Pharmacological Profiles of Acute Myeloid Leukemia Treatments in Patient Samples by Automated Flow Cytometry: A Bridge to Individualized Medicine

Teresa A. Bennett,1 Pau Montesinos,2 Federico Moscardo,2 David Martinez-Cuadron,2 Joaquin Martinez,3 Jorge Sierra,4 Raimundo García,5 Jaime Perez de Oteyza,6 Pascual Fernandez,7 Josefina Serrano,8 Angeles Fernandez,9 Pilar Herrera,10 Ataulfo Gonzalez,11 Concepcion Bethancourt,12 Gabriela Rodríguez-Macias,13 Arancha Alonso,14 Juan A. Vera,15 Begoña Navas,16 Esperanza Lavilla,17 Juan A. Lopez,18 Santiago Jimenez,19 Adriana Simiele,20 Belen Vidrales,21 Bernardo J. Gonzalez,22 Carmen Burgaleta,23 Jose A. Hernandez Rivas,24 Raul Cordoba Masuñano,25 Guiomar Bautista,26 Jose A. Perez Simon,27 Adolfo de la Fuente,28 Consolación Rayón,29 Iñaki F. Troconiz,30 Alvaro Janda,30 Andrew G. Bosanquet,31 Pilar Hernandez-Campo,1 Daniel Primo,1 Rocio Lopez,1 Belen Liebana,1 Jose L. Rojas,1 Julian Gorrochategui,1 Miguel A. Sanz,2 Joan Ballesteros1

Abstract

We have estimated the pharmacological sensitivity and synergism of 125 individual patient samples for all drugs and combination treatments for acute myeloid leukemia in the context of the overall patient population. Each ex vivo pharmacological profile identifies drugs and treatments for which the patient’s malignant cells are particularly sensitive or resistant, assisting in the selection of individualized treatments.

Background: We have evaluated the ex vivo pharmacology of single drugs and drug combinations in malignant cells of bone marrow samples from 125 patients with acute myeloid leukemia using a novel automated flow cytometry—based platform (ExviTech). We have improved previous ex vivo drug testing with 4 innovations: identifying individual...
leukemic cells, using intact whole blood during the incubation, using an automated platform that escalates reliably data, and performing analyses pharmacodynamic population models. **Patients and Methods:** Samples were sent from 24 hospitals to a central laboratory and incubated for 48 hours in whole blood, after which drug activity was measured in terms of depletion of leukemic cells. **Results:** The sensitivity of single drugs is assessed for standard efficacy (E_max) and potency (E_C50) variables, ranked as percentiles within the population. The sensitivity of drug-combination treatments is assessed for the synergism achieved in each patient sample. We found a large variability among patient samples in the dose-response curves to a single drug or combination treatment. **Conclusion:** We hypothesize that the use of the individual patient ex vivo pharmacological profiles may help to guide a personalized treatment selection.

**Introduction**

Acute myeloid leukemia (AML) is the most common acute leukemia affecting adults. AML is an extremely heterogeneous disease, with > 50 cytogenetic and molecular genetic markers identified to date. These genetic markers, along with patient-related factors, are used to define several subtypes of AML, with treatment and prognosis varying among subtypes. Genomic and molecular findings have helped stratify patients to guide treatment selection, and new strategies are necessary to individualize treatments.

Current efforts to personalize treatments in hematological neoplasms, such as AML, rely mostly on genomic and genetic prognostic factors, which stratify rather than individualize patients for treatments. A more direct approach would be to evaluate the pharmacological activity of drugs directly in the individual patient’s bone marrow sample (ex vivo). Ex vivo assays for detecting cell death inducible by drugs for hematological neoplasms have been in development for over 35 years. There now exist several functional assays for detecting activity in ex vivo samples, collectively known as individualized tumor response testing (ITRT). The term refers specifically to studies that measure the effect of different treatments against cancer on live tumor cells from an individual patient, excluding measurements in subcellular fractions, animal samples, or cell lines. However, current ITRT methods have significant limitations that have restricted their clinical usefulness.

