Network-based protein response profiling to define mechanisms of ara-C resistance in normal and leukemic myeloid progenitor cells

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The goal of this project is to define the protein network responses to ara-C treatment that drive ara-C sensitivity or resistance in acute myeloid leukemia (AML). Ara-C is the most common chemotherapy drug used to treat AML. 30% of AML patients are initially resistant to ara-C and do not achieve remission; despite this, the mechanisms of ara-C resistance are poorly understood and we cannot predict which AMLs are likely to resist ara-C. Ara-C triggers a complex set of signaling and transcriptional protein responses; however, it is unclear which proteins drive ara-C sensitivity or resistance and are molecular targets that could be exploited clinically to modulate ara-C sensitivity. Network-based protein analysis can infer these driving factors given broad information about ara-C response in sensitive and resistant cells, but a comprehensive dynamic evaluation of ara-C protein response has been limited by the technical inability to accurately measure a large number of proteins in a sample. We propose the first broad, unbiased analysis of the dynamic protein response to ara-C treatment. Our lab developed the microwestern array (MWA), a novel high-throughput proteomics assay that allows us to simultaneously quantify hundreds of proteins in cell lysates. We will define dynamic protein response profiles (PRPs) to ara-C treatment using MWAs. We will determine which proteins and pathways are most influential ara-C responses and cell fate. Our **central hypothesis** is that **ara-C sensitive and resistant cells have distinct ara-C PRPs driven by key proteins, and targeting these key proteins sensitizes cells to ara-C.** Understanding drivers of ara-C responses is a critical first step in developing rational therapy that sensitizes AML to ara-C based on its biological features. To this end, we propose 3 specific aims:

AIM 1: To test the hypothesis that isogenic ara-C sensitive and resistant cell lines have different PRPs and distinct proteins and pathways drive their differences in cell fate, and that PRPs convey distinct information from gene expression profiles. Use of isogenic cell lines removes the confounding variable of genetics and improves our power to detect informative protein differences. In the isogenic AML cell lines CEM and CEM/ara-C, we will measure ~500 protein species by MWA at various time points after ara-C treatment to obtain dynamic PRPs. We will build data-driven regression models to identify key driver proteins of resistance, and use RNAi and targeted inhibition to causally link these proteins to differences in ara-C sensitivity. We expect these cell lines will have distinct PRPs driven by a few key proteins that determine cell fate after ara-C treatment.

AIM 2: To test the hypothesis that normal hematopoietic and AML cells with differential ara-C sensitivity have different PRPs with distinct protein drivers. We will measure ara-C cytotoxicity and PRPs in lymphoblastoid cell lines (LCLs), AML cell lines, primary myeloid progenitor cells (MPCs), and primary AML cells as in aim 1. We will use unsupervised hierarchical clustering to define classes of cells with similar PRPs, and we will correlate these classes with cytogenetic, mutational, and ara-C sensitivity characteristics of the samples. We will use data-driven modeling to identify key driver proteins of resistance in these classes and use RNAi to causally link these proteins to differences in ara-C sensitivity. We expect sensitive and resistant cells will have different PRPs driven by different proteins and that multiple classes of PRPs can result in sensitivity or resistance.

AIM 3: To test the hypothesis that efficacy of various ara-C potentiating drugs can be predicted from PRPs. We will treat the cell types from aim 2 with ara-C and a panel of known ara-C potentiating drugs. We will use cytotoxicity assays to assess the effect of ara-C potentiating drugs on ara-C sensitivity. We will relate these different drug responses to ara-C PRPs to identify PRP signatures significantly associated with response to a given potentiating drug, and we will compare our results to aim 2 to determine whether specific driver proteins confer susceptibility to a particular drug. We will validate the predictive power of our PRP signatures on an additional cohort of LCLs and AML cell lines. We expect PRPs can predict how a given drug will affect ara-C sensitivity of a particular cell. We also expect that cells with specific driver proteins will have ara-C sensitivity selectively modulated by drugs that target that driver or related proteins in the network.

SIGNIFICANCE AND INNOVATION

Overview

Acute myeloid leukemia (AML) is a malignancy of the myeloid lineage of hematopoietic cells. AML has the worst prognosis of the leukemias with a 5-year overall survival of 35%. (Figure 1) It is therefore crucial to find better treatments for AML. Ara-C (cytarabine, 1-β-arabinofuranosylcytosine) is the most common chemotherapy drug used to treat AML. Ara-C resistance is common in AML and a leading cause of treatment failure, but the mechanisms of ara-C resistance are poorly understood and we cannot predict which patients will be resistant to ara-C. If the mechanisms of ara-C resistance were better understood, we could identify ways to undermine these resistance mechanisms and sensitize resistant AML to ara-C. Study of individual proteins in response to ara-C reveal many differences in protein response in ara-C resistant and sensitive AML and suggest molecular targets to sensitize AML to ara-C; however, none of these proteins have proven to be useful targets for combination chemotherapy in clinical trials.

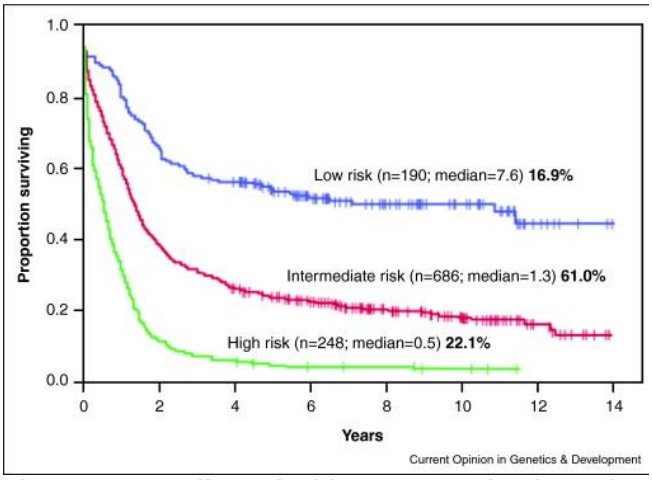


Figure 1. Overall survival in AML remains low. The 5-year overall survival rate in AML hovers at 35%. Current risk stratification gives a general estimate of prognosis, but the low risk group still has a 5-year survival under 60%. Many of these deaths, particularly in higher risk groups, are due to ara-C resistance and could be avoided if resistance mechanisms were better understood. *Figure from reference 1.*

Protein response profiles (PRPs) can be used to identify drivers of ara-C resistance. New methods such as the microwestern array (MWA) make it feasible to measure PRPs for many proteins across a large number of samples and conditions. We intend to measure ara-C PRPs in normal and AML cells and infer protein drivers of ara-C resistance in various contexts. We also aim to use PRPs to predict how a specific ara-C modulating drug affects ara-C induced cell death in a given cell line. These methods could be extended to predict which patients would benefit from a given ara-C modulating drug.

Ara-C is the main chemotherapy drug used to treat AML. Ara-C is a component of every standard AML induction chemotherapy protocol: 100% of AML patients receive ara-C regardless of the molecular features of their cancer. However, 30% of AML is initially resistant to ara-C and refractory to induction therapy.[2] These patients have a very poor prognosis and are often cross-resistant to other cytotoxic chemotherapy drugs. Of those patients who respond to ara-C and achieve remission, over 70% will relapse with ara-C resistant disease.[2] It would be highly beneficial to identify patients likely to resist ara-C before starting treatment. These patients could be given an alternative chemotherapy regimen. Ideally, the mechanisms by which these patients resist ara-C would be known. In this case, we could prescribe combination chemotherapy that circumvents these resistance mechanisms and sensitizes AML cells to ara-C. There is currently no accurate way to predict an individual patient's likelihood of response to ara-C. Although several cytogenetic aberrations and recurrent mutations are known to affect overall risk,[3] their effects specifically on ara-C resistance are not known and these prognostic factors are not used for stratification of induction therapy. Until the mechanisms of ara-C response and resistance are understood, we cannot effectively use our knowledge to properly prescribe ara-C or rationally design combination chemotherapies that sensitize particular cells to ara-C.

