeutic targets of the DNA damage pathway in neuroblastoma, we focused our efforts on Wee1, for which there are inhibitors in clinical trials (NCT00648648) and evidence of synthetic lethalities (11). Inactivation of Wee1 would contribute to a malignant phenotype. However, many cancer types harbor G1 checkpoint deficiencies, such as TP53 inactivation, necessitating reliance upon G2 checkpoint signaling for continued survival. In particular, cancers with G2 checkpoint hyperactivation may be more efficient at repairing the genetic lesions generated by genotoxic insults (17, 18). Therefore, small-molecule inhibitors targeting the G2 checkpoint are intriguing as both single-agent and combination therapies aimed at enhancing conventional chemotherapy with the rationale that abrogation of Chk1/Wee1 signaling forces mitotic progression with incompletely replicated or damaged DNA, leading to mitotic catastrophe and subsequent cell death (11, 18, 19).

Therefore, with the goal of identifying additional therapeutic targets of the DNA damage pathway in neuroblastoma, we focused our efforts on Wee1, for which there are inhibitors in clinical trials (NCT00648648) and evidence of synthetic lethality with Chk1 (20). We found that Wee1 is highly expressed and phosphorylated in neuroblastoma, with single-agent sensitivity reminiscent of what we previously observed for Chk1. In addition, we provide evidence that combination therapy with small-molecule inhibitors of Chk1 and Wee1 exhibits synergistic efficacy in vitro and in vivo models of neuroblastoma. Therefore, attenuation of Chk1 and Wee1 signaling may be a rational therapeutic approach for the treatment of neuroblastoma.

Materials and Methods

Cell culturing

All neuroblastoma cell lines were obtained from the CHOP neuroblastoma cell line bank. They are routinely mycoplasma and identity tested using AmpFLSTR Identifier (Applied Biosystems), last done in October 2011. The non-neuroblastoma cell line bank. They are routinely mycoplasma and identity tested using AmpFLSTR Identifier (Applied Biosystems), last done in October 2011. The non-neuroblastoma cell lines were purchased directly from American Type Culture Collection (where they do short tandem repeat testing) within 6 months of use in this study.

siRNA transfection

Transfections were conducted in triplicate as previously described (21) using ON-TARGET SMARTpool siRNAs (Thermo Scientific) specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Plk1, Wee1, and Chk1. Cell viability was quantified at 72 hours by use of Cell Titer-Glo assay (Promega). Gene knockdown was confirmed to be more than 90% by quantitative real-time PCR.

Immunohistochemistry

Following standard antigen retrieval protocol, phospho-Wee1 antibody (Cat Number: 3786) was used to stain formalin-fixed, paraffin-embedded sections at a 1:1,000 dilution for 1 hour at room temperature. Slides were rehydrated and then incubated with biotinylated anti-rabbit IgG (Vector Laboratories BA-1000) at a 1:200 dilution for 30 minutes at room temperature, followed by avidin–biotin complex (Vector Laboratories PK-6100) for 30 minutes at room temperature. Slides were then rinsed and incubated with DAB (DAKO Cytomation K3468). Counterstaining was conducted for 1 minute in Harris Hematoxylin (Fisher Scientific 6765001). Slides were rinsed and dehydrated through a series of ascending concentrations of ethanol and xylene and then coverslipped. After drying, slides were digitally scanned at ×20 magnification on an Aperio OS slide scanner (Aperio Technologies Inc.).

Pharmacological inhibition

MK-8776 (also known as SCH 900776) and MK-1775 were provided by Merck & Co. Twenty-four hours after plating, cells were treated in triplicate over a 4-log dose range (10–10,000 nmol/L) and a dimethyl sulfoxide (DMSO) control. Cells were cultured for 72 hours, and cell viability was measured using Cell Titer-Glo assays (Promega). IC50 determination was made using a nonlinear log inhibitor versus normalized response curve fit function (GraphPad). Caspase activation assays and the cleaved PARP Western blot analyses were conducted at 16 hours and quantified by use of the Caspase-Glo 3/7 assay (Promega).

