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Toward Predicting Acute Myeloid Leukemia Patient Response to 7 + 3 Induction Chemotherapy via Diagnostic Microdosing

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Abstract

Acute myeloid leukemia (AML) is a rare yet deadly cancer of the blood and bone marrow. Presently, induction chemotherapy with the DNA damaging drugs cytarabine (ARA-C) and idarubicin (IDA), known as 7 + 3, is the standard of care for most AML patients. However, 7 + 3 is a relatively ineffective therapy, particularly in older patients, and has serious therapy-related toxicities. Therefore, a diagnostic test to predict which patients will respond to 7 + 3 is a critical unmet medical need. We hypothesize that a threshold level of therapy-induced 7 + 3 drug-DNA adducts determines cytotoxicity and clinical response. We further hypothesize that *in vitro* exposure of AML cells to nontoxic diagnostic microdoses enables prediction of the ability of AML cells to achieve that threshold during treatment. Our test involves dosing cells with very low levels of ^{14}C-labeled drug followed by DNA isolation and quantification of drug-DNA adducts via accelerator mass spectrometry. Here, we have shown proof of principle by correlating ARA-C- and DOX-DNA adduct levels with cellular IC_{50} values of paired sensitive and resistant cancer cell lines and AML cell lines. Moreover, we have completed a pilot retrospective trial of diagnostic microdosing for 10 viably cryopreserved primary AML samples and observed higher ARA-C- and DOX-DNA adducts in the 7 + 3 responders than nonresponders. These initial results suggest that diagnostic microdosing may be a feasible and useful test for predicting patient response to 7 + 3

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Author Contributions

T.M.S., P.T.H., and B.A.J. designed the experiments. T.M.S. performed the experiments, analyzed the data, and wrote the manuscript. M.M., K.H., and K.W.T. analyzed the DNA samples by AMS. M.M., C.X.P., and B.A.J. provided guidance. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

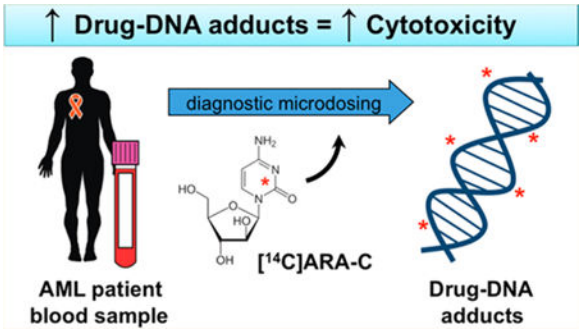
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.8b00107. Raw data from AMS experiments, clinical characteristics of primary AML samples, *ex vivo* dose optimization and ARA-C and IDA dual dosing data (PDF)

The authors declare the following competing financial interest(s): C.X.P. and P.T.H. are shareholders of Accelerated Medical Diagnostics Incorporated, a company dedicated to commercializing diagnostic microdosing. The other authors have no relevant conflicts of interest.

induction chemotherapy, leading to improved outcomes for AML patients and reduced treatment-related morbidity and mortality.

Graphical Abstract



INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive, clonal cancer of the blood and bone marrow that is characterized by high morbidity and mortality with an estimated 19,520 new cases and 10,670 deaths in the United States in 2018.[1] The majority of AML patients are treated with 7 + 3 induction chemotherapy, consisting of 7 days of continuous infusion (CIV) with the antimetabolite, cytarabine (ARA-C), and 3 days of bolus infusions with an anthracycline, such as idarubicin (IDA). The standard of care for AML is evolving as four new therapy regimens were approved in 2017, and several others are currently being tested in clinical trials; however, 7 + 3 remains as the backbone of AML care.[2] In addition to 7 + 3, a subset of AML patients, including younger and relapsed or refractory (R/R) patients, can be treated with a more intensive combination of these drugs consisting as a high-dose bolus of both ARA-C and IDA, known as 4 + 3.[3–6] Patients who are not eligible for 7 + 3 or 4 + 3 are typically placed on a less toxic, low-intensity regimen. Importantly, treatment is typically started within 5–7 days of diagnosis.[7–10]

ARA-C is a pyrimidine analogue that is incorporated into DNA, while IDA intercalates into and can covalently bind to DNA. Both drugs initiate cell death by inhibiting DNA replication. Although treatment with 7 + 3 and 4 + 3 can result in complete remission rates of 50–70%, they are associated with significant toxicity and treatment-related mortality, especially in patients >60 years old, which includes the majority of AML patients.[8,11–15] Despite the many known clinical factors that can contribute to patient response and toxicity, there are currently no drug-specific predictive tests available to guide the decision to prescribe AML patients to the 7 + 3 or 4 + 3 induction chemotherapy regimens or to a less intensive treatment regimen.[16,17]

Based on previous studies demonstrating a correlation between ARA-C incorporation into DNA and cytotoxity or patient response,[18–20] we hypothesized that the level of microdose-induced ARA-C- and IDA-DNA incorporation is predictive of patient response to induction chemotherapy. Here, we present *ex vivo* “diagnostic microdosing” as a promising strategy to rapidly predict which AML patients will respond to 7 + 3. Diagnostic microdosing involves

treating primary AML cells with a “microdose,” defined as 1% of the therapeutic dose, of radiolabeled ARA-C or doxorubicin (DOX) (Figure 1), followed by quantification of DNA-bound drug via accelerator mass spectrometry (AMS), an ultrasensitive method for quantifying rare isotopes such as C-14.[21] We have used DOX, an economically feasible and commercially available anthracycline that is structurally and functionally similar to IDA. Diagnostic microdosing mimics the 7 + 3 or 4 + 3 induction chemotherapy regimen that AML patients receive and allows measurement of the pharmacodynamic effect of drug, the formation of drug-DNA complexes. Importantly, microdoses do not induce cell death during the experimental procedure, which could confound the quantification of drug-DNA incorporation.

MATERIALS AND METHODS

ARA-C/DOX.

ARA-C (100 mg/mL) and DOX (2 mg/mL) were generously supplied by the UC Davis Medical Center and Comprehensive Cancer Center pharmacy. ^{14}C-labeled ARA-C (specific activity of 57.8 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA), and ^{14}C-labeled DOX (specific activity of 56 mCi/mmol) was purchased from PerkinElmer (Waltham, MA). ^{14}C-labeled drugs were mixed with unlabeled drugs at indicated ratios to reduce the amount of radiocarbon used and to achieve the desired specific activities required for measurement via AMS. All drug mixtures were freshly prepared immediately prior to use.

Cell Culture.