Ara-C causes DNA damage and triggers apoptotic cell death. Ara-C is an antimetabolite that interferes with DNA synthesis and repair. (Figure 2) The basic paradigm for cell killing by ara-C is that ara-C causes DNA damage, which triggers the DNA damage response; this leads to signaling and transcriptional changes that initiate apoptosis. Ara-C is a sugar-modified cytosine nucleoside. In most cases the cell handles ara-C as it would a cytosine due to their structural similarity. Nucleoside metabolizing enzymes activate ara-C by converting it to ara-CTP, which can then act as a CTP nucleotide anywhere that deoxycytidine is usually found. Most of the cytotoxic effects of ara-C are from its incorporation into DNA and resulting DNA damage. These effects activate the DNA damage response, which can attempt to fix the damage or initiate apoptosis. Cell death after ara-C treatment is a fairly slow process. Our preliminary data show no increase in apoptosis within 12 hours of ara-C treatment in LCLs. This is consistent with clinical data showing apoptotic cells are absent from bone marrow and peripheral blood for at least 24 hours after beginning ara-C treatment.[4-6] Apoptosis is

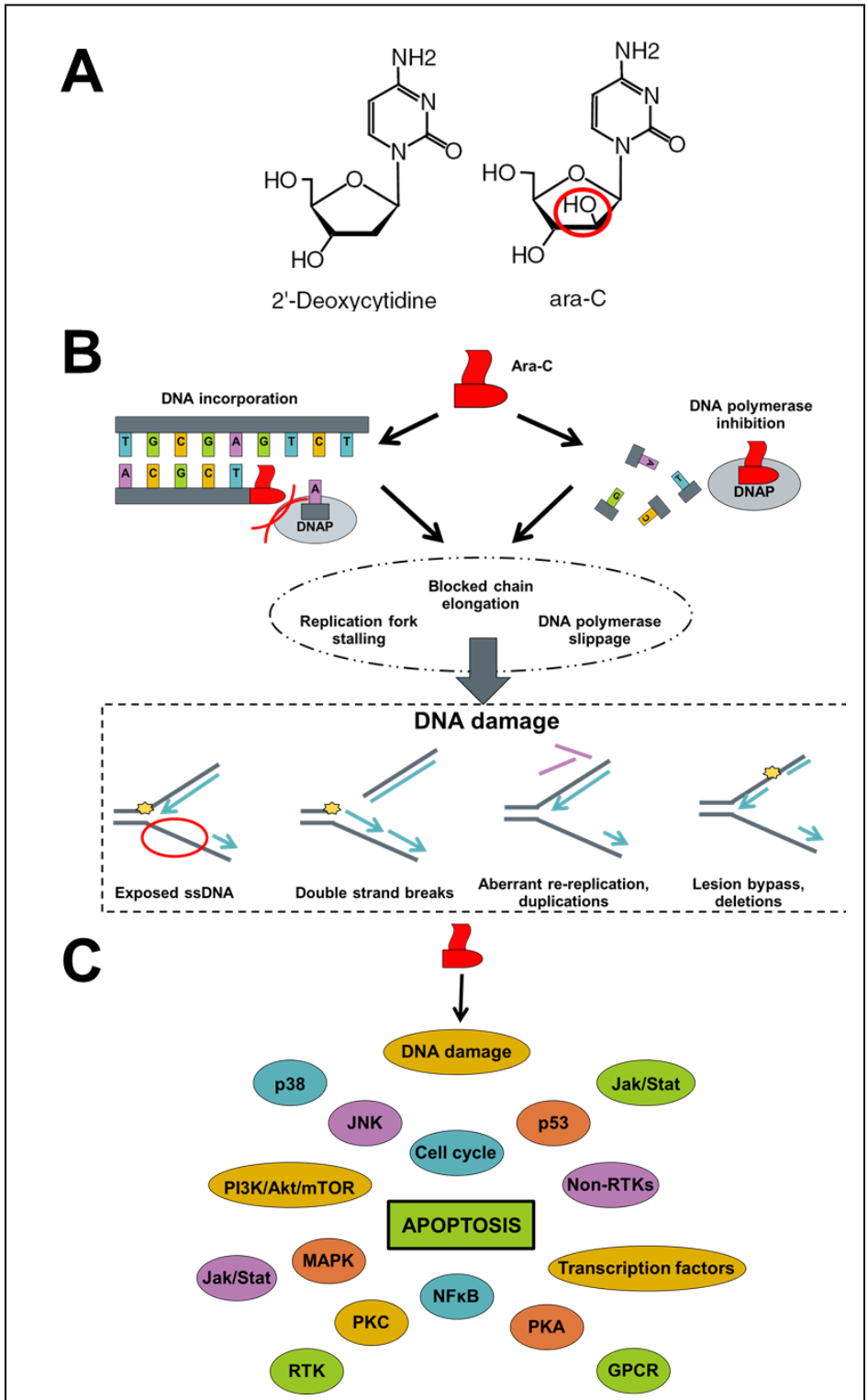


Figure 2. Ara-C causes DNA damage and triggers apoptotic cell death. (A) Ara-C is a sugar-modified cytosine nucleoside. (B) Ara-C becomes incorporated into DNA and causes DNA damage. This is the source of its cytotoxicity. (C) Ara-C induced DNA damage activates a variety of signaling and transcriptional pathways. All of these protein groups are reported to change in response to ara-C. The balance of pathway activation determines whether a cell will survive treatment or undergo apoptosis, and thus regulates ara-C sensitivity.

therefore a delayed outcome of ara-C treatment, and pro-apoptotic signaling and cell fate decision making occur long before apoptosis begins.[7] These characteristics suggest ara-C induced apoptosis is controlled by a variety of processes and that some mechanisms can keep cells alive, at least temporarily, following DNA damage from ara-C treatment.

Ara-C treatment activates a variety of protein-level responses. Many pathways are activated in response to ara-C (figure 2C) but the contribution of any given pathways to ara-C sensitivity is unclear. Cell fate is an emergent property of protein responses. Many pro-apoptotic and pro-survival protein responses occur following ara-C treatment. Pathways interact in non-linear, combinatorial ways to determine cell behavior, and it is unclear how pathways interact to direct cell fate towards survival or apoptosis. Changes in an individual protein do not always intuitively affect the protein response, and response is determined by the combined action of many protein pathways. Furthermore, proteins are linked in causative networks, and changes in one protein affect many other proteins. Most observed changes are downstream consequences of other changes. Many of these changes are responses to the same upstream signal. This means protein network changes are highly collinear: many changes reflect the same causative event and do not provide unique information about cell behavior. As a result, it is difficult to determine which protein-level changes are drivers of a response.

Attempts to modulate AML ara-C sensitivity in patients have been unsuccessful. A variety of non-metabolic proteins implicated in ara-C response pathways have been shown to modulate ara-C sensitivity *in vitro*.[8] Many drugs clearly potentiate the response of AML cell lines and primary cells to ara-C.[9-14] However, clinical trials combining ara-C with these drugs show little improvement in patient response rate and long-term survival, and no combinations

have significantly improved AML outcome.[15] Why do drugs that modulate ara-C response and sensitivity *in vitro* tend to fail in patients? One likely explanation for the clinical failure of these drugs is the problem of context: in clinical trials, a drug is given to patients indiscriminately, but only a subset of these patients likely has biological features that permit ara-C modulation by that drug. If the drug was given to the correct patients, it might be clinically successful. Another explanation is the problem of driver and passenger effects. Targeting drivers with drugs should be effective, since they are causative of the observed response and should therefore have a strong effect on cell fate following ara-C treatment. Most of the differences, however, will be passengers: secondary, downstream consequences of the causative changes. Targeting these proteins will likely be ineffective, since the drivers of resistance are left intact. Mathematical modeling is the most reliable way to identify drivers of network-wide effects, but these techniques only work if there is broad data about many network components. Low-throughput experimental approaches generally do not provide sufficient data to support this type of inference on biological networks. The best data for network-based analysis measure a large number of nodes over several time points. We refer to changes in protein expression level or activation state as protein responses. We refer to network-wide measurements of dynamic protein responses as protein response profiles (PRPs).