Combination studies

Following single-agent IC50 determination, neuroblastoma cells were plated in duplicate in 96-well plates and treated with 2 agents at doses ranging in a 2-fold difference above and below each individual IC50 (i.e., ×0.25, ×0.5, ×1, ×2, and ×4). Combination indices were determined using CalcuSyn software via the Chou–Talalay method (22). All combination studies were repeated at least once (total of n ≥ 4 for each cell line).

Western blotting

Cell lysates were prepared as described previously (21, 23). Neuroblastoma cell lines or primary tumor lysates (40 μg) were separated on 4% to 12% gradient polyacrylamide gels via SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Primary antibody dilutions included 1:1,000 Chk1, Chk1S296, Wee1, Wee1S642, p-H2A.X(S139), Cdc2(Y15), cleaved PARP (Asp214) and 1:3,000 β-actin (Cell Signaling).

In vivo studies

CB17SC-M SCID−/− mice were used to propagate subcutaneously implanted neuroblastoma xenografts. Caliper measurements were obtained, and tumor volumes were calculated using the formula, \( \pi d^2 \), where \( d \) represents the mean diameter. Once the tumor was greater than 200 mm3, mice bearing neuroblastoma tumors were randomized to treatment arms of: (i) 30 mg/kg/dose twice daily intraperitoneal MK-8776, (ii) 30 mg/kg/dose twice daily per os MK-1775, (iii) the 2 compounds combined, or (iv) vehicle control administered for 5 consecutive days. Published OnlineFirst November 7, 2012; DOI: 10.1158/0008-5472.CAN-12-2669
days for 2 weeks. Tumors were measured twice weekly for a total of 28 days or until tumor volume reached 3 cm³. The Children’s Hospital of Philadelphia (Philadelphia, PA) Institutional Animal Care and Use Committee approved all animal studies.

**Statistical analysis**

Group comparisons were determined with a 2-tailed *t* test. For the xenograft studies, a linear mixed-effects model was used to test the difference in the rate of tumor volume changing over time between different the vehicle group and treatment groups.

**Results**

**Neuroblastoma harbors elevated Wee1⁵⁶⁴₂ phosphorylation**

We previously showed that Chk1, a DDR kinase, is highly expressed and aberrantly activated in neuroblastoma, leading to cellular dependence on this pathway. To elucidate mechanisms underlying sensitivity to Chk1 inhibition, we interrogated Wee1—a serine/threonine kinase that signals through a pathway complimentary to Chk1. In a manner similar to Chk1, we observed robust expression of Wee1 protein levels with extensive phosphorylation in a comprehensive panel (n = 18) of neuroblastoma lines. In addition, canonical signal transduction through the Wee1 pathway was intact in these neuroblastoma lines, as phosphorylation of Cdc2 at Y15 often phenocopied Wee1 activation (Fig. 1A; Supplementary Fig. S1A). In contrast, substantial Wee1 expression or activation was not detected in DAOY medulloblastoma or the h-tert immortalized nontransformed, retinal pigmented epithelial (RPE-1) cells. Similarly, the several adult tumor types we investigated revealed very low basal Wee1 activity in comparison to what was observed for neuroblastoma (Supplementary Fig. S1B).

![Figure 1](image1.png)