The 5637, THP-1, and MV-4–11 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The 5637R-resistant cell line was developed as previously described.[22] The A2780 and A2780ADR cell lines were purchased from Sigma-Aldrich (St. Louis, MO). MOLM-13 cells were purchased from AddexBio (San Diego, CA). All cell lines were maintained in the recommended medium at 37 °C in a humidified incubator.

Blood samples were collected with informed consent and under IRB-approved protocols from AML patients prior to treatment with 7 + 3 induction chemotherapy at UC Davis Medical or Comprehensive Cancer Center. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples using Ficoll (GE Healthcare Bio-Sciences, Pittsburgh, PA) gradient separation, de-identified, and viably cryopreserved in a liquid nitrogen biobank. Normal PBMC were not separated from the primary AML cells because the majority of isolated cells were AML blasts due to the patient donors having high white cell and blast counts. Patient response to 7 + 3 was determined upon hematologic recovery, typically 28–42 days following therapy initiation. Patients were considered responders to 7 + 3 if they reach CR, as defined by <5% blasts in the bone marrow, absolute neutrophils >1000/mm^3, and platelets >100,000/mm^3.[23] Table S1 describes the patient characteristics and clinical details for each primary sample. Primary cells were cultured in primary cell media: IMDM (ATCC) + 20% BIT9500 serum substitute (Stemcell Technologies, Vancouver, Canada), 20 ng/mL IL-3, 10 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL GM-CSF (Gold Biotechnology, St.

Louis, MO), and 50 ng/mL SCF (PeproTech, Rocky Hill, NJ) at 37 °C in a humidified incubator.

Cell Viability Assay.

Approximately, 4000–8000 cells from each cell line were seeded per well in 96-well plates and were treated with increasing concentrations of ARA-C or DOX. Adherent cells were seeded 1 day prior to treatment to allow for attachment. After 72 h of continuous treatment, the cells were incubated with viability/ cytotoxicity reagent (Advanced BioReagents, Hayward, CA), and fluorescence was measured at Ex/Em = 530/590 on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). GraphPad Prism software (La Jolla, CA) was used to determine cellular IC_{50} values for each drug.

Cell Treatment and AMS Analysis.

When feasible, all cell lines and primary cells were treated with a “high dose” that is equivalent to the drug C_{max} observed in patients (ARA-C CIV: 1 μM; ARA-C bolus: 300 μM; DOX bolus: 400 nM) and a “low dose” that is equivalent to 1% of the C_{max} (ARA-C CIV: 0.01 μM; ARA-C bolus: 3 μM; DOX bolus: 4 nM).[24–27] In some cell lines, these concentrations were high enough to induce cell death during the treatment incubation period, which would confound the measurement of drug-DNA adducts. As indicated, these cell lines were treated with empirically determined lower doses to prevent cell death.

To quantify ARA-C-DNA incorporation in the bladder cancer cell lines, 1×10^6 cells were seeded on 60 mm dishes, allowed to attach overnight, and treated the following day. To simulate the ARA-C continuous infusion (CIV) in the 7 + 3 dosing regimen, the bladder cancer cell lines were exposed to a high dose of 1 μM ARA-C or a low dose of 10 nM, each supplemented with 8 nM (1000 dpm/mL) ^{14}C-labeled ARA-C for 24 h.[24] To simulate the ARA-C bolus (bolus) in the 4 + 3 dosing regimen, the bladder cancer cell lines were exposed to a high dose of 300 μM ARA-C or a low dose of 3 μM each supplemented with 8 nM (1000 dpm/mL) ^{14}C-labeled ARA-C for 4 h, washed three times with PBS, and incubated in drug-free medium for an additional 20 h. Following ARA-C exposure, the cells were washed three times with PBS and subjected to cell lysis and DNA isolation using the Wizard Genomic DNA Purification system (Promega, Madison, WI). To quantify DOX-DNA incorporation in the ovarian cancer cell lines, 1×10^6 cells were seeded on 60 mm dishes, allowed to attach overnight, and were treated the following day. To simulate a DOX bolus in both the 7 + 3 and 4 + 3 dosing regimens, the ovarian cancer cell lines were exposed to a high dose of 0.1 μM DOX or a low dose of 0.01 μM each supplemented with 0.8 nM (100 dpm/mL) ^{14}C-labeled DOX for 4 h, washed three times with PBS, and incubated in drug-free medium for an additional 20 h. Following the DOX exposure, the cells were washed three times with PBS and subjected to cell lysis and DNA isolation using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), followed by two phenol and one chloroform extractions to remove intercalated DOX as previously described.[28] This protocol left intact covalent DOX-DNA adducts that are known to form as a consequence of DOX metabolism. Removal of intercalated DOX is important, since it is noncovalently bound and would otherwise partially diffuse out from the DNA sample causing variable results. Five to 10 μg of DNA from triplicate samples were converted to graphite followed by AMS analysis

to determine the ^{14}C/total C ratio, which was used to calculate the concentration of drug in each DNA sample.[29]

For AMS analysis in the AML cell lines, 2×10^6 AML cells from each cell line were seeded in suspension culture in 60 mm dishes and were treated the same day. To simulate the ARA-C CIV regimen, the AML cell lines were treated with a low (1.6 nM) or a high (16 nM) dose of ARA-C supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled ARA-C for 24 h. To simulate the ARA-C bolus regimen, the AML cell lines were treated with a low (0.48 μM) or a high (4.8 μM) dose of ARA-C supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled ARA-C for 4 h, washed three times with PBS, and incubated in drug-free media for an additional 20 h. To simulate the DOX bolus regimen, the AML cell lines were treated with a low (0.8 nM) or a high (8 nM) dose of DOX supplemented with 0.8 nM (100 dpm/ mL) of ^{14}C-labeled DOX for 4 h, washed three times with PBS, and incubated in drug-free media for an additional 20 h. Following treatment, DNA was isolated as above and analyzed by AMS. The AML cell lines were dosed in primary cell media to maintain consistency with the more specialized media required for culturing primary AML cells.

For AMS analysis in primary AML cells, the primary sample was removed from liquid nitrogen storage, viably thawed and resuspended in primary cell media, and treated the same day. Two million primary AML cells were treated with 10 nM ARA-C supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled ARA-C (ARA-C CIV microdose), 3 μM ARA-C supplemented with 8 nM (1000 dpm/mL) of ^{14}C-labeled ARA-C (ARA-C bolus microdose), or 4 nM DOX supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled DOX (DOX bolus microdose) for 1 h in primary cell media, followed by DNA isolation and AMS analysis as above. The primary AML cells were treated for a shorter time to reduce the influence of cell death from the thawing process and to minimize the turnaround time needed for a potential *ex vivo* predictive assay.

Statistical Analysis.

