Investigation of Cytarabine Resistance: Targeting the Cell Cycle Checkpoints and Strategies for Overcoming Resistance of Acute Myeloid Leukemia to Cytarabine

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Abstract

Patients diagnosed with Acute myeloid leukemia (AML) often become resistant to standard chemotherapeutic regimens. Cytarabine, a nucleoside analog, is the standard of care therapy for AML treatment. We hypothesized that by using an siRNA platform to inhibit 572 kinases in combination with Ara-C (cytarabine) in two AML cell lines (THP-1 and TF-1) we would be able to identify potential therapeutic targets to improve sensitivity to Ara-C (cytarabine). Our siRNA screen identified CHK1 as the most potent sensitizer to Ara-C. However, other kinases involved in DNA repair and checkpoint activation also improved sensitivity of cells to Ara-C. Checkpoints are present at the G1/S transition, within S phase and at the G2/M transition. Within the G2/M checkpoint, CHK1 functions to halt the transition to mitosis when DNA damage is detected. Additional siRNA screening of proteins that function in the G2/M checkpoint identified WEE1 as a potent sensitizer as well. It is hypothesized that abrogation of the G2/M checkpoint prevents repair pathways from repairing genotoxic damage caused by chemotherapeutics. Therefore, a literature review of the checkpoint targeting and rational therapeutic targets for future treatments was conducted. Both WEE1 and CHK1 are currently

being targeted in order to enhance activity of various genotoxic chemotherapeutics in many different cancers and present rational targets for further investigated in combination with Ara-C in AML.

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Introduction

It is estimated in 2011 that 12,950 people will be diagnosed with Acute Myeloid Leukemia and 9,050 will die.[1] While a more rare form of cancer, patients with poor cytogenetic features and the elderly have an increased risk of failing standard treatment. Standard treatment for AML consists of a "7+3" regimen consisting of 3 days of daunorubicin and 7 days of cytarabine.[2] In order to retain remission most patients will still undergo consolidation chemotherapy and possibly stem cell transplantation depending on factors such as initial bone marrow response, WHO cytogenetic classification, and life expectancy. Only 40-45% of younger patients (<60 years old) will achieve a complete remission without relapse and patients >60 years old have a far worse prognosis.[2]

While multiple different chemotherapy combinations and doses have been tested in AML, the only treatment advance that confers survival advantage over the standard 7+3 regimen has been optimization of daunorubicin dosage (to 90mg/m^2) in those <60 years old.[3-4] However, majority of the disease burden remains in those >60 years old.[1] The poor response to standard induction therapy in this age group is likely due to a variety of factors including more adverse

cytogenetic profile, increasing comorbidities, and a reduction in organ function. These factors limit their tolerance to the extreme cytotoxic doses required for induction therapy.

Patients that relapse are often treated with the initial regimen if they have sustained a clinical response of >12months, are treated with salvage cytotoxic chemotherapy regimens, or are referred to clinical trials.[5-6] However, chemoresistance, including resistance to Ara-C has presented limitations to such strategies. Previous research has identified that alterations in the import and metabolism of Cytarabine are involved in resistance. Specifically mutations in the human nucleoside transporter (*SLC29A1),* deoxycytidine kinase (DCK) an enzyme involved in the activation of the cytarabine pro-drug, and alterations in multiple-drug resistance (MDR) efflux pumps have been shown to alter cellular response to cytarabine.[7] Patients with overexpression of p-glycoprotein efflux pumps show increased rates of relapse to cytarabine treatment.[8] However, clinical trials with MDR targeting agents have been unsuccessful mostly due to toxicity.[9-10]

An alternative approach to targeting resistance is to look at not what makes cells resistant to Ara-C but rather what pathways may be targeted to make cells more sensitive to Ara-C treatments.

Recently RNA-interference methodologies have been employed to investigate potential targets for sensitization to standard chemotherapeutic treatments.[11-13] RNA-interference encompasses many different laboratory techniques whose end result is a decrease in protein translation. Our laboratory focused on the use of small-interfering RNA (siRNA). Small interfering RNA (siRNA) utilizes short targeted RNA sequences to cause a reduction in the targeted protein. Libraries of these short RNA sequences have been developed making it possible for a high through-put assay looking at the effects of knocking down specific proteins.

The goal of this study was to use an siRNA library of the human kinome (572 different kinases) in leukemia cell lines in order to identify targets that increase Ara-C sensitivity. We hypothesized that if these identified kinases can be inhibited with currently available small molecule inhibitors that a rational drug combination may be translated into the clinic and be able improve Ara-C response and decrease the development of Ara-C resistance. This combination may also allow for decreased dosage of Ara-C to be given to those patients that may not be able to tolerate the cytotoxicity of higher Ara-C doses.