Figure 1. Wee1 kinase is highly expressed in neuroblastoma. A, Western blot analysis of neuroblastoma (NB) cell lines shows that Wee1 is highly expressed at the protein level and constitutively activated compared with non-NB lines such as DAOY medulloblastoma cells or nontransformed RPE-1 cells. Phosphorylated Wee1⁵⁶⁴₂ is present in the majority of neuroblastoma lines and often coincides with downstream phosphorylation of Cdc2⁷⁷⁵ (Cdk1). B, Wee1 is also highly expressed in diagnostic patient tumor samples, with increased Wee1 activity in 67% (8 of 12) of tumors derived from high-risk patients, compared with 28.5% (2 of 7) of tumors derived from low-risk patients. C, neuroblastoma tissue microarray (TMA) also stained positively for phospho-Wee1 (S642), confirming higher expression levels in high-risk, including MYCN-amplified tumors (red triangles), as compared with low-risk samples. Representative staining for each tumor risk group is shown (top). HR, high-risk; INSS, International Neuroblastoma Staging System; LR, low-risk. P-Wee1 score = antibody intensity (0, 1+, 2+, 3+) × % of cells positive.
To exclude the possibility that robust Wee1 activity was solely an artifact of DNA damage induction during cell culturing, we examined the Wee1 status in patient primary tumor samples obtained at diagnosis. As shown in Fig. 1B, 7 of 12 high-risk samples (58.3%) had appreciable p-Wee1 expression compared with 2 of 7 low-risk patient samples (28.5%). These findings were supported by immunohistochemical evidence of Wee1 phosphorylation (Ser642) obtained by staining neuroblastoma tissue microarray representing tumors from 91 patients. We again found that high-risk neuroblastoma tumors had increased levels of Wee1 phosphorylation compared with low-risk tumors (P < 0.0001; Fig. 1C). Finally, quantification of Wee1 mRNA expression levels in a large number of neuroblastoma tumor samples (n = 251) confirmed, such as Chk1, there was significantly higher expression in high-risk, MYCN-amplified tumors (Supplementary Fig. S2). Taken together, these data show that Wee1 expression and activity appears significantly elevated in neuroblastoma—particularly in MYCN-amplified, high-risk disease.

**Neuroblastoma is sensitive to targeted Chk1/Wee1 inhibition**

To ascertain the contribution of Wee1 signaling in neuroblastoma, we depleted Wee1 in 3 neuroblastoma lines using siRNA. Depletion of Wee1 substantially inhibited the growth and viability of neuroblastoma cell lines (Fig. 2A). To assess the effect of pharmacologic kinase inhibition, we tested small-molecule inhibitors of Chk1 (MK-8776) and Wee1 (MK-1775) on neuroblastoma growth and viability. The majority of neuroblastoma lines were sensitive to single-agent inhibition of Chk1 (82%) or Wee1 (91%), with median IC_{50}s of roughly 900 and 300 nmol/L, respectively (Fig. 2B). We subsequently confirmed that the observed decrease in cell viability was due primarily to apoptosis, rather than cell-cycle arrest as we observed activation of caspase-3/7 or cleavage of PARP in 8 neuroblastoma lines (Fig. 2C). We also tested 2 murine tumor lines derived from the MYCN transgenic mouse model to examine the consequence of MYCN expression on the potency of these inhibitors (24). Both single-agent Wee1 and Chk1

![Graph showing the effect of Chk1/Wee1 inhibition on neuroblastoma cell lines.](image-url)
inhibition were cytotoxic in these MYCN-derived lines, with the MYCN homozygous line roughly twice as sensitive to both Chk1 and Wee1 inhibition than the heterozygous line, although this was not statistically significant (Fig. 3A). We confirmed target engagement in these murine cells, with the expected ATR compensatory Chk1S345 phosphorylation in response to MK-8776 administration (25) and a decrease in Cdc2Y15 phosphorylation following exposure to MK-1775 (Fig. 3B).