Statistical analyses were performed using GraphPad Prism software. The data were analyzed by *t* test or ANOVA with Tukey's multiple comparison test, as indicated. *P* values <0.05 were considered significant.

RESULTS

To show proof of principle, we began by determining the levels of ARA-C-DNA incorporation in a pair of ARA-C-sensitive and -resistant bladder cancer cell lines that were previously described, 5637 and 5637R (Table 1).[22] We designed the cell treatment protocol to simulate the two most common ARA-C-containing regimens received by AML patients: either a low dose ARA-C continuous infusion (CIV) as in 7 + 3 induction chemotherapy or a high dose ARA-C bolus (bolus) as in 4 + 3 induction chemotherapy. The treatment concentrations were chosen so that the high dose was equivalent to the approximate C_{max}. observed in AML patients and the low dose was equivalent to 1% of the C_{max}. One million cells from each cell line were seeded on 60 mm dishes, allowed to attach overnight, and treated the following day. To mimic the ARA-C CIV regimen, the cells were treated with 10 nM (low) or 1 μM (high) of ARA-C that was supplemented with 8 nM (1000 dpm/mL) ^{14}C-

labeled ARA-C for 24 h.[24] To mimic the ARA-C bolus regimen, the cells were treated with 3 μM (low) or 300 μM (high) of ARA-C that was supplemented with 8 nM (1000 dpm/mL) ^{14}C-labeled ARA-C for 4 h, washed, and grown in drug-free media for an additional 20 h.[25] Following the ARA-C treatment, the cells were washed to remove excess radioactive drug from the media, and DNA was isolated. AMS was used to analyze the DNA and calculate the amount of ARA-C incorporated into triplicate DNA samples in units of adducts per 10 million (10^7) nucleotides. As shown in Figure 2A,B and Table S2A, significantly higher levels of ARA-C were incorporated into the DNA of the sensitive cell line, 5637, compared to the resistant cell line, 5637R, at both the high and low doses of the CIV regimen [24.5 ± 0.0 vs 3.51 ± 0.12 ARA-C-DNA adducts/10^7 nt, $p < 0.0001$, *t* test (low dose) and 1560 ± 81 vs 258 ± 11 ARA-C-DNA adducts/10^7 nt, $p < 0.0001$, *t* test (high dose)]. A similar trend was observed in the cells treated using the ARA-C bolus regimen [532 ± 10 vs 358 ± 4.7 ARA-C-DNA adducts/10^7 nt, $p < 0.0001$, *t* test (low dose) and 1130 ± 56 vs 860 ± 57 ARA-C-DNA adducts/10^7 nt, $p < 0.05$, *t* test (high dose)] (Figure 2C,D, Table S2B).

In addition to ARA-C, the 7 + 3 and 4 + 3 induction chemotherapy regimens also contain a bolus treatment with an anthracycline, commonly IDA. However, due to limits on commercial availability, we have used an anthracycline that is structurally and functionally similar to IDA, DOX, to measure anthracycline-DNA incorporation. To show proof of principle, we began by determining the levels of DOX-DNA incorporation in a pair of DOX-sensitive and -resistant ovarian cancer cell lines, A2780 and A2780ADR (Table 1). To mimic the DOX bolus regimen, 1×10^6 cells were seeded on 60 mm dishes and allowed to attach overnight. The next day, the cells were exposed to 0.01 μM (low) or 0.1 μM (high) of DOX that was supplemented with 0.8 nM (100 dpm/mL) ^{14}C-labeled DOX for 4 h, washed, and grown in drug-free media for an additional 20 h.[26,27] Note that these doses are lower than the patient C_{max} to avoid cell death. At 24 h, the cells were washed to remove excess radioactive drug in the media, and DNA was isolated using a protocol developed to remove intercalated DOX, which could confound results.[28] Similar to the results for ARA-C-DNA incorporation, significantly higher levels of DOX were incorporated into the DNA of the sensitive cell line, A2780, compared to the resistant cell line, A2780ADR, at both the low and high doses [5.61 ± 0.12 vs 0.0676 ± 0.0072 DOX-DNA adducts/10^7 nt, $p < 0.0001$, *t* test (low dose) and 24.7 ± 5.0 vs 0.910 ± 0.051 DOX-DNA adducts/10^7 nt, $p < 0.01$, *t* test (high dose)] (Figure 2E,F, Table S2C).

We next adapted the above dosing protocols to measure drug-DNA incorporation in three AML suspension cell lines with a range of cell sensitivities to both drugs: MV-4–11, THP-1, and MOLM-13 (Table 1). Two million cells from each AML cell line were seeded into 60 mm dishes in primary cell media (a specialized medium used for culturing the primary AML cells) and treated the same day. To simulate the ARA-C CIV regimen, the AML cell lines were treated with a 1.6 nM (low) or a 16 nM (high) dose of ARA-C supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled ARA-C for 24 h. To simulate the ARA-C bolus regimen, the AML cell lines were treated with a 0.48 μM (low) or a 4.8 μM (high) dose of ARA-C supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled ARA-C for 4 h, washed, and incubated in drug-free media for an additional 20 h. And to simulate the DOX bolus regimen, the AML cell lines were treated with an 0.8 nM (low) or an 8 nM (high) dose of DOX supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled DOX for 4 h, washed, and

incubated in drug-free media for an additional 20 h. Note that these values are lower than the patient C_{max} values to limit confounding results from cell death, as these cell lines are quite sensitive to ARA-C and DOX. Following treatment, the cells were washed to remove radioactive drug from the media and DNA was analyzed by AMS. As shown in Figure 3, all of the dosing regimens showed a trend of the most sensitive cell line having the highest level of drug-DNA adducts and the least sensitive cell line having the lowest number of drug-DNA adducts, though the separation between the cell lines differed based on the dosing regimens. For example, in the cell lines treated with the ARA-C CIV regimen, the most sensitive cell line (MOLM-13) had the highest levels of ARA-C-DNA incorporation, followed by the intermediate sensitivity cell line (MV-4–11), while the least sensitive cell line (THP-1) had the lowest levels of ARA-C incorporation [18.6 ± 0.78 vs 6.08 ± 0.20 vs 3.16 ± 0.14 ARA-C-DNA adducts/10^7 nt (low dose) and 373 ± 6.0 vs 88.7 ± 4.1 vs 52.4 ± 2.4 ARA-C-DNA adducts/10^7 nt (high dose)] (Figure 3A,B, Table S3A). The overall differences in ARA-C DNA adduct levels between the three cell lines were significant at both doses [$F(2,6) = 485.5$, $p < 0.0001$ (low), $F(2,6) = 1673$, $p < 0.0001$ (high)] as determined by ANOVA. Further, the ARA-C-DNA adduct levels for each cell line were significantly different from each other cell line as determined by Tukey's multiple comparison test. A similar trend was observed after treating the cell lines with the ARA-C bolus regimen [169 ± 11 vs 67.8 ± 2.2 vs 56.0 ± 3.1 ARA-C-DNA adducts/10^7 nt, $F(2,6) = 84.60$, $p < 0.0001$ (low dose) and 417 ± 15 vs 137 ± 2.8 vs 105 ± 3.6 ARA-C-DNA adducts/10^7 nt, $F(2,6) = 351.7$, $p < 0.0001$ (high dose)], with the overall differences between the cell lines being significant as determined by ANOVA and the differences between the MOLM-13 and MV-4–11 cells being significant as determined by Tukey's multiple comparison test (Figure 3C,D, Table S3B). The DOX bolus treated cells showed the same trend [0.581 ± 0.073 vs 0.558 ± 0.12 vs 0.340 ± 0.012 DOX-DNA adducts/10^8 nt (low dose) and 5.22 ± 0.21 vs 5.07 ± 0.26 vs 4.42 ± 0.57 DOX-DNA adducts/10^8 nt (high dose)], but the overall differences between the three cell lines did not reach significance (Figure 3E,F, Table S3C).