Research Materials and Methods (adapted from Tibes et al.[14])

Cell Lines, Reagents and Culture Conditions: The human cell lines TF-1, THP-1 were obtained from ATCC (Manassas, VA, USA) or DSMZ (www.dsmz.de). All tissue culture reagents were obtained from Invitrogen (Carlsbad, CA). Cells were maintained in RPMI-1640 media supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37˚C in a humidified 5% CO_2 atmosphere. Cytarabine was obtained from EMD Chemicals (Gibbstown, NJ, USA).

To prepare the appropriate Ara-C concentration, 9.72 mg of Ara-C was dissolved in 4 mL of RNAse-free water to create a 10 mM stock solution. Working solutions of Ara-C were then prepared by diluting stock Ara-C in serum-free RPMI-1640 to arrive at appropriate concentrations . 10 uL of appropriate working solution of Ara-C was added to each well of the 384-well plates to impart a final concentration of 1.42 uM and 1.31uM for TF-1 and THP-1, respectively.

High-Throughput siRNA Screens: The transfection conditions and RNAi assay platform were previously reported and adapted to myeloid suspension cells.[11,14-18]. Briefly, an siRNA library targeting 572 kinases (Qiagen's Kinome siRNA library version 1.0, Valencia, California) with 2 different siRNA sequences per kinase was printed onto 384-well plates (Fisher Scientific, Pittsburgh, PA). All siRNAs were validated by Qiagen for target-specific silencing. Cationic lipid-based transfection reagents were diluted in Opti-MEM (Invitrogen, Carlsbad, CA) and added to assay plates using a μFill liquid dispenser (Bio-Tek, VA). Subsequently TF-1 and THP-1 cells were added followed by Ara-C at the corresponding EC_{30} or EC_{70} concentrations for each individual cell line at 48 hours. EC_{30} refers to the concentration of Ara-C needed to cause a decrease of 30% in cell viability (EC_{70}, to 70% reduction). After an additional 48 hours total viability/proliferation was measured using Cell Titer Glo (CTG, Promega, Madison,WI) according to the supplier's instructions. Relative luminescence units were measured using an EnVision plate reader (Perkin-Elmer, Waltham, MA). Log_2 ratios of siRNA+Ara-C/siRNA were calculated (see hit selection criteria below). Screens in THP-1 cells were performed in duplicate aiming for a target EC_{30} concentration. In TF-1 cells, one screen was performed at the

target EC_{30} dose and one screen at the EC_{70} dose to mirror a low versus high dose Ara-C treatment approach.

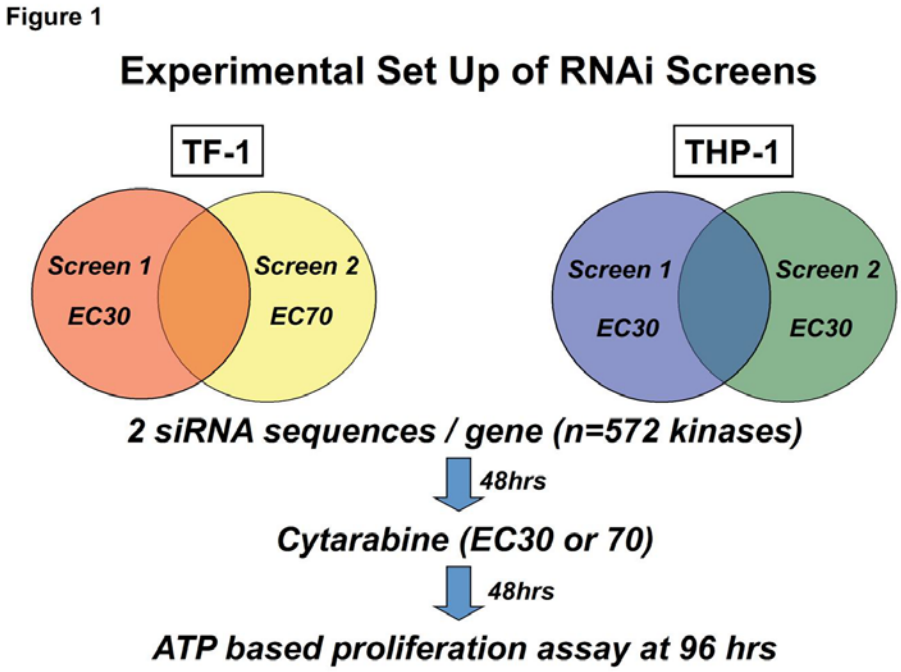

Figure 1: Experimental Overview of siRNA Screens

Figure taken from Tibes et al.[14]