Chk1 and Wee1 inhibition acts synergistically with chemotherapy in neuroblastoma cells

Current development strategies for Chk1/Wee1 inhibitors have been focused on their chemosensitizing properties, leading us to assess the ability of these compounds to potentiate both a topoisomerase inhibitor (irinotecan) and a nucleoside analog (gemcitabine) in our neuroblastoma cell line panel. Before combination experiments, we calculated single-agent IC_{50}s for both SN-38 (the active metabolite of irinotecan) and gemcitabine for our neuroblastoma cell line panel (n = 10) using a 4-log dose–response cell viability assay with a 72-hour time point. We subsequently combined either MK-1775 (Wee1) or MK-8776 (Chk1), with these chemotherapeutic agents as well as each other, to generate a combination index (CI) value denoting the level of observed synergy (Table 1; Supplementary Table S1). Nearly all of our neuroblastoma lines had a pronounced synergistic effect when combining these inhibitors with either SN-38 (7 of 10 cell lines for both MK-1775 and MK-8776) or gemcitabine (8 of 10 lines for MK-1775, 10 of 10 cell lines for MK-8776; Supplementary Table S1). In addition, there is potent cytotoxicity in almost all neuroblastoma lines when combining MK-1775 and MK-8776 (9 of 10), suggesting that the dual inhibition of Chk1 and Wee1 was a robust synergistic combination.

Simultaneous inhibition of Chk1 and Wee1 results in substantial DNA double-strand breakage

In an effort to elucidate downstream events responsible for the synergistic cytotoxicity in the MK-8776/MK-1775 combination group, we examined pathway signaling following pharmacologic disruption. As expected, there was a substantial inhibition of Cdc2 phosphorylation downstream of Wee1 following administration of MK-1775 and decreased CHK1 phosphorylation following administration of MK-8776, at doses at or below the respective IC_{50}s in sensitive lines (Fig. 4; Supplementary Fig. S3). In addition, inhibition of Wee1 resulted in a modest accumulation of DNA double-strand

Figure 3. Murine neuroblastoma lines derived from MYCN transgenic mice are sensitive to Chk1/Wee1 inhibition. A, cells homozygous (844) or heterozygous (282) for the MYCN oncogene were derived from MYCN transgenic murine tumors and were found to be sensitive to single-agent MK-1775 (bottom) and MK-8776 (top). n = 9 for each cell type. B, target engagement was confirmed via Western blotting 6 hours after treatment with increasing concentrations of MK-1775 or MK-8776. Increasing phosphorylation of Chk1S345 has been shown to be a biomarker of Chk1 inhibition (15).
breaks as evidenced by sustained phosphorylation of histone H2A.X (Fig. 4A). Similarly, although inhibition of Chk1 alone did result in activation of histone H2A.X, MK-8776 in combination with chemotherapy potentiated the DNA-damaging aspect of both SN-38 and gemcitabine (Fig. 4B). The dual combination of MK-1775 and MK-8776 resulted in greater than additive histone H2A.X phosphorylation (lane 6) than either inhibitor alone (lanes 4 and 7) and caused DNA damage equivalent to that induced by the cytotoxic chemotherapeutic agents. (Fig. 4C). Furthermore, despite the presence of DNA damage, cells were unable to inhibit the cell cycle as evidenced by Cdc2 phosphorylation. This suggests that mitotic progression is occurring in the presence of damaged DNA, and this has been shown to be indicative of mitotic catastrophe (18).

**Dual Chk1/Wee1 inhibition is efficacious in vivo**

On the basis of the dual efficacy of MK-8776 and MK-1775 in our in vitro models, we administered Wee1 and Chk1 combination therapy to mice bearing neuroblastoma tumors. Mice harboring xenografts from NB-1643 or SKNAS cell lines were treated with vehicle control, MK-1775, or MK-8776 (each dosed at approximately half the single-agent maximum tolerated dose) or the combination of MK-1775 and MK-8776, dosed daily 5 times for 2 weeks, with 1 week off. The regimen was well tolerated. Mice receiving both the Wee1 and Chk1 inhibitors had a reduction in tumor growth rate as compared with control mice receiving vehicle alone (P < 0.001 for NB-1643, P < 0.05 for SKNAS; Fig. 5A; Supplementary Fig. S4). Mice with NB-1643 tumors showed growth inhibition with single-agent MK-1775 (P = 0.042), whereas mice harboring SKNAS tumors responded to single-agent MK-8776 (P = 0.023), which with Bonferroni correction does not meet the significant P value of P ≤ 0.017, presumably due to the lower single-agent doses chosen. Tumor resection following 48 hours of treatment (4 doses) with these inhibitors showed the expected reduction of the Chk1 (S296) autophosphorylation site in the MK-8776 group, as well as abrogation of Cdc2 (Y15) phosphorylation in the MK-1775-treated mice bearing NB-1643 tumors (Supplementary Fig. S4) and EBC1 tumors (Fig. 5B). These in vivo markers of pathway inhibition mimic those seen in our in vitro experiments and provide a rationale for the biologic effect of tumor growth inhibition seen in these experiments.