We next adapted the diagnostic microdosing procedure for *ex vivo* microdosing of primary AML patient samples in a retrospective pilot study as depicted in Figure 4. PBMCs were isolated by Ficoll gradient separation from clinically annotated AML patient blood samples collected prior to the start of treatment with 7 + 3 induction chemotherapy. The samples were de-identified and viably cryopreserved in a liquid nitrogen biobank. Ten primary AML samples (five responders and five nonresponders) were viably thawed, and 2×10^6 cells per sample were treated *ex vivo* with an ARA-C CIV microdose (10 nM) supplemented with 0.8 nM (100 dpm/mL) ^{14}C-labeled ARA-C, ARA-C bolus microdose (3 μM) supplemented with 8 nM (1000 dpm/mL) of ^{14}C-labeled ARA-C, or a DOX bolus microdose (4 nM) supplemented with 0.8 nM (100 dpm/mL) of ^{14}C-labeled DOX for 1 h in 60 mm dishes in primary cell media followed by AMS analysis. Consistent with our hypothesis, the responsive patients had higher mean drug-DNA incorporation levels compared to the nonresponders for all three microdosing groups (ARA-C CIV: 1.68 ± 0.27 vs 0.797 ± 0.15 ARA-C-DNA adducts/10^7 nt, $p < 0.05$; ARA-C bolus: 36.2 ± 3.3 vs 29.4 ± 5.6 ARA-C-DNA adducts/10^7 nt, $p = 0.3326$; DOX bolus: 4.37 ± 1.4 vs 0.998 ± 0.44 DOX-DNA adducts/10^8 nt, $p = 0.0503$, Figure 5A–C, Table S4). As shown in Figure 5D,E, the patient

ARA-C-CIV and DOX-DNA adduct levels can be further categorized into high (all responders), low (all nonresponders), and intermediate (mixed response) as an initial stratification scheme for predicting patient response. If these two metrics (ARA-C CIV and DOX bolus) are combined, as in Figure 5F, a clear separation in adduct levels between the patient responders and non-responders can be observed.

Importantly, for the diagnostic microdosing assay, the radiocarbon levels in the DNA from three replicates of each drug-exposed primary AML sample fell in a small range (Figure S1A,C,E, Table S4). Furthermore, the drug-DNA adducts were at least 5–10-fold above the background (Figure S1B,D,F). The 1 h dosing time was chosen to prevent confounding results from cell death due to thawing and growing primary cells in culture and to reduce processing time for any potential diagnostic microdosing assay. Moreover, increasing the *ex vivo* dosing time to 4 or 24 h did not improve separation between responders and nonresponders (Figure S2, Table S5). Furthermore, we observed that the addition of an unlabeled IDA microdose to the ARA-C CIV dosing regimen did not influence the level of ARA-C-DNA adducts under these conditions (Figure S3, Table S6).

DISCUSSION

Though 7 + 3 is currently the most commonly used first-line therapy for AML, it is not effective for all patients and is often accompanied with significant toxicities. Thus, a test that predicts 7 + 3 responders and nonresponders has the potential to improve the treatment of AML patients and reduce therapyrelated morbidity and mortality. We hypothesized that we could use the formation of ARA-C and DOX-DNA adducts as a biomarker to predict patient response.

ARA-C kills cells by incorporating into cellular DNA and halting DNA replication. Therefore, factors that reduce the intracellular concentration of triphosphorylated cytarabine (ARA-CTP), the active form of ARA-C, may induce chemoresistance in AML patients. These factors include reduced influx of ARA-C by the human equilibrate nucleoside transporter 1 (hENT1), reduced phosphorylation by deoxycytidine kinase (dCK), and increased degradation by cytoplasmic 5′-nucleotidase (5NT) and/or cytidine deaminase (CDD). Increased levels of DNA polymerase α (DNA POL) and reduced levels of topoisomerase I (TOPO I) and topoisomerase II (TOPO II) have also been detected in ARA-C-resistant cell lines (reviewed by Galmarini et al.).[30] Many of the ARA-C resistance mechanisms modulate the degree to which the drug is metabolized and incorporated into DNA. Therefore, it is logical to utilize ARA-C-DNA levels as a potential biomarker of response to treatment.

Similar to ARA-C, DOX kills cells by diffusing into the nucleus, interacting with cellular DNA, and initiating a series of signaling events that culminates in apoptosis. The best understood of these events involves the interaction between DOX and topoisomerase IIα (TOP2A).[31] TOP2A is involved in separating entangled DNA strands, and as part of its function, it transiently generates and then repairs protein-bound double-strand DNA breaks (DSBs).[32] DOX stabilizes the cleaved-strand intermediate, suppressing the completion of the process, resulting in numerous DSBs.[31] DSBs have numerous negative consequences for

cells and notably trigger caspase-dependent apoptosis programs. This process involves the activation of master regulators p53 and forkhead box O3 (FOXO3) and suppression of pro-growth signaling pathways, leading to changes in the ratio of anti/pro-apoptotic Bcl-2 family proteins.[33] Multiple other mechanisms have been observed to be involved in DOX cytotoxicity, including the formation of TOP2A-independent DNA adducts,[34] inhibition of DNA and RNA synthesis, and mitochondrial reactive oxygen species (ROS) production triggering apoptosis.[35] Furthermore, a reduction in the amount of DOX diffused into the cells, which is controlled by many mechanisms, including the ATP-binding cassette (ABC) family of transporters (also known as multiple drug resistance (MDR) proteins), could lead to reduced DOX sensitivity. Our assay protocol features exhaustive phenol/chloroform extraction of the [^{14}C]DOX-exposed DNA, which removes essentially all of the noncovalently bound intercalated drug from the DNA sample. Therefore, the resulting radiocarbon signal is predominantly due to covalent DOX-DNA adducts, which act as a surrogate measure of total drug-target engagement.