Many mechanisms likely drive ara-C resistance. Since many components are involved in ara-C response and cells differentially express and activate these components, cell fate following ara-C treatment is affected by a variety of components and there are potentially many diverse mechanisms leading to ara-C resistance or sensitivity. Most studies have focused on mechanisms by which cells resist damage from ara-C treatment, and the mechanisms by which cells tolerate ara-C induced stress are largely unexplored. The main mechanism for minimizing ara-C damage is modified ara-C metabolism, and the only known prognostic factors for ara-C response are variations in metabolic enzymes. Importantly, however, ara-CTP metabolism is not the only factor determining ara-C sensitivity. Cells with identical intracellular ara-CTP metabolism can have different ara-C sensitivities.[16] Non-metabolic mechanisms of ara-C resistance are of particular interest, as nucleoside metabolism pathways are difficult to modulate with drugs without considerable toxicity. Thus, drivers of these alternative mechanisms are appealing targets for combination therapy. Little study has gone into non-metabolic resistance mechanisms, and the mediators of ara-C survival strategies are not clearly known.

Protein profiles are potentially highly informative, but their use is limited by available technology. Proteins are the functional units of cell behavior and do most of the biochemical work necessary for life, from cell signaling and metabolism to cell structure and transcriptional regulation. Genetic, epigenetic, transcriptional and regulatory alterations ultimately converge at the protein level to modify cell behavior. In theory, high-throughput protein data can be used to build dynamic protein expression and activation profiles that reflect cell behavior and can reveal mechanisms of ara-C resistance in AML.

Innovation: **Microwestern array technology allows reproducible high-througput protein quantification.** Most high-throughput data on ara-C response comes from gene expression profiling, but protein data is more directly reflective of changes in cell behavior and thus should have a greater predictive value. The majority of protein data is low-throughput due to technical limitations,[4] though high-throughput data is essential for understanding network-wide cell behaviors and decision processes. All existing high-throughput protein

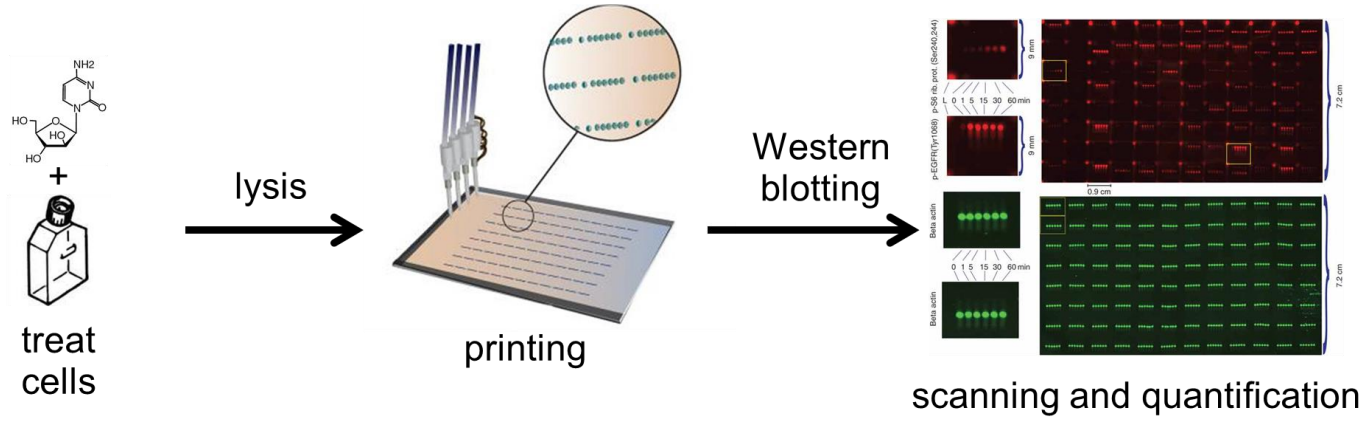


Figure 3. The microwestern array (MWA) allows accurate high-throughput protein quantification. MWAs are reproducible, inexpensive protein arrays that give data comparable to standard Western blots. MWAs generate >100x as much data as a standard Western in the same time frame with only nLs of lysate and nLs of antibody. IR fluorescence protein quantification is linear over several orders of magnitude. We will use MWAs to measure several hundred proteins following treatment with ara-C, siRNA, and ara-C modulating drugs. Adapted from ref. 17.

expression data in AML comes from reverse phase lysate array (RPLA) studies, which are notoriously noisy and inconsistent.[18] Our lab developed the microwestern array (MWA), an adaptation of the RPLA that gives higher quality signal.[17] (Figure 3) We will use MWAs to measure ara-C PRPs across several hundred proteins in numerous cell lines and use these data to model the cell death decision network in response to ara-C.

Innovation: Quantifying ara-C induced changes in many proteins simultaneously. Many proteins and post-translational modifications (PTMs) change in response to ara-C, but with previous techniques only a few proteins could be dynamically studied in a sample.[8,18] We will evaluate the network-wide changes in protein response to ara-C for the first time. By looking at many proteins, we minimize the bias of our antibody-based approach and maximize our chance of finding significant protein-level differences in ara-C sensitive and resistant cells. All existing high-throughput protein expression data in AML measures only basal protein levels.[8] By observing dynamic changes, we can make stronger inferences about the mechanisms involved in these changes and better infer drivers of protein responses.

Innovation: Using data-driven modeling to identifying mechanisms and drivers of ara-C resistance by network-based ara-C PRP analysis. Data-driven modeling techniques allow identification of driver proteins of cell death from PRPs. Partial least squares regression (PLSR) is a data-driven modeling technique useful in determining how signaling molecules work together to control cell decisions.[19] A PLSR model takes in dynamic quantitative measurements of protein abundance and can distinguish highly informative changes from redundant, uninformative changes, yielding a reduced system model of the protein expression and activation measurements most significantly correlated to differences in viability. Consequently, model components are the proteins most relevant for driving cell fate decisions. We have used PLSR on MWA data to successfully identify key proteins driving cell survival in response to EGF. (Figure 4) Our PLSR model accurately predicted synergistic drug combinations that promote cell death following EGF treatment (Ciaccio et al 2013, submitted)