Hit Selection Criteria and Definition of Hits: Published criteria for analysis and hit selection were adapted[19] in a multi-step analysis algorithm. First, the $\log_2$ ratio for each individual siRNA per gene was calculated according to [$siRNA_x$ + drug : $siRNA_x$ + vehicle], both for individual plates and individual screens. The $\log_2$ ratio describes a Sensitization Effect (SE) by which silencing of a kinase with a particular siRNA sequence in combination with Ara-C treatment reduces relative cell number as assessed with CTG. In a second step, a specific siRNA was now called a hit within a screen, if the $\log_2$ ratio of that individual siRNA was greater than two standard deviations below the median of the $\log_2$ ratio of all siRNA + Ara-C treatment; both for individual plates and the entire screen. This plate- and screen-based normalization allows intra-plate and intra-screen comparison and hit selection. In a third step, a gene was now defined as a candidate (hit) if the same siRNA met the hit selection criteria across screens. A kinase was called a hit only if both siRNA sequences (2/2 siRNA) met hit selection criteria in the same cell line. Also, a kinase was also a hit if 3 of 4 or all 4 siRNA for a particular kinase met hit selection criteria across all four screens. It is important to note that the RNAi approach

screens for a loss-of function in combination with Ara-C and every hit accounts for the siRNA only effect.

siRNA Drug-Dose Response Validation: Selected candidates from initial RNAi screens were validated by siRNA drug-dose response (siDDR) assays. Following essentially the same protocol as for the siRNA screens and as previously published,[11-12] four different validated siRNA sequences (Qiagen) against selected candidate genes were assessed. Control wells with non-silencing siRNA, a lethal positive control (Qiagen), and buffer/transfection reagent were included on each plate. Serial dilutions of Ara-C across a ten-point concentration range were added at 48 h and relative cell number was measured with CTG at 96 h. Relative cell number was calculated by dividing the average RLU values for the drug-treated wells of individual siRNA/genes by the average of RLU values for the control wells. EC_{50} values were calculated using GraphPad Prism Software (GraphPad, La Jolla, CA).

Results

Reproducibility and Transfection Efficiency: Two leukemic cell lines THP-1 and TF-1 were screened by for RNAi knockdown of 572 different kinases in two separate screens. To examine reproducibility, a Pearson's correlation coefficient (R=0.65) was calculated for the THP-1 screens demonstrating good reproducibility between the two screens. Transfection efficiency was calculated using a universally lethal siRNA and showed a transfection efficiency of 97% and 65% for TF-1 and 96% and 79% for THP-1 in the two RNAi kinome screens (data not shown).[14] In addition, we looked at the effect of the transfection reagent and a non-silencing siRNA. It was found that the non-specific toxicity from the siRNA was minimal (5% and 1% on TF-1 and 14% and 1% for THP-1).[14]

Sensitizers to Ara-C: For each siRNA used in the kinome screen a $\log_2$(siRNA+Ara-C/(siRNA)) value was calculated and those values ≥2 standard deviations from the median $\log_2$ ratio of the plate were siRNA's that decreased cell viability. Each kinase was represented by 2 separate verified siRNA's in 2 different screens and therefore each

kinase had 4 data points. A kinase that sensitized cells to Ara-C was defined as ≥2/4 points meeting the above criteria.

In the TF-1 screens, ATR was the most common sensitizer (4/4 siRNA sequences). Additionally CHK1 and HGS were common sensitizers (3/4 siRNA's). The TF-1 screens were conducted at two different concentrations of Ara-C (EC_{30} and EC_{70}). Interestingly, the four kinases that were identified as "hits" at the lower concentration of Ara-C (AURKA, AURKB, BUB1B, and PKMYT1) are all involved in cell cycle regulation. In THP-1 screens, RPS6KA5 and RYK were the most common sensitizers to Ara-C (3/4 siRNA's showing sensitization). Both of the screens with THP-1 were conducted at EC_{30} of Ara-C.

THP-1 and TF-1 are cell lines that represent two different subtypes of AML cells. THP-1 is a MLL re-arranged cell lines and TF-1 is an immature, growth factor-dependent cell line. While, both cell lines have very different molecular properties, there were two kinases from the screen that identified as Ara-C sensitizers in both screens: CHK1 and HGS.