Here, we have demonstrated the ability to quantitate the incorporation of ARA-C and DOX into the DNA of cancer cell lines and primary AML cells that have a range of ARA-C and DOX sensitivities. We observed a positive correlation between higher ARA-C-DNA incorporation and lower cellular IC_{50} values of a paired set of sensitive and resistant non-AML cancer cell lines and in three AML cell lines with a range of ARA-C IC_{50} values. Furthermore, we observed a positive correlation between ARA-C- and DOX-DNA incorporation levels and response in primary AML cells from a cohort of ten patients (five 7 + 3 responders and five nonresponders). Our results are consistent with previous leukemia studies that observed a correlation between ARA-C-DNA incorporation and cellular cytotoxicity and patient response, though we have improved the technique.[18–20] Kufe et al. (1980) and Major et al. (1981) measured the incorporation of [^{3}H]ARA-C into the DNA of two leukemia cell lines, as well as blast cells from an AML patient, and observed a significant correlation with clonogenic survival of the cells and blasts but did not relate this to patient clinical response. Using a different technique, Raza et al. (1992) were able to correlate the level of [^{3}H]ARA-C incorporation into the DNA of pretherapy AML cells isolated from bone marrow samples and patient clinical response to high-dose ARA-C. This group incubated the AML cells with [^{3}H]ARA-C followed by plating the cells on slides and measuring the number of radioactive grains per S-phase cell by autoradiography. Though there was some overlap in the ranges, they observed a higher mean ARA-C incorporation in AML patients who had a complete remission after treatment with single agent high-dose ARA-C compared to non-responders, and this difference reached significance in the subset of relapsed/refractory AML patients. However, the ARA-C incorporation levels were nearly identical between the responders and nonresponders to treatment with high-dose ARA-C plus mAMSA, indicating the level of ARA-C incorporation may not predict patient response to all ARA-C-containing therapies, at least with this method. These results led them to conclude that there may be a minimum amount of ARA-C that needs to be incorporated in order for a patient to respond, but that a high level of ARA-C incorporation does not necessarily indicate a patient will respond. Though we have presented data from fewer patients here, we similarly observed a higher level of ARA-C- and DOX-DNA adducts in the 7 + 3 responders compared to the nonresponders. Furthermore, we observed less overlap in

the level of ARA-C incorporation between the responders and nonresponders, and by combining the incorporation levels of both ARA-C and DOX, we are able to observe a clear separation of the two groups. Additionally, these data bolster our group's documented correlations between diagnostic microdosing-induced carboplatin-DNA adduct levels and clinical response and overall survival in lung and bladder cancer patients.[36,37]

In contrast to the previous leukemia studies and our previous microdosing studies, the diagnostic microdosing assay described here is feasible for use as a clinical tool because it is highly sensitive, has a rapid turnaround time, and can be performed *ex vivo*. We have shown the feasibility of using ultrasensitive AMS to analyze ARA-C incorporation, which allows us to minimize the radioactivity used in our assay to 1.35 nCi per sample, compared to the 10–15 μCi used per sample to measure ARA-C incorporation via liquid scintillation counting or autoradiography in the previous studies. In addition to reducing the amount of radioactivity needed, the use of AMS for the measurement of drug-DNA adduct formation allows us to use nontoxic doses of chemotherapy. The physiologically relevant concentrations of chemotherapy used for measurement via less sensitive techniques often lead to a decrease in cell viability, which can obscure the differences in drug sensitivity for primary AML cells, at least from the perspective of using drug-DNA adducts as predictive biomarkers. Another advantage of C-14 labeling and AMS includes the use of simple catalysts to convert the DNA samples to graphite, which does not rely on enzyme-mediated hydrolysis of phosphodiester linkages or cleavage of phosphates that can be inhibited by the presence of adducts.[29] However, analysis via AMS requires a C-14 labeled drug and does not provide information, such as the specific adduct type, that may help improve the predictive ability of the assay. These concerns are minimal in the case of ARA-C and DOX, since ARA-C is incorporated as an alternative to the canonical 2′-deoxynucleosides, and covalently bound DOX is known to occur specifically at the exocyclic amine of guanine residues.[38]

Moreover, our test can be completed in less than a week, which is similar to or faster than the previous protocols that can require up to a week of exposure to measure the radioactivity alone. Finally, an improvement from our previous microdosing studies is the ability to perform diagnostic microdosing on the cells *ex vivo* through isolation of cells from a standard of care blood draw or bone marrow biopsy, which eliminates the need to expose patients to the radioactive drug. Furthermore, *ex vivo* cytotoxicity studies on AML cells isolated from R/R patients have recently been shown to correlate with patient response and show promise for the use of *ex vivo* assays for personalizing and improving AML therapy.[39–41]

Although we tested a limited number of patient samples, the initial data presented herein provide proof-of-concept for a predictive biomarker test specific to 7 + 3 chemotherapy in AML. Limitations of the study include: (1) use of doxorubicin as a surrogate for IDA or DNR in the test protocol, (2) use of white blood cells without separation of blasts (the samples we tested were 50–90% blasts, determined by complete blood counts and, in some cases, flow cytometry), (3) lack of examination of older versus younger patients and those with relapse/refractory disease in previously treated patients and other patient subpopulations such as FLT3 mutant blasts, and (4) lack of comparison of fresh and frozen samples from the same patients and lack of validation of the test with respect to accuracy,

precision, robustness and other parameters common to clinical diagnostics tests. These limitations may cause confounding factors, such as differential cellular uptake and DNA adduct formation from IDA and DNR compared to DOX, the selection of hardier cells after freeze/thaw that have an altered phenotype, and small but significant contributions to the biomarker levels from normal PBMCs and others. These potential limitations will be examined in a larger confirmatory study that will include sufficient patient samples to determine a statistically significant and clinically testable threshold values that differentiate AML patients according to 7+ 3 response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