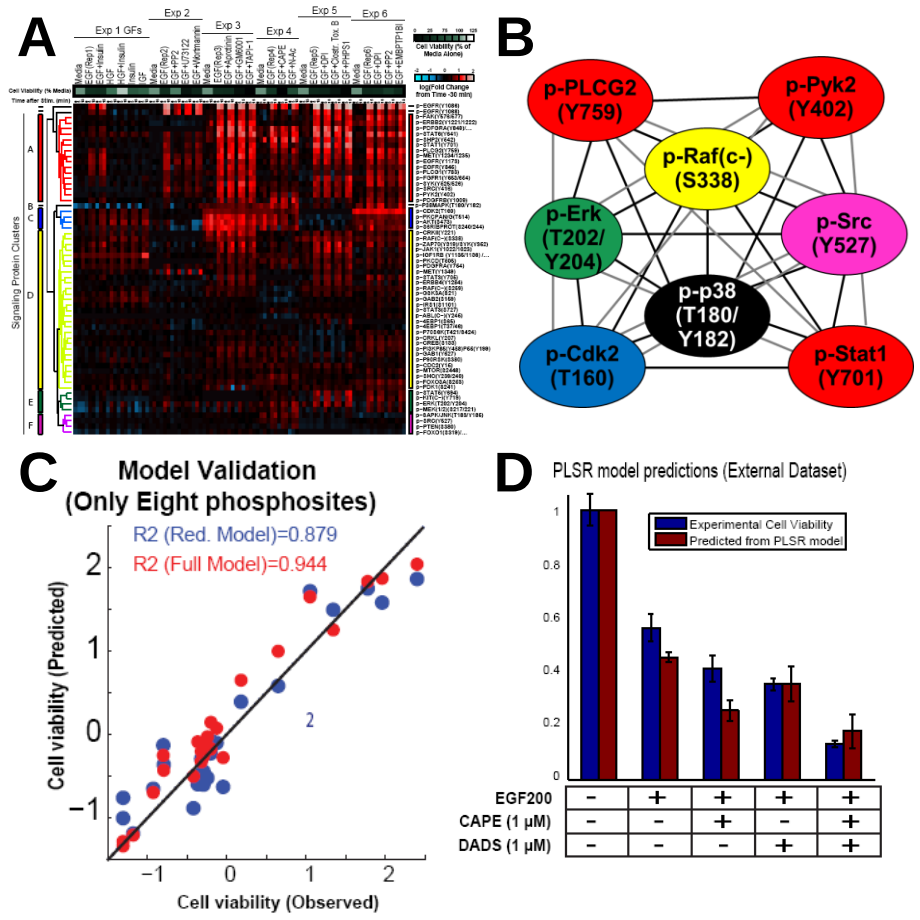


Figure 4. PLSR effectively predicts cell behavior and drivers of cell death after EGF stimulation. (A) Hierarchical clustering yields groups of proteins with similar dynamic profiles after stimulation. These proteins have highly collinear behavior. (B) PLSR reduces the model network to a smaller number of descriptive protein species. These proteins are those most important to cell fate decisions and thus strongest drivers of cell fate. (C) PLSR models are highly predictive of cell behavior, including cell viability in response to a stimulus. (D) PLSR model accurately predicts drugs that modulate cell death in response to EGF. Data from Ciaccio et al., 2013 (submitted).

Innovation: Studying the range of ara-C responses and associated ara-C resistance mechanisms. Most dynamic studies of ara-C look at a very small number of cell lines or samples. However, data consistently show that ara-C response differs significantly across cell lines and patients.[2,20] These data suggest that intrinsic differences in cells affect PRPs and drug response, and that a one-size-fits-all approach will not successfully potentiate ara-C response in diverse cellular backgrounds. We will account for this complexity by identifying classes of samples with similar PRPs. These classes likely have different response mechanisms driven by different proteins. We intend to correlate PRP classes to resistance mechanisms and to identify ara-C modulating drugs effective against any given class.

This analysis is clinically relevant because each patient's cancer is driven by unique molecular lesions and has a distinct PRP; this type of approach could be used to determine the optimal combination chemotherapy for a given patient based on PRPs.

EXPERIMENTAL APPROACH

Experimental Overview: **Cytotoxicity.** We will use an Alamar Blue cytotoxicity assay as in Wen et al., 2011 to measure percent survival after 72h in response to a range of ara-C concentrations (0, 0.1, 0.5, 1, 5, 10, 20, 40, 80, 160 μM). This concentration range is physiologically relevant as ara-C plasma concentrations typically range from 0.1-100 μM depending on the chemotherapy protocol.[21] 72h is a standard time point for measuring ara-C cytotoxicity,[22] and percent cell viability after ara-C treatment reaches a minimum around 72h in HL-60 AML cells.[23] From resulting survival curve data, we will quantify cytotoxicity by area under the curve (AUC) and estimate the LD_{50} for each cell line.

MWAs. We will measure the response of several proteins to ara-C using MWAs. We will treat cells with 5uM ara-C in DMSO, or with DMSO as a control; in some experiments, cells will be pre-treated with siRNA or concurrently treated with ara-C modulating drugs. We will collect cell lysates at 0, 1, 2, 4, 6, 8, 12, and 24h after treatment. These time points are appropriate because ara-C induced apoptosis is slow, with little occurring by 24h in most AML cell lines[24,25] and AML patients during induction chemotherapy.[4-6] Cellular commitment to survival or apoptosis occurs significantly earlier than loss of viability, and eventual cell fate is evident in cell behavior by 24h after ara-C treatment.[7] Therefore, protein data up to 24h after treatment should be sufficient to distinguish ara-C sensitive and resistant cells by PRPs. MWAs will be performed as in ref. 17. We will use antibodies against about 500 proteins shown to be expressed in untreated LCLs by MWA. (Hause et al., 2013, manuscript in preparation) Data will be preprocessed and normalized as in ref. 17. Cell treatment and MWAs will be performed in triplicate.

PLSR modeling. We will use PLSR modeling to reduce the complexity of our ara-C cell fate decision model and identify putative drivers of ara-C sensitivity and resistance. PLSR will be performed as in Ciaccio et al 2013 (submitted) using custom MatLab code. We will determine the optimal number of principal components (PCs) to include in the model such that adding another PC does not significantly increase the variance explained by the model. We will identify which proteins contribute non-redundant information regarding cell death. This reduces multiple measurements of collinear variables that contribute the same information regarding cell death. From this subset, we will identify a reduced model consisting of the minimal number of protein variables needed to accurately describe the viability data. The proteins remaining in the model are those most important to determining cell fate following ara-C treatment. These proteins are putative drivers of cell fate decisions.

siRNA transfection. siRNA from Cell Signaling or Santa Cruz will be transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol for suspension cells. This protocol is only appropriate for cell lines; if we decide to also transfect primary cells, we will use a lentiviral delivery method as in Chevrier et al., 2011. 24 hours after transfection, we will treat cells with ara-C and harvest cells as usual.

SPECIFIC AIM 1: To test the hypothesis that isogenic ara-C sensitive and resistant cell lines have different PRPs and distinct proteins and pathways drive their differences in cell fate.

Overall Rationale: We aim to show that cells with different ara-C sensitivity have different ara-C PRPs driven by distinct proteins. To do so, we need to detect protein differences in sensitive and resistant cells that meaningfully contribute to ara-C sensitivity. However, many factors other than resistance can affect cellular protein expression and behavior. One potential confounding variable is genetic background: inherited differences could affect protein levels or the magnitude of responses but have no bearing on resistance. To have the greatest chance of detecting informative protein differences that directly relate to sensitivity, we will initially study an isogenic pair of cell lines with different ara-C sensitivity. By beginning with isogenic cell lines, we will refine our ability to identify driver proteins within distinct PRPs. This will prepare us to detect meaningful changes in the noisier setting of genetically distinct cells. We will use CEM and CEM/ara-C (an ara-C resistant clone of CEM) leukemia cells.[16] These cells are a great isogenic model to study ara-C resistance because their ara-C metabolic properties have been extensively investigated: same levels of all ara-C metabolic enzymes known to be prognostic of AML ara-C response.[26,27] We will measure ara-C PRPs in these cells by MWA and