**Discussion**

Children with high-risk neuroblastoma have a poor prognosis despite toxicity-limiting chemotherapy, emphasizing the need to develop novel targeted therapies for the treatment of this disease (1–3). Because an unbiased siRNA screen previously identified unique sensitivity to DDR pathway interference in neuroblastoma and because Wee1 ultimately impacts Cdc2 via pathways complementary to Chk1 signaling, we focused our current research efforts on combination therapies targeting this pathway. Elevated expression and phosphorylation of Wee1 was observed in neuroblastoma cell lines, particularly in contrast to other cancer cell types. In addition, both

![Table 1. Chk1 and Wee1 inhibitors combine synergistically with chemotherapy, and each other, in neuroblastoma cells](image)

Table 1. Chk1 and Wee1 inhibitors combine synergistically with chemotherapy, and each other, in neuroblastoma cells

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CI value        Symbol        Observed synergy
<0.1            ++++           Very strong
0.1–0.3         +++            Strong
0.3–0.7         +              Synergy
0.7–0.85        ++             Moderate synergism
0.85–0.9        +              Slight synergism
0.9–1.1         ±              Additive
>1.1            –              Antagonistic
Chk1 and Wee1 expression were significantly elevated in samples obtained from high-risk, MYCN-amplified tumors, suggesting that these tumors may be particularly sensitive to pathway perturbation (Fig. 1; Supplementary Figs. S1 and S2). We previously showed that induction of MYCN activity resulted in Chk1S296 phosphorylation, which we reasoned to be a DDR due to replicative stress (6). In support of this theory, 2 lines derived from MYCN transgenic mice were both highly sensitive to single-agent Chk1 or Wee1 inhibition, with the MYCN homozygous line twice as sensitive as the heterozygous line (Fig. 3). These results support the hypothesis that MYCN-driven replicative stress may underlie aberrant activation of the DDR pathway in neuroblastoma, resulting in a dependence on Chk1/Wee1 signaling to maintain cell viability. Indeed, siRNA-mediated depletion of Wee1 significantly impairs cell viability, and the majority of neuroblastoma cells show single-agent sensitivity to Wee1 or Chk1 inhibition, with IC50s in physiologically attainable ranges (Fig. 2). Consequently, sustained inhibition of Chk1 or Wee1 results in apoptosis as evidenced by PARP cleavage and activation of caspase-3/7.

Rationale for the development of Chk1 and Wee1 inhibitors as chemosensitizers is based on their role as integrative regulators of the cell cycle and DDR pathways. To assess chemopotentiation in neuroblastoma, combination strategies using gemcitabine and SN-38 (the active metabolite of irinotecan) were tested across a panel of neuroblastoma cell lines. Gemcitabine, although not used for the treatment of neuroblastoma (26), has been previously shown to be potently chemosensitized by Chk1 and Wee1 inhibition (9, 20), whereas irinotecan is used clinically for treatment of relapsed neuroblastoma (27). Nearly all neuroblastoma cell lines were found to show synergy in a variety of combinations (Table 1). Dual inhibition of Chk1 and Wee1 was highly synergistic in nearly every line—equivalent to, or exceeding many of the chemotherapy combinations. In support of these findings, recent data from Davies and colleagues showed that dual Chk1/Wee1 inhibition was synergistic in several different cancer lines and suggested that further studies be done to identify sensitive cancer cell types or if p53 status dependency were necessary (10). Here, we have shown that Chk1 and Wee1 inhibition in combination with chemotherapy is synergistic in virtually all neuroblastomas tested and is likely due to the sensitivity to DDR pathway interference in this disease.