4 + 3,	4 days high dose ARA-C plus 3 days IDA bolus
5NT 5′	nucleotidase
7 + 3	7 days continuous infusion ARA-C plus 3 days IDA bolus
ABC	ATP-binding cassette
AML	acute myeloid leukemia
AMS	accelerator mass spectrometry
ANOVA	analysis of variance
ARA-C	cytarabine
ARA-CTP	triphosphorylated cytarabine
CDD	cytidine deaminase
CIV	continuous infusion
C_{max}	maximum serum concentration of a drug
CR	complete response/remission

dCK	deoxycytidine kinase
DNA	deoxyribonucleic acid
DOX	doxorubicin
DSB	double-strand break
FDA	Food and Drug Administration
FOXO3	forkhead box O3
hENT1	human equilibrate nucleoside transporter 1
IDA	idarubicin
mAMSA	amsacrine
MDR	multiple drug resistance
MRC	myelodysplasia-related changes
NOS	not otherwise specified
nt	nucleotide
PBMC	peripheral blood mononuclear cells
ROS	reactive oxygen species
R/R	relapsed or refractory leukemia
TOPO	I/II/2a topoisomerase I/II/2a
WHO	World Health Organization

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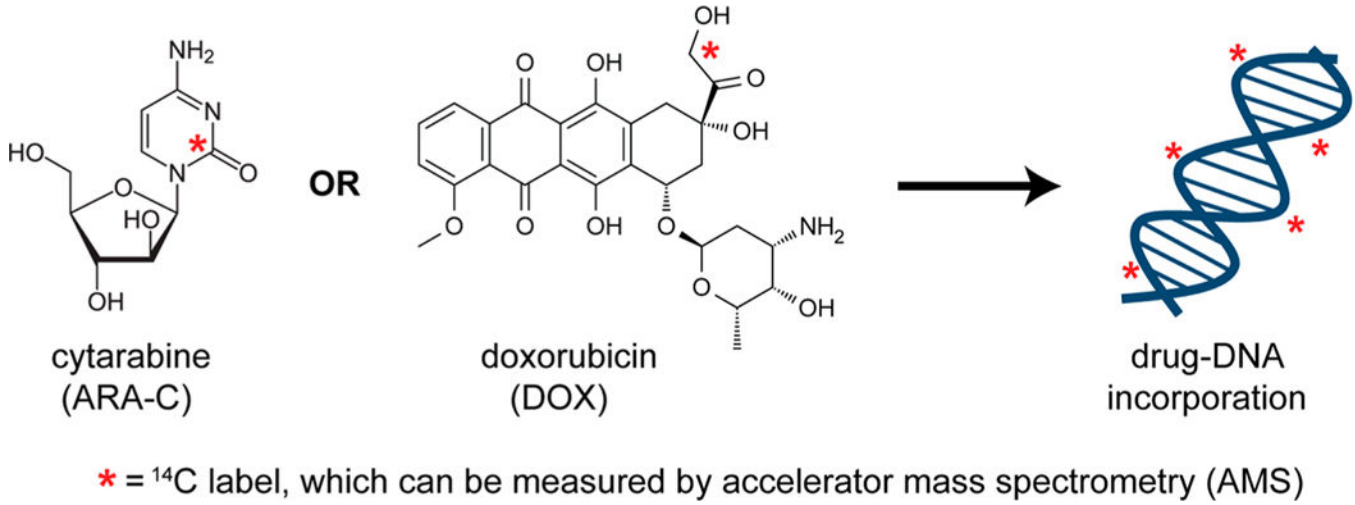

Figure 1.
Structure of radiolabeled cytarabine and doxorubicin and schematic of DNA incorporation that can be measured via AMS.

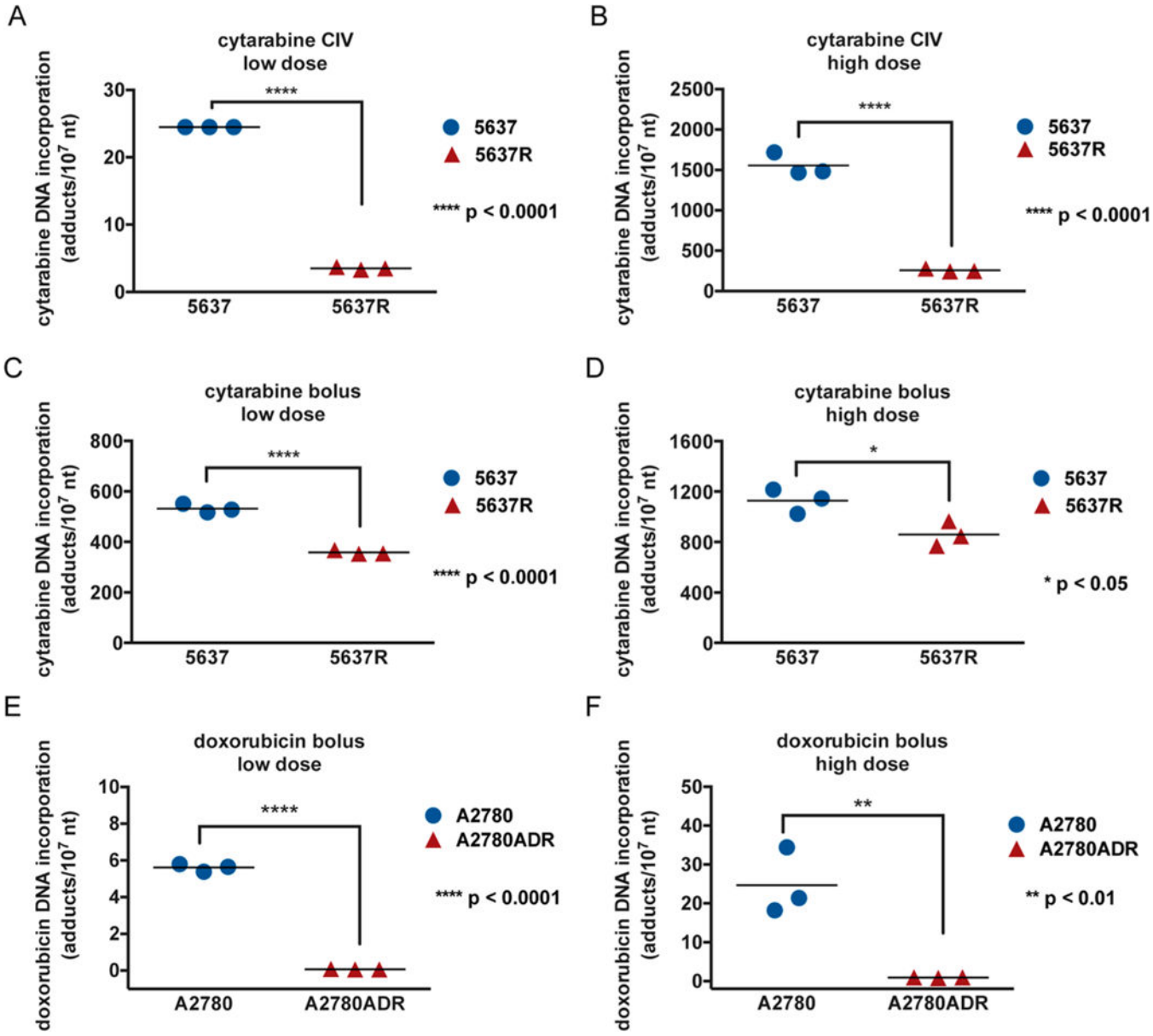

Figure 2.