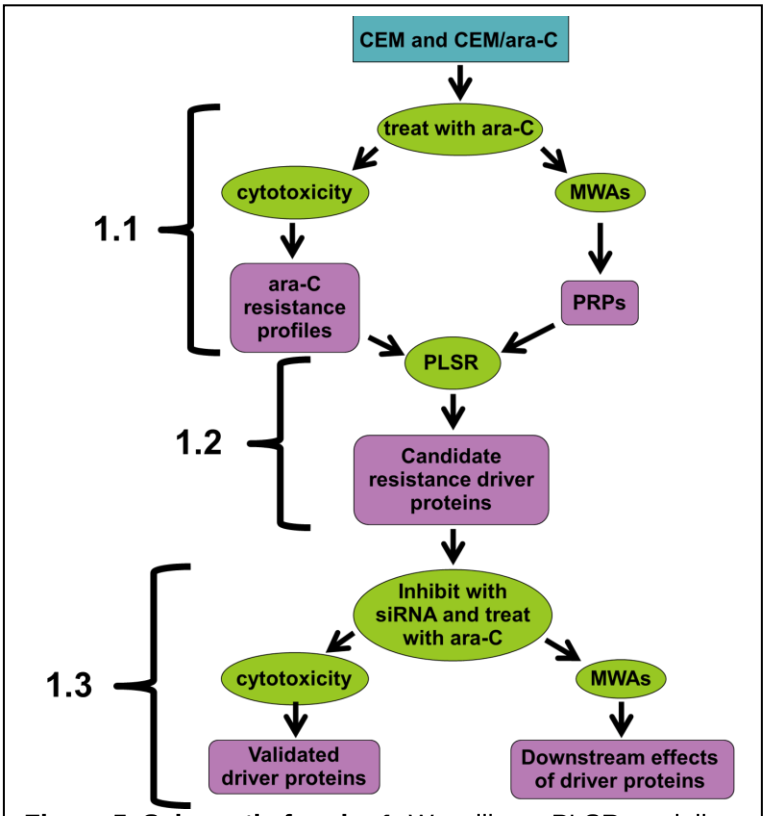


Figure 5. Schematic for aim 1. We will use PLSR modeling to relate PRPs to ara-C response and identify driver proteins of resistance. We will validate these proteins by RNAi.

identify putative driver proteins by PLSR. We will confirm their causative role in determining resistance by siRNA transfection to show that driver protein knockdown alters ara-C sensitivity. (Figure 5)

Sub-Aim 1.1: Obtain dynamic ara-C PRPs in CEM and CEM/ara-C cells.
Experimental Design: We will obtain CEM (ATCC) and CEM/Ara-C (a kind gift of T. Ueda, University of Fukui) leukemia cell lines. We will confirm differential ara-C sensitivity in CEM and CEM/ara-C cells by ara-C cytotoxicity assay. Cells will be treated with ara-C and proteins will be quantified by MWA. We will identify proteins differentially expressed or activated in ara-C sensitive and resistant cells. For each protein, we will calculate fold enrichment over control DMSO at that time point. We will compare fold enrichment over control in resistant and sensitive lines and identify proteins with significantly different levels in sensitive and resistant cell lines. For each time point, we will also calculate fold change of protein relative to baseline to identify proteins that change in response to ara-C. We will compare fold change from baseline in resistant and sensitive lines to identify proteins that have significantly different dynamics in resistant and sensitive cells.
Data Interpretation: If CEM/ara-C have significantly higher percent survival and AUC after ara-C treatment than CEM, we will conclude that CEM/ara-C are more resistant to ara-C than CEM, consistent with ref. 16. Otherwise, we will test other isogenic cell lines to find a pair with differential ara-C sensitivity. If PRPs differ significantly in CEM and CEM/ara-C, we will conclude that PRP differences are basic features that distinguish ara-C sensitive and resistant cells and infer that some differentially expressed or activated proteins directly affect ara-C sensitivity. We expect sensitive and resistant cells to have different ara-C PRPs as this outcome is consistent with their different cell fates; however, if both sensitive and resistant cells happen to share PRPs, this would also be an intriguing outcome as it would suggest common response mechanisms in all cells that could be targeted to universally manipulate ara-C response. Nevertheless, this outcome is highly unlikely.
Limitations: Our overall question involves ara-C sensitivity in AML cells, but CEM and CEM/ara-C cells are T-cell ALL cells, not myeloid cells. Despite this lineage difference, this is the most interesting of several known isogenic pairs with differential ara-C sensitivity. All others had significantly different expression of at least one ara-C metabolizing enzyme known to be prognostic for outcome in AML.[16] In particular, since CEM and CEM/ara-C cells are known to have identical ara-C metabolism properties, the causes of ara-C resistance are likely protein-level changes downstream of metabolism and DNA damage. These protein changes are ideally suited to be identified in our proteomic assay. As our goal here is to show we can identify protein changes in isogenic cell lines that cause differential ara-C sensitivity, it is not essential to use AML cell lines.

Sub-Aim 1.2: Identify driver proteins of sensitivity and resistance by data-driven modeling.
Experimental Design: We will input CEM and CEM/ara-C data into a PLSR model relating cell death to PRPs. We will determine which proteins contribute unique information to our cell death model and identify the proteins that contribute most strongly to cell fate following ara-C treatment. We will further reduce our model to the minimal number of proteins required to accurately reflect cell fate.
Data Interpretation: PLSR will yield a reduced model of the protein expression and activation measurements most significantly correlated to differences in viability. We will interpret these proteins as the putative drivers of ara-C response and proceed to validate them in aim 1.3.
Limitations: This set of one resistant and one sensitive cell line may be insufficient to build a reliable PLSR relating protein signatures to cell death. With so few samples, our resulting model may be overfit to the

peculiarities of these two cell lines. If our model is clearly flawed – for example, if it returns only one protein essential to distinguishing cell survival from cell death in response to ara-C – we will instead use proteins identified as significantly different in sensitive and resistant in aim 1.1 as our putative drivers to validate in aim 1.3. Nevertheless, even in this case our PLSR model will be useful in figuring out which protein changes are collinear and carry identical information. Proteins with collinear response patterns are likely to be in common pathways, and only part of that pathway likely drives response, so this analysis is still worth doing to focus our validation on non-collinear proteins. We will build a PLSR model with a much more extensive set of samples in aim 2, which will have a much lower risk of overfitting. Another limitation is that mathematically significant contributors to viability may be downstream consequences of the causative variables rather than the drivers. There is no way to avoid this, as PLSR will pull out the proteins most highly correlated to survival differences. It is often a good inference that these are the causative variables, but downstream responses unrelated to survival may be highly correlated with survival if they are collinear with causative proteins. The causative role of all putative driver proteins will be validated in aim 1.3.

Sub-Aim 1.3: Validate driver proteins by RNAi and determine how loss of significant components affects ara-C response.

Experimental Design: We will transfect CEM and CEM/ara-C with siRNA against putative driver proteins identified in aim 1.2. 24 hours later, we will split the cells into two groups. On one group, we will perform a cytotoxicity assay to determine how siRNA affects survival following ara-C treatment. On the second group, we will perform MWAs to obtain PRPs. We will compare ara-C PRPs in siRNA knockdown cells with original cells as in Chevrier et al., 2011 to identify which proteins significantly change as a result of the knockdown. In particular, we will evaluate the behavior of proteins collinear to the siRNA target in our PLSR model. We will compare PRPs of original CEM cells to knockdown CEM/ara-C cells to determine whether knockdown makes resistant cells respond with PRPs similar to sensitive cells. We will also put these PRPs and cytotoxicity results in our original PLSR model to determine whether our model accurately predicts cell fate following siRNA perturbation.

Data Interpretation: If siRNA against a protein significantly reduces viability of CEM/ara-C, we will conclude that the targeted component is a driver of ara-C resistance in CEM/ara-C. We expect that proteins collinear to the target in our PLSR model will also be affected by knockdown, reflecting the causative role of the target in regulating various downstream effects in response to ara-C. If siRNA against a target does not significantly change behavior of collinear proteins, we will infer that the target is not the causative driver of this set of responses. Nevertheless, if the siRNA still sensitizes cells to ara-C, we will conclude that the target contributes to cell fate decisions in response to ara-C, and the target could still be useful in treatments that potentiate ara-C response. If siRNA against a component does not significantly affect viability, we will infer the targeted component is not a driver of ara-C resistance on its own. However, it is also possible that this target affects cell fate but that the cell compensates for changes in this protein by activating other pathways. To identify this case, we will compare siRNA treated and original ara-C PRPs and note significant changes in other proteins. These protein changes may compensate for pro-apoptotic effects from the siRNA knockdown and may be more appealing protein targets.