Investigating the cellular signaling events following pathway perturbation yielded some insights to explain the potency observed in the Chk1/Wee1 combination (Fig. 4). Exposure to single-agent MK-1775 results in a continuous abrogation of Cdc2Y15 signaling concurrent with sustained H2A.X phosphorylation indicative of double-stranded DNA breakage (28). Although MK-8776–mediated inhibition of Chk1 resulted in DNA damage, exposure to chemotherapy-induced activation of Chk1, and therefore concomitant inhibition of Chk1 resulted in increased DNA damage compared with chemotherapy alone. Finally, simultaneous inhibition of Chk1 and Wee1 signaling resulted in robust accumulation of DNA damage as evidenced by H2A.X phosphorylation. Therefore, it appears that dual Chk1/Wee1 inhibition induces DNA strand breakage similar to that observed in combinations with chemotherapy,
accounting for the comparable synergy we observed in many of our neuroblastoma lines. Furthermore, as we also observed a complete absence of Cdc2 phosphorylation following Chk1/Wee1 inhibition, it is likely that mitotic progression is proceeding with damaged DNA, leading to mitotic catastrophe and resultant apoptosis. In support of this hypothesis, data from Potapova and colleagues recently showed that simultaneous inhibition of Wee1 and the Cdc25 family members results in irreversible mitotic progression (29).

On the basis of the cumulative results of these in vitro experiments, we chose a highly sensitive (SKNAS) and moderately sensitive (NB-1643) neuroblastoma line to investigate the in vivo efficacy of targeted Chk1/Wee1 combination therapy, noting that the SKNAS xenograft is highly chemoresistant in vivo. We chose a dosing strategy intended to recapitulate a clinically relevant schedule of 2 weeks on and 1 week off and our linear mixed-effects analysis accounted for data through the entire cycle, and not just during dosing. For NB-1643, there remained a statistically significant reduction in tumor burden with dual Chk1/Wee1 inhibition, even after treatment had stopped. For SKNAS, however, there was significant reduction while being dosed, but regrowth after treatment was stopped, suggesting that for some tumors it may be necessary to continually dose, or combine with chemotherapy in the manner with which these inhibitors were designed and are being developed. Nonetheless, these results argue strongly for further optimization of a Chk1/Wee1 inhibition strategy in neuroblastoma.

Conclusion

In conclusion, these results strengthen previous findings that neuroblastoma is particularly susceptible to therapies targeting the DDR pathway. In addition, because many relapsed neuroblastomas are refractory to conventional chemotherapy, targeted inhibition of Chk1 and Wee1 also serves to sensitize these tumors to currently used agents. The finding that Wee1 is highly expressed in neuroblastoma, particularly in high-risk, MYCN-amplified tumors, provides rationale for targeting this kinase. We have shown that neuroblastoma is sensitive to single-agent inhibition of Chk1 and Wee1 through the use of 2 small-molecule inhibitors, MK-8776 and MK-1775. These compounds act synergistically when combined with gemcitabine and irinotecan (SN-38), or with each other, by inducing DNA double-strand breaks and forcing cells to undergo mitotic catastrophe and subsequently, apoptosis. Furthermore, dual inhibition therapy of Chk1 and Wee1 is able to substantially inhibit the growth of neuroblastoma xenografts in vivo. Taken together, these data show the effectiveness of dual inhibition therapies aimed at targeting the DNA damage repair pathways in pediatric neuroblastoma.

Disclosure of Potential Conflicts of Interest

K.A. Cole has ownership interest as a patent application is being prepared. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Dual Inhibition of Chk1/Wee1 in Neuroblastoma

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