Proof-of-principle experiments in paired sensitive and resistant cancer cell lines show drug-DNA adduct levels correlate to cellular IC_{50}. (A, B) ARA-C-DNA adduct levels in paired bladder cancer cell lines after a 24 h exposure to a low or a high dose of ARA-C (CIV). (C, D) ARA-C-DNA adduct levels in paired bladder cancer cell lines after a 4 h exposure to a low or a high dose of ARA-C, followed by 20 h in drug-free media (bolus). (E, F) DOX-DNA adduct levels in paired ovarian cancer cell lines after a 4 h exposure to a low or a high dose of DOX, followed by 20 h in drug-free media. Drug-DNA incorporation levels are significantly higher in the sensitive cell line for both drugs. Values are shown as replicates with line indicating mean.

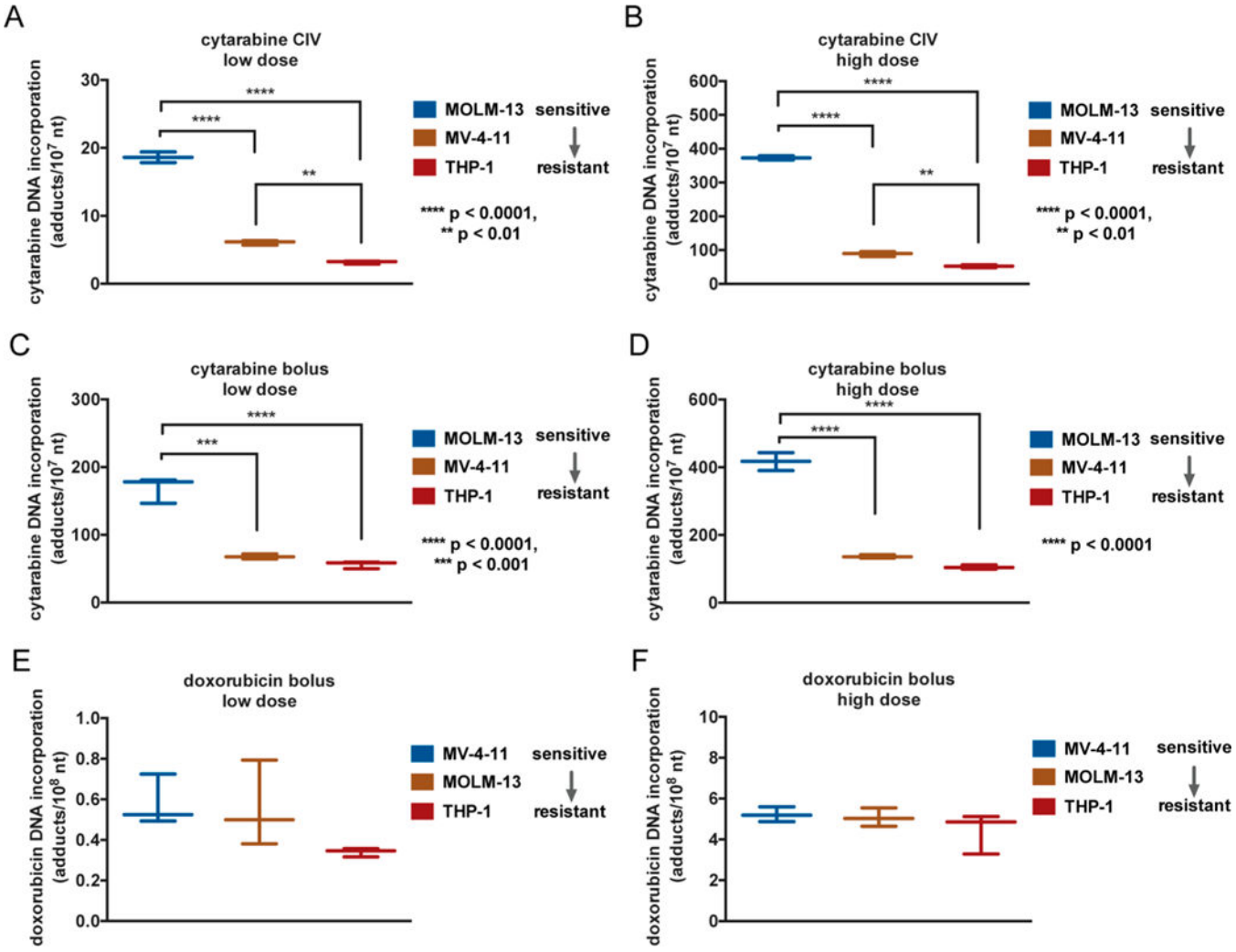

Figure 3.

ARA-C- and DOX-DNA adduct levels correlate to cellular IC_{50} of three AML cell lines. (A, B) ARA-C-DNA adduct levels after a 24 h exposure to a low or a high dose of ARA-C (CIV). (C, D) ARA-C-DNA adduct levels after a 4 h exposure to a low or a high dose of ARA-C followed by 20 h in drug-free media (bolus). (E, F) DOX-DNA adduct levels after a 4 h exposure to a low or a high dose of DOX followed by 20 h in drug-free media. Mean drug-DNA incorporation levels are highest in the most sensitive cell line and lowest in the least sensitive cell line for both drugs with statistically significant differences noted. Values are shown as medians (line) with min to max (whiskers) of three replicates.