Validation of Top Kinases: As CHK1 was one of the most potent sensitizers, CHK1 siRNA sensitization was validated with additional

siRNA sequences. Using additional sequences improves likelihood that siRNA effect is specific to the targeted gene and not due to off target effects. With the 4x siRNA sequences we saw similar patterns of sensitization likely due to the decrease in CHK1 protein levels. In addition, the amount of CHK1 sensitization with Ara-C was investigated with siRNA drug-dose response (siDDR) assays. Serial dilutions of Ara-C with and without 4 chk1 siRNA's were graphed and EC_{50} were calculated (figure 2). A 1.3-13 and 1.9 -29 EC_{50} fold shifts were observed with TF-1 and THP-1 respectively.

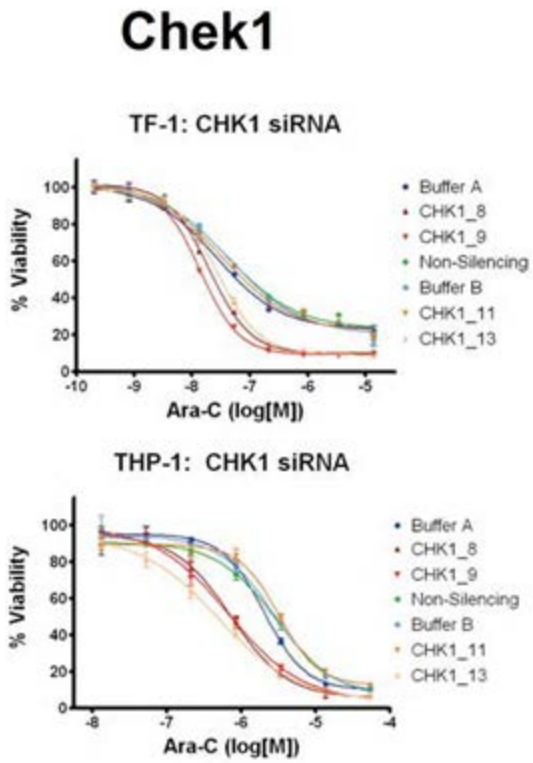

Figure 2: Drug Dose Response Curves: TF-1 and THP-1 cell lines were incubated with 4 different CHK1 siRNA sequences at various concentrations of Ara-C for 48 hours and then cell titer glo was used to measure cell viability. EC_{50} was calculated for the various chk1 siRNA curves and then compared to controls (Ara-C with transfection reagent non-silencing siRNA).

Review of Literature: Many of the targets identified (CHK1, ATR, ABL 1/2, NIK11, PKMYT1) are involved in cellular checkpoints and are activated in response to DNA damage. A literature search was performed to look at the current treatment strategies targeting proteins involved in DNA repair and cell cycle checkpoint regulation in leukemia.

Checkpoints are present at the G1/S transition, within S phase, and at the G2/M transition and can be activated in response to DNA damage.[5,20] Most of the proteins that were identified as sensitizers to Ara-C are involved in the G2/M checkpoint. The G2/M checkpoint is a complex and incompletely understood pathway that regulates entry into mitosis via the regulation of the CDK1-Cyclin B complex. The activation of this checkpoint can be initiated by ATM/ATR proteins in response to DNA damage leading to downstream activation of CHK1. CHK1 can subsequently inhibit CDC25 phosphatases. These phosphatases are responsible for activating the CDK1-Cyclin B complex. Therefore, activation of CHK1 can prevent entry into mitosis via inhibition of the CDK1-Cyclin B complex. WEE1 and PKYMT are the only known kinases that phosphorylate amino acid resides Y15 and

T14 of CDK1 respectively, causing to inhibit CDK1-cyclin B complex and are key regulators in this checkpoint. [21]

Targeting these checkpoints in order to sensitize cells to genotoxic therapies is a novel concept in cancer therapeutics and is best explained by a theory known as "synthetic lethality."[22-24] Within cells there are multiple redundant pathways involved in DNA repair and checkpoint activation.[5] Cancer cells often lose some inherent DNA repair machinery in order to propagate mutations; however, they maintain or upregulate other redundant pathways in order to maintain genomic stability.[22-24] If cancer therapeutics can target these redundant pathways, the cell can no longer maintain its genomic stability and apoptosis is induced when exposed to genotoxic agents like Ara-C. One of the most prominent examples of the exploitation of synthetic lethality involves BRCA mutated cancer cells and treatment with poly(ADP) ribose polymerase (PARP) inhibitors.[25] The results of a phase 2 trial using the PARP-1 inhibitor Olaparib with patients that have BRCA mutated breast cancer patients showed clinical benefit in patients who were originally resistant to conventional chemotherapy.[26] However, more recent data from a phase III randomized control trial

did not show survival benefit.[27] However, this theory is still being investigated in many different clinical settings.