Limitations: siRNA knockdown may not be successful. In our MWA results, we will check target protein levels at 0h (no ara-C) time point to make sure protein levels are down. If not, we will try a different transfection reagent or a different siRNA against the same target. If a specific targeted inhibitor is available against the protein of interest, we can treat cells with this in the event that siRNA knockdown is unsuccessful. siRNA knockdown may not be an appropriate way to alter protein drivers that work by changes in activation status, as knockdown would reduce all related protein species rather than only the target of interest. If a specific targeted inhibitor is available against a particular PTM, we will treat cells with this instead. If this is not available, we will knock down the endogenous protein and transfect a protein variant with a mutated PTM site. This will require overexpression of the mutant construct, which may have non-specific effects, but if the driver role of the protein species cannot be evaluated by siRNA then this is an acceptable alternative. Lastly, our PLSR mode may no longer accurately predict cell fate after knockdown. This suggests our model from aim 1.2 is overfit, as discussed earlier, and that additional variables affect survival in different contexts. To build a more accurate predictive model, we could build a new PLSR including the full set of knockdown PRPs and sensitivity profiles, then make predictions for individual samples by a leave-one-out strategy as in Ciaccio et al., 2013. As this model would include more perturbations, it would likely be more predictive of cell fate determinants across a variety of conditions. As this model would still consist only of isogenic cell line data, it may still be limited in its

ability to predict behavior of other cell types. We will follow up on this issue in aim 2 by generating a PLSR model from a much larger and genetically heterogeneous set of cells.

SPECIFIC AIM 2: To test the hypothesis that normal hematopoietic and AML cells with differential ara-C sensitivity have different PRPs with distinct protein drivers.

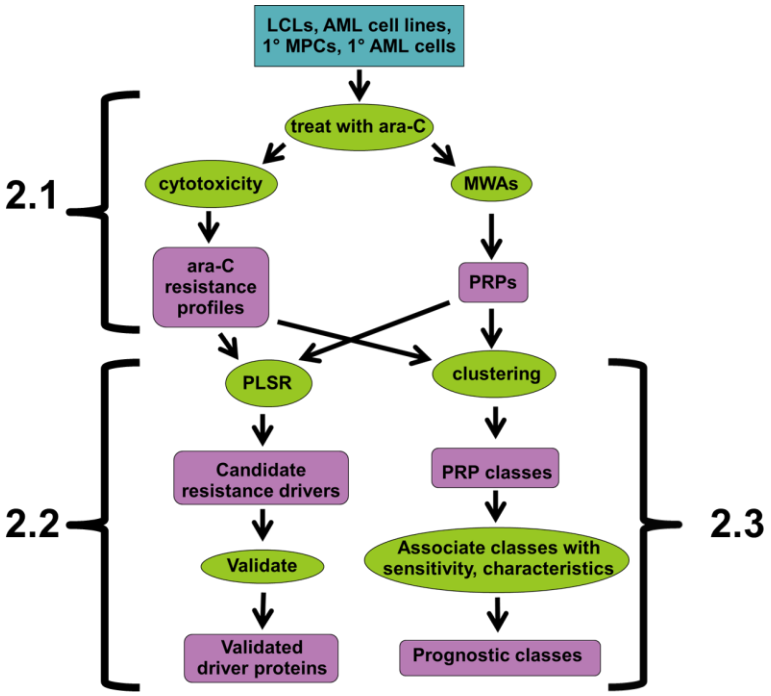


Figure 6. Schematic for aim 2. We will use ara-C resistance profiles and PRPs to identify resistance driver proteins and to identify prognostic PRP classes associated with ara-C sensitivity.

Overall Rationale: Ara-C sensitive and resistant cells have different responses to ara-C that lead to their differences in resulting cell fate. We will measure ara-C PRPs in normal and malignant hematopoietic cells, and we will define the range of ara-C response mechanisms by clustering the observed PRPs into classes with shared features. Normal cells have a variety of mechanisms to respond to genotoxic stress such as ara-C, and it is clear which of these response mechanisms promote survival and resistance versus apoptosis and sensitivity in the context of ara-C treatment. AML cells likely have a similar variety of mechanisms, but because they are malignant and driven by abnormal mutations and gene expression, AML cells may have unique resistance or sensitivity mechanisms not seen in normal cells. Using ara-C PRPs, we will define the differences between ara-C sensitive and resistant cells and infer the driver proteins of these responses. We will cluster cells into classes with similar PRPs and thus similar driving mechanisms. We will disrupt these driver proteins by RNAi and then test ara-C cytotoxicity to determine whether these proteins causally affect ara-C sensitivity, and if so in what context. (Figure 6)

Sub-Aim 2.1: Measure ara-C cytotoxicity and ara-C PRPs in LCLs, AML cell lines, primary MPCs, and primary AML cells.

Experimental Design: We will obtain a variety of LCLs from the Pharmacogenomics of Anticancer Agents Research (PAAR) group through our active collaboration with Dr. M. Eileen Dolan. The ara-C sensitivity of Yoruban (YRI) HapMap cell lines has been extensively characterized .[22] We will take the top 1/4 most sensitive and bottom 1/4 most resistant cell lines from this cohort for our studies. (Figure 7) We will obtain AML cell lines from the ATCC and DSMZ cell culture repositories that represent a variety of clinical subtypes of AML, as we expect AML subtype will impact PRPs. We will obtain primary MPCs and AML cells with linked pathology data through the University of Chicago Human Tissue Resource Center; all samples will be obtained with informed consent. We will perform an ara-C cytotoxicity assay to determine the sensitivity of each sample to ara-C. We will collect protein data by MWA. We will identify significant differences in individual protein responses as in aim 1.1.

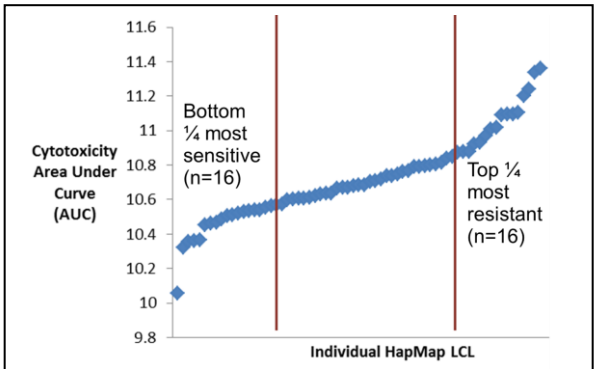


Figure 7. YRI HapMap LCLs have a range of ara-C sensitivity. Data from ref. 22 (n=66). We will limit our studies to the ¼ most sensitive and ¼ most resistant cell lines,

Data Interpretation: We expect ara-C sensitive and resistant cells to have different ara-C PRPs. We expect that some proteins will significantly differ with ara-C sensitivity across our whole set of samples; we will conclude that these proteins are important contributors to ara-C induced cell fate decisions in many cellular contexts.

Since different cells have different PRPs, however, we expect some proteins will only significantly affect ara-C sensitivity in select contexts. These proteins will likely emerge from our analysis of distinct PRP classes in aim 2.2.

Limitations: Our overall study focuses on AML, a cancer of the myeloid lineage of hematopoietic cells, but LCLs are lymphoid lineage cells. Therefore, protein differences and resistance mechanisms in LCLs may not reflect the behavior of normal myeloid cells. As there are no stable non-malignant myeloid cell lines, LCLs are the best cell line alternative because they are derived from the same precursors as myeloid cells. We are also testing primary normal myeloid precursor cells, so we will have a set of data from normal myeloid cells and can determine whether LCL ara-C responses accurately reflect normal myeloid cell behavior. Additionally, we may not have sufficient primary material to measure our whole set of proteins in triplicate. We will prioritize proteins from the reduced PLSR model in aim 1.2, proteins that were significantly different in sensitive and resistant cells in aim 1.1, and proteins that dynamically change in response to ara-C in aim 1.1.We will also aim to measure at least one protein from every major pathway included in the antibody set, as different pathways may be relevant in these cells compared to the isogenic lines from aim 1.