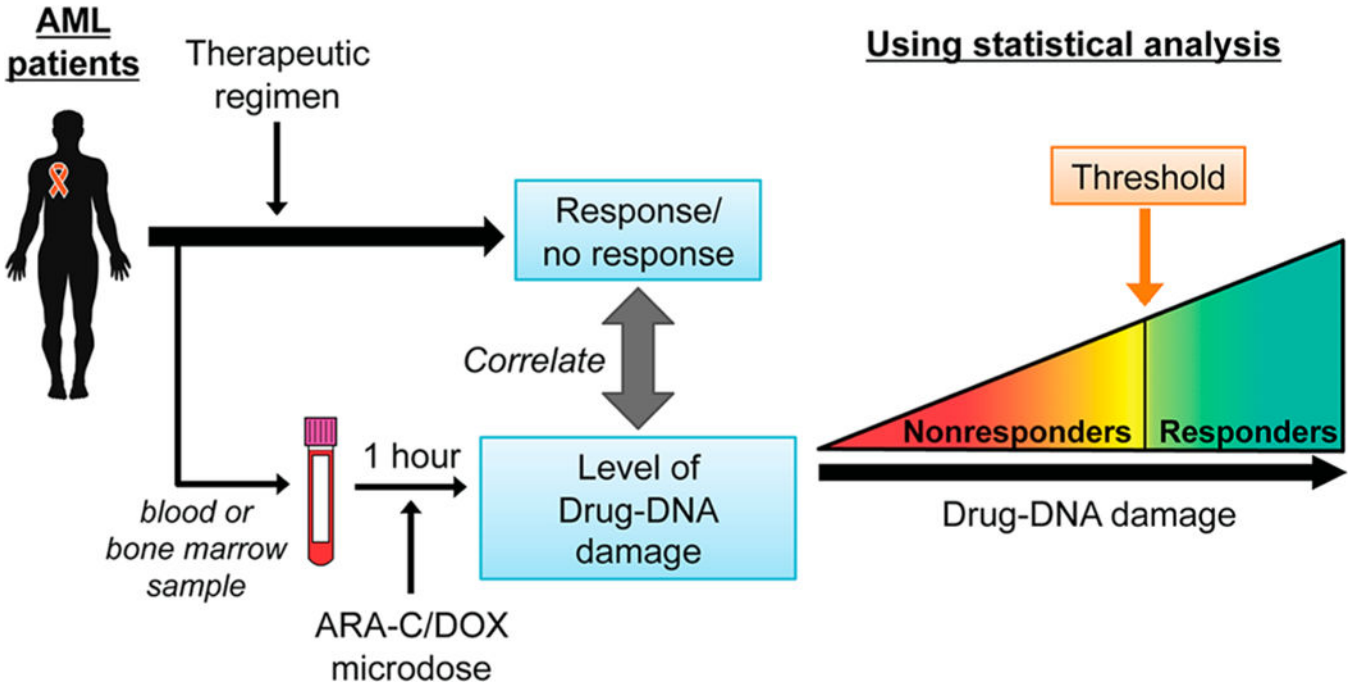

Figure 4.

Strategy for developing an *ex vivo* microdose-based diagnostic test to predict ARA-C and DOX response in AML patients.

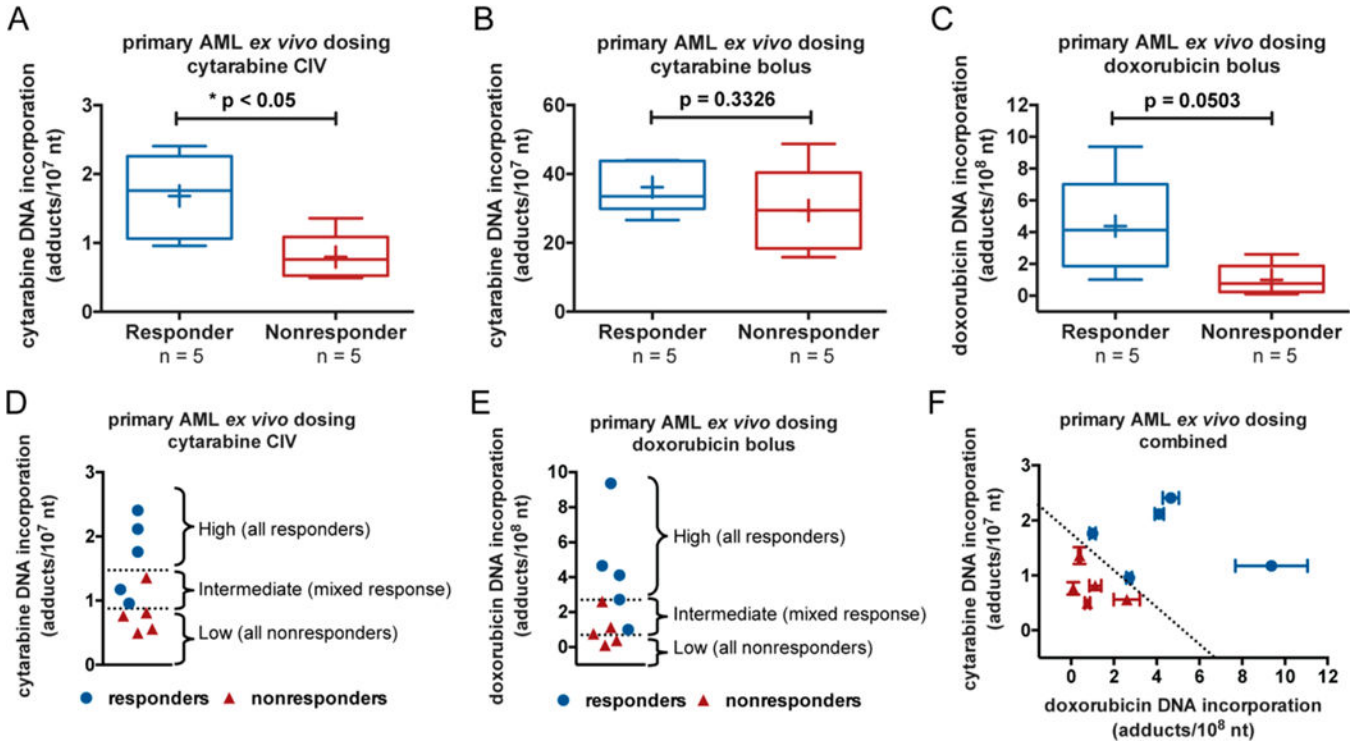

Figure 5.

ARA-C- and DOX-DNA adduct levels correlate to patient response. (A–C) ARA-C- or DOX-DNA incorporation levels after a 1 h *ex vivo* microdosing of PBMCs isolated from primary AML patient samples. AML cells from the responsive patients had significantly higher drug-DNA damage levels in the ARA-C CIV dosing condition and nearly significant difference in the DOX bolus dosing condition. Values are shown as median (line), mean (+), and 25th to 75th percentiles (box) with min to max (whiskers), for the groups of responders or nonresponders. (D, E) ARA-C- (CIV) and DOX-DNA adduct levels were categorized into high (all responders), low (all nonresponders), and intermediate (mixed response) as well as plotted together (F). Values are shown as the mean of three replicates for each primary sample with SEM displayed in (F).

Table 1.

Cell Line IC_{50} Values with 72 h Continuous Treatment (μM)

cell line	5637	5637R	MV-4–11	THP-1	MOLM-13	A2780	A2780ADR
cytarabine	5.687	60.12	0.353	4.400	0.018	–	–
doxorubicin	–	–	0.003	0.024	0.004	0.021	11.99

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