In AML both in pre-clinical models and clinical trials the targeting of cell cycle checkpoints and DNA repair is being investigated. Inhibition of cyclin-dependent kinases (CDK) is one example where disruption of cell cycle checkpoints may sensitize cells to Ara-C treatment. CDK inhibitors including flavopiridol and UCN-01 have been investigated in AML patients. Falvopiridol is a semisynthetic flavonoid that inhibits multiple CDK's and has shown promising clinical activity when combined with Ara-C and mitoxantrone.[5, 28-31] This combination is now being tested in a phase II trial (NCT01413880). UCN-01 has been shown to affect many proteins, however, at physiological doses primarily abrogates the G2/M checkpoint.[5,28,32-35] Despite trials being terminated due to toxicities, it has shown promising results in a phase I trial with CLL and fludarabine monophosphate (FAMP) thereby showing proof of principle. [36-38]

In addition to targeting CDK's, CHK1 and WEE1 have emerged as important therapeutic targets in AML. Inhibition of both WEE1 and CHK1 have been shown to abrogate the G2/M checkpoint.[39-41] Without

this critical checkpoint, it is hypothesized that cells are unable to recover from the genotoxic stress of Ara-C .[39-41] Initially, both CHK1 and WEE1 were found to be potent sensitizers to chemotherapeutic agents in p53 deficient cells.[42-47] p53 is activated in response to DNA damage and involved in stopping cell cycle progression. It is primarily involved in G1/S cell cycle checkpoint, and therefore cells deficient in p53 rely heavily on the G2/M checkpoint for maintenance of genomic stability. Therefore, inhibition of the G2/M checkpoint by inhibition of CHK1 or WEE1 would eliminate the cells ability to repair DNA damage after exposure to genotoxic stress.[42-48]

While CHK1 was originally described in p53 deficient cells, recent studies have shown that inhibition of these proteins may not necessarily rely on the status of p53.[49] Various CHK1 small molecule inhibitors have been developed and are now being investigated in clinical trials.[50] The results of a phase I dose escalation study of SCH 900776 in combination with Ara-C in relapse and refractory AML was presented recently at the ASH 2011 annual meeting.[51] 13/24 (54%) patients with relapsed and refractory AML showed a complete clearance of bone marrow on day 14. A plan for a randomized phase II trial with a dose of 100mg is now being planned.[51]

While CHK1 inhibition in combination with Ara-C shows promising clinical evidence, recent pre-clinical models have shown that WEE1 may be an equally or even more valuable target to sensitization to chemotherapeutics.[5,14,52] Our original kinome siRNA library did not include WEE1; however, after additional RNAi screening of proteins in the G2/M checkpoint, this protein was found to be a more universal sensitizer than CHK1 in leukemic cell lines.[14] Additional groups have also identified WEE1 as a mediator of Ara-C response in AML cell lines by using shRNA inhibition.[53]

While WEE1 is a relatively new target in AML, it has been studied more extensively in solid tumors. WEE1- inhibition also has been shown to sensitize other solid tumor cell lines to various chemotherapeutic agents.[45-46,52,54-55] In addition, MK-1775 (a small molecule WEE1 inhibitor) has been shown to radiosensitize lung, breast, prostate cancer, and glioblastoma cells lines.[56-57]

The growing pre-clinical evidence that WEE1 sensitizes cells to both chemotherapy and radiotherapy, had led to MK-1775 being tested in phase 1 clinical trials. It is being investigated in advanced solid tumors in combination with gemcitabine, cisplatin, or carboplatin.[58] Preliminary results were presented at the ASCO meeting in 2011

showing that of the 134 patients being tested for safety and tolerability, 46% showed stable disease, while 5 patients had a partial response.[58]

Interestingly while literature supports both the advancement of CHK1 and WEE1 inhibition to sensitize tumors to chemotherapeutics, there only recently has been research focused on the combination of both CHK1 and WEE1 inhibition.[59] Pre-clinical models suggest that the combination of CHK1 and WEE1 inhibitors combine synergistically to increase apoptosis and deregulation of the G2/M checkpoint.[59] In addition to this combination, there has recently been research into the combination of CHK1 and PARP inhibitors in p53 deficient pancreatic cells. The authors of this study believe that CHK1 inhibition may create a BRCA 1/2 mutational state in cells, making them responsive to PARP inhibition.[60]

Overall this literature search supports our results that targeting the G2/M checkpoint by CHK1 and WEE1inhibition sensitize AML to Ara-C. It also highlights the need for future clinical research into the efficacy of small molecule inhibitors of both WEE1 and CHK1 in relapsed and refractory AML patients.