Sub-Aim 2.2: Identify classes of cells with similar ara-C PRPs and identify key protein-level and AML characteristic differences between these classes

Experimental Design: Using MWA data from aim 2.1, we will perform unsupervised hierarchical clustering of samples by their PRPs and build clustered heatmaps. With these results, we will identify the number of main PRP classes. We will define the protein-level differences that distinguish these classes, which will provide insight into the mechanisms driving each response profile. We will overlay ara-C sensitivity data from 2.1 onto these PRP clusters to determine whether certain classes associated with sensitivity or resistance? We will also overlay clinical AML characteristics for AML cell lines and patient samples, including FAB subtype, cytogenetics features such as fusion proteins and chromosomal gains or losses, and key mutations, to determine whether clinical features associate with particular ara-C PRP patterns.

Data Interpretation: We expect clustering will split samples into several classes with similar PRPs. We will infer these similarities reflect common underlying biological mechanisms, and that the protein-level differences between classes reflect these different mechanisms. We also expect samples with similar clinical characteristics will have similar PRPs and cluster in similar classes, and that certain classes are associated with ara-C sensitivity or resistance.

Limitations: As our clustering method is unsupervised, the emergent PRP classes may not reflect meaningful biological characteristics. Clustering may split classes on factors unrelated to ara-C sensitivity. For example, profiles could be separated based on lymphoid versus myeloid lineage, cell line versus primary cell status, or benign versus malignant origin. These distinctions are still interesting, but not informative of mechanisms regulating ara-C sensitivity. They could suggest factors that differ between these classes, which contribute noise to the rest of our data; these features could be regressed out of our data and make our data more reflective of meaningful differences in ara-C sensitivity. These distinctions could also be informative for other studies like how AML cells differ from primary myeloid precursors. Clustering could also identify biologically irrelevant variables in our data, like the day we treated particular cells. While it would be useful to know the magnitude of experimental variance in our measurements, these data would not be informative about biological factors regulating ara-C sensitivity.

Sub-Aim 2.3: Identify driver proteins of ara-C response in sensitive and resistant cells using data-driven modeling and validate causative role of driver proteins in determining sensitivity.

Experimental Design: Using PRP data from aim 2.1, we will perform PLSR modeling to infer main protein determinants of ara-C response. We will validate the causative role of driver proteins by siRNA knockdown as in aim 1.3 using a subset of our most sensitive and resistant LCLs and AML cell lines. We will also make smaller PLSR models using cells in the same PRP class to infer the driver proteins of these specific classes.

Data Interpretation: PLSR will yield a reduced model of protein response most significantly correlated to cell fate following ara-C treatment. We will interpret these proteins as the putative drivers of ara-C response. If siRNA against a protein significantly reduces cell viability, we will conclude that the targeted component is a driver of ara-C resistance. We expect proteins collinear to the target in our model will respond to knockdown in a manner that reflects the causative influence of the target on those proteins. If siRNA against a component does not significantly affect viability, we will infer that the targeted component does not drive ara-C response in the tested cell lines. Various points discussed in aim 1.3 are also relevant here. We expect our overall PLSR model will better match perturbation data and outside data sets than the PLSR from aim 1.2, since it will be built from a more extensive catalog of sensitive and resistant cell responses. In aim 3, we will explicitly test the

predictive capacity of this model by comparing responses drug-treated cells and new cell lines to our model's cytotoxicity predictions. We expect our class-specific PLSR models will identify specific drivers for individual PRP classes, and that these drivers will selectively impact survival in cell lines where they were identified as causative factors.

Limitations: Ara-C responses may be so diverse between classes that the PLSR model cannot identify protein groups universally predictive of ara-C response. In this case, we will focus exclusively on proteins defined as drivers in specific PRP classes. As in aim 1.3, our siRNA strategy may be unsuccessful or inappropriate for certain target proteins; we will address these limitations as described above.

SPECIFIC AIM 3: To test the hypothesis that efficacy of various ara-C potentiating drugs can be predicted from PRPs.

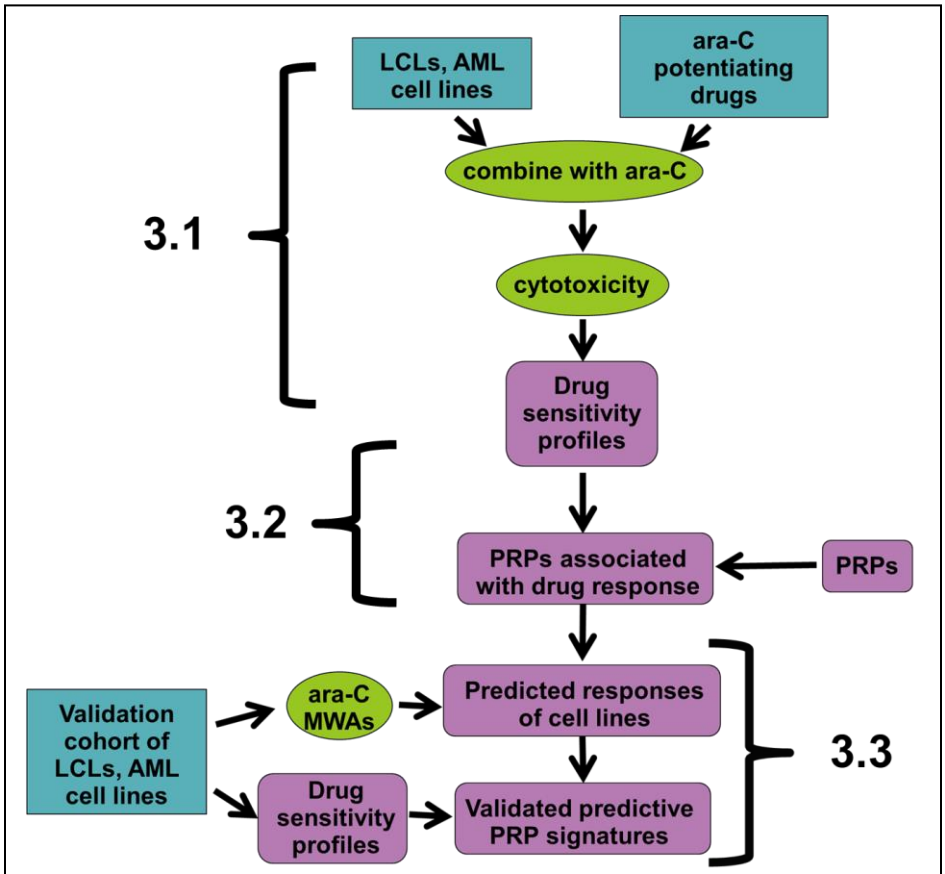


Figure 8. Schematic for aim 3. We will test the efficacy of various ara-C potentiating drugs on LCLs and AML cell lines. We will correlate sensitivity with PRPs to identify signatures associated with response to a given ara-C potentiating drug. We will validate our signatures on an additional cohort of cell lines.

Overall Rationale: Ara-C potentiating drugs are likely effective when they target a component that drives ara-C response. PRP classes reflect different biological mechanisms used by cells and thus reflect the different driver components. These different classes likely respond differently to ara-C potentiating drugs as a result of their different response mechanisms. It follows that cells with different PRPs will respond differently to drugs. If this is true, we should be able to associate PRPs with drug sensitivity. We will test the sensitivity of LCLs and AML cell lines to ara-C potentiating drugs and determine which drugs effectively modulate ara-C in various cell lines. We will correlate drug efficacy with PRPs to obtain predictive signatures for drug response. We will validate our signatures by collecting PRPs for a new cohort of LCLs and AML patients, predicting their response to various drugs, and then testing their drug sensitivity to evaluate our predictive power. (Figure 8)

Sub-Aim 3.1: Measure cytotoxicity of LCLs and AML cell lines treated with ara-C and several ara-C potentiating drugs.