Discussion

Given the high rates of relapsed and refractory AML, this study has looked at key targets that sensitize leukemic cell lines to Ara-C treatment. The screen presented a high-throughput siRNA platform to investigate key regulators that modulate Ara-C response in AML. In the screen of 572 kinases we found that inhibition of CHK1 showed the most potential for sensitizing AML cells to Ara-C. Given that other kinases involved in DNA checkpoint and DNA damage repair were identified as sensitizers, including PKMYT1, our lab proceeded with testing WEE1 kinase and additional proteins that may represent potential sensitizers of Ara-C in AML.[14] This revealed that inhibition of WEE1 by siRNA and MK-1775 potentiated the effects of Ara-C in both AML cell lines and primary AML/MDS/CML samples.[14]

Both CHK1 and WEE1 are critical components of the G2/M checkpoint. Given our results, a literature search was performed to investigate checkpoint targeting in leukemia treatment. The results of this literature review and from published trials support the results from our original kinome screen and validation experiments. The report published by Tibes et al,[14] together with the publication of C. Porter et al,[53] are the first to document that WEE1 is a crucial target

in combination with Ara-C in AML. Many small molecule inhibitors to both CHK1 and WEE1 are in early clinical trials in both AML and other advanced solid tumors. The combination of the WEE1 inhibitor MK-1775 with Ara-C has been shown to enhance Ara-C activity in pre-clinical AML models and further clinical research is necessary to investigate its effects in relapsed and refractory AML.

Many targeted therapies are most effective in a specific clinical context as with PARP inhibitors in BRCA mutated breast cancer. Another area of potential further research will be needed to delineate if CHK1 and WEE1 inhibitors are most effective in specific populations of patients with AML. Identification of biomarkers to help predict and monitor treatment response may potentially allow for improved response rates to these combination treatments. In addition, further research into unique combination such as combining WEE1 and CHK1 inhibitors or CHK1 and PARP inhibitors could be used to sensitize AML cell lines to cytarabine.

The identification of possible therapeutic targets to sensitize cells to Ara-C has important clinical relevance. AML is primarily a disease that affects elderly patients who are unable to tolerate the high doses of Ara-C that are frequently needed for treatment. If the combination

of a lower Ara-C dose can achieve similar remission rates in combination with small molecule inhibitors, additional patients may be able to tolerate treatment. In our in vitro experiments we were giving Ara-C concentrations of approximately 1.42uM and 1.31uM (Approximately the EC30 for both TF-1 and THP-1). Current *in vitro* models do not permit an exact replication of *in vivo* conditions. While it is possible to replicate the plasma concentrations, it is not possible to replicate the microenvironment and metabolism that occurs within the body. However, the peak plasma concentrations achieved after both continuous infusion and high dose Ara-C treatments range from 0.284uM-169uM.[61] As these drug combinations advance to *in vivo* studies, it will need to be investigated if a lower Ara-C dose can achieve similar remission rates in combination with small molecule inhibitors of CHK1 or WEE1. The ultimate goal is to have patients that currently only qualify for palliative treatment to be able to tolerate treatment and achieve similar remission rates as their younger counterparts.

In addition, many patients with AML that originally achieved a complete remission will relapse. Relapsed and refractory and newly diagnosed leukemia can be resistant to Ara-C treatment. With the

identification of targets that sensitize AML cells to Ara-C, chemoresistance may be overcome, especially as WEE1 targets complementary pathways to Ara-C resistance. The ultimate goal is to overcome the dismal prognosis that AML patients currently face.

In summary, this high-throughput screen of 572 kinases by siRNA identified CHK1 and later WEE1 as potential therapeutic targets to improve Ara-C sensitivity in AML. These targets, as well as additional kinases involved in checkpoint regulation and DNA repair, continue to be investigated in AML. Clinical trials with both CHK1 and WEE1 in combination with Ara-C will hopefully help elucidate the clinical impact that these targets will have on future treatment of AML.

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