Experimental Design: We will treat LCLs and AML cell lines from aim 2 with a variety of ara-C potentiating drugs. (Table 1) We will treat each cell line with DMSO only, 5 uM ara-C only, ara-C potentiating drug only, and ara-C plus potentiating drug. We will only use one dose of the ara-C potentiating drug based on existing literature regarding effective concentrations of that drug. If the literature suggests a drug is most effective if given at a certain time before or after ara-C addition, we will treat with that drug at the suggested time. We will perform cytotoxicity assays 72 hours after ara-C addition to evaluate the ability of various drugs to potentiate ara-C response in a variety of cell lines.

Data Interpretation: If a drug reduces survival in response to ara-C in a set of cells, we will conclude it potentiates ara-C in those cells. We will compare the combination treatment to treatment with the potentiating drug alone to ensure the observed toxicity is not purely a result of the drug in question, but is due to synergy with ara-C. We expect that each drug will effectively potentiate ara-C in a subset of cell lines, and that these

Drug	Target	Citation
aliesrtib	Aurora A kinase inhibitor	Kelly et al 2012
SCH 900776	Chk1 inhibitor	Schenk et al 2012
valproic acid	HDAC inhibitor	Lane et al 2012
MK1775	Wee1 inhibitor	Tibes et al 2012
AKN-028	tyrosine kinase inhibitor, including FLT3 and KIT	Eriksson et al 2012
sorafenib	tyrosine kinase inhibitor - many RTKs and intracellular	Hu et al 2011
forskolin	adenylyl cyclase activator (PP2A activator)	Cristobal et al 2011
SNS-032	cyclin-dependent kinase inhibitor (cdk2, 7, 9)	Walsby et al 2011
niclosamide	NFkB inhibitor, through blocking TAK1 to IKK to IkBa	Jin et al 2010
tandutinib	FLT3 inhibitor (best in FLT3 ITD)	Schittenhelm et al 2009
AZD6244	MEK inhibitor	Nishioka et al 2009
PFWT	AF4-AF9 inhibitor (transcription activation machinery, effective in MLL-rearranged)	Bennett et al 2009
triptolide	HSP70 inhibitor, possibly indirect	Pigneux et al 2008
bortezomib	proteasome inhibitor (sequence dependent, must be after ara-C)	Weigert et al 2007
genistein	gene expr changes…MAPK, PI3K, PKA, PKC, KIT, TGFbetaR all downreg	Shen et al 2007
7-hydroxystaurosporine (UCN-01)	PKC and Chk1 inhibitor…(potentially good for reducing Chk1-Cdk2-Akt inhibition and JNK activation followed by apoptosis)…possibly works through intrinsic apoptosis, but partly through extrinsic maybe bc Bcl-2 overexpr does not fully rescue apoptosis	Sampath et al 2006, Wang et al 2003, Tang et al 2000
17-AAG	HSP90 inhibition (Chk1 depletion…causative role implied by siRNA. PI3K blocked too but siRNA against it not useful)	Mesa et al 2006
Deguelin	PI3K/Akt inhibitor (active in leukemias w active PI3K/Akt)	Bortul et al 2005
imatinib mesylate	tyrosine kinase inhibitor	Bornhauser et al 2004
HA14-1	Bcl-2 inhibitor (overexpressing lines)	Lickliter et al 2003
bryostatin 1	PKC activator and downregulator (works partly through TNFa release but also through PKC)	Wang et al 2003, Wang et al 2002
lovastatin	potentially downregulating MAPK activity?	Holstein and Hohl, 2001
GM-CSF	pre-treatment activates growth pathways	Reuter et al 1997
N-phosphon-acetyl-L-aspartate	inhibits de novo pyrimidine synthesis	Noordhuis et al 1996
aphidicolin	inhibits ara-CTP incorporation into DNA by some mechanism	Kuwakado et al 1995
G-CSF	activates growth pathways	Gandhi et al 1995
IL-3	activates growth pathways	te Boekhorst et al 1994,

TABLE 1. Ara-C potentiating drugs to be tested in aim 3.

cell lines will have common PRPs that implicate common driver proteins in determining ara-C response in that context. This will suggest that matching PRPs are biomarkers for efficacy of a given ara-C potentiating drug. Some drugs may not effectively potentiate ara-C response in our cells; we will conclude that in the contexts tested, those drug targets are not primary drivers of ara-C response, and we expect these results will match our driver protein data from aim 2.

Limitations: The initial drug dose we test may be toxic to cells in the absence of ara-C. The initial dose will be based on the literature, but this is no assurance of drug safety in our cells. We will test lower doses of the drug alone in a dose-response cytotoxicity assay similar to our ara-C assays from aims 1 and 2. We will identify a

relatively non-toxic dose and use that to test drug synergy with ara-C. Additionally, we will only test the response of cell lines to potentiating drugs in this sub-aim, but cell line behavior may not reflect primary cell behavior in response to a drug. As primary samples are hard to come by, low volume, and can't be expanded in vitro, it is best not to use them in a screening phase and save them for validation. If initial results are promising and we get consistent results in cell lines, we will test primary cells as part of 3.3.

Sub-Aim 3.2: Associate drug responses with PRPs to identify signatures for sensitivity to ara-C potentiating drugs.

Experimental Design: We will overlay our drug synergy data with PRPs obtained in aim 2. We will perform unsupervised hierarchical clustering to determine common PRP features of samples with similar drug response profiles. We will compare the putative driver proteins for each cell line with the drug sensitivity data to determine whether effective drugs preferentially target driver proteins.

Data Interpretation: If resistance driver proteins are frequent targets of effective ara-C modulating drugs, we will conclude that these proteins actively drive ara-C response in cells and are good targets for ara-C modulating combination therapy. We expect that cells will be sensitive to drugs that inhibit their resistance driver proteins. If PRPs correlate well with drug efficacy, we will conclude that PRPs are good biomarkers for response to ara-C potentiating drugs. We expect that cells with similar PRPs will have similar drug sensitivity profiles.

Limitations: Drugs commonly have off-target effects that could affect ara-C response. If this is the case, then our predicted driver proteins may not match the drugs with efficacy in cell lines. It is difficult to eliminate this possibility, but we can attempt to detect off-target effects by examining protein-level changes induced by drug in the absence of ara-C.

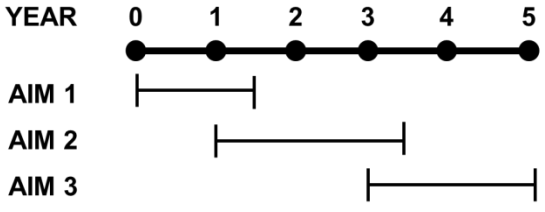
Sub-Aim 3.3: Validate the power of PRPs to predict cell response.

Experimental Design: We will obtain a validation set of cell lines composed of additional LCLs and AML cell lines. We will obtain ara-C PRPs by MWA. To minimize the number of arrays, we will only use antibodies shown to be informative for cell death in aims 1 and 2. We will use our PLSR model to predict cell viability in response to various ara-C potentiating drugs. We will then treat cells with ara-C and potentiating drugs, measure cytotoxicity, and determine whether our results match our predictions.

Data Interpretation: If our PRPs accurately predict cells most sensitive to certain drugs, we will conclude that PRP is a biomarker for response to the given drug. We will determine what PRP classes the sensitive cells have and check against aim 2.3 whether the targets of the successful drugs were identified as drivers of cell fate in their respective classes.

Limitations: Our discovery set may be too narrow to identify all classes of potential ara-C PRPs and thus all response mechanisms. Some cells may have such a distinct signature we cannot accurately predict their response from our pre-existing data. This is somewhat expected since we can only test a limited number of samples in our discovery set, although we hope the mechanisms regulating ara-C response are shared across a large enough fraction of samples that our signatures will be clinically applicable. Our model can be refined by using PRP signatures from the second set of cell lines to expand the data used to train the model. We could include all of our samples in the discovery set and then "predict" a given sample's response by a leave-one-out strategy as in Ciaccio et al., 2013. This would maximize set of discovery samples while still allowing us to make and test predictions for any given sample.

TIMELINE



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