We have developed a method to test a patient’s bone marrow sample ex vivo, using a novel automated flow cytometry-based screening system called ExviTech (ex vivo Technology), which may overcome previous barriers for these assays. The purpose of this study was to examine the ex vivo pharmacology of single drugs used to treat AML, and combinations of these drugs, against the malignant cell population in bone marrow samples from 86 to 125 AML patients, characterizing the specific-patient pharmacodynamic parameters to guide treatment individualization.

**Patients and Methods**

**Patients**

Vivia-PMAML (Study of the correlation between the ex vivo response to antineoplastic drugs and their efficacy in the treatment of AML), a noninterventional and prospective study, included bone marrow (BM) samples from adult patients over 18 years of age who were diagnosed with de novo AML in Spanish centers from the Programa Español de Tratamientos en Hematología (PETHEMA) group. All patients gave informed consent for study participation. Bone marrow samples from 177 patients from 26 hospitals participating in the study were enrolled from September 2011 to August 2012 at the moment of this interim analysis. Of these, 52 samples were not evaluable by the laboratory. Finally, 125 bone marrow samples from adult AML patients were successfully incubated for 48 hours and analyzed to characterize the cytotoxic effect of drugs used for the treatment of AML. We have received clinical response information for 78 of the 125 samples reported. These 78 samples were from patients with an average age of 57 years (range, 26-88 y); 45 patients were male and 33 female.

**Methods**

Figure 1 displays the overall method of data acquisition: On day 1, the patient sample was received. A small part was separated for validation, and the majority was diluted with culture media and plated into 96-well plates previously prepared with the desired drugs and drug combinations. The number of live leukemic cells seeded in each well was fixed between 8000 and 32,000, depending on the percentage of leukemic cells for each sample. These plates were incubated for 48 hours and analyzed on day 3. Antibodies shown were added to identify leukemic cells using a gating strategy based on forward scatter (FSC) or side scatter (SSC) and expression or lack of expression of different surface markers. The monoclonal antibodies selection was performed to optimize the identification of leukemic cell in each sample. The aim of our analysis is not the phenotypic characterization but only the identification of these cells. According to this, the markers that Euroflow has pointed out as the “backbone markers” for AML, CD34, CD45, CD117, and human leukocyte antigen—DR (HLA-DR), were included in our combination. They allowed us to identify the leukemic cells in almost 90% of AML patients. This allowed for the selection of the 2 best antibodies for unequivocal identification of the pathological cell population in each particular sample. We used 3 antibody combinations: CD117/CD45 for 56% of samples, CD34/CD45 for 31%, and CD117/CD45 for 13%. Live leukemic cells were identified by their light scatter properties classified as high, intermediate, or low (FSC^high/SSC^low) in the absence of annexin-
Ve fluorescein isothiocyanate (FITC) staining. FSC/SSC selection was performed to exclude debris. The average percentage of cell viability on receipt of the sample was 80%, and samples were only processed if the viability was greater than 50%.

Sample Validation. BM samples were extracted under sterile conditions and were received in the laboratory within 24 hours of extraction. Initial analysis evaluated the number of pathological cells and their viability. All reagents and drugs were obtained from Sigma (St. Louis, MO) unless otherwise noted. Briefly, different volumes of sample (1 mL, 3 mL, 5 mL, and 7 mL) were aliquoted in duplicate into a 96-well plate. To lyse red blood cells, 180 mL of ammonium chloride lysis solution was added to each well (2 g KHCO₃, 16.58 g NH₄Cl, 0.074 g Na₂-ethylenediaminetetraacetic acid [EDTA]/C₁₅₂H₂₂O, H₂O to 1 L). Following a 10-minute incubation period at 4°C, each plate was centrifuged for 5 minutes at 1200 rpm and the supernatant removed. The lysis step was performed twice. To analyze, 20 mL of a combination of annexin-V–FITC (Immunostep, Salamanca, Spain), binding buffer (BB) (2.4 g 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], 8.19 g NaCl, 0.37 g Cl₂Ca, H₂O to 1 L), and the following monoclonal antibodies (MoAbs) were added to each well: CD117 (clone 104D2)-PE (Becton Dickinson, San Jose, CA), CD34 (clone 581)-PerCP (BioLegend, San Diego, CA), HLADR (clone L243)-PB (BioLegend), and CD45 (HI30)-PO (Life Technologies, Carlsbad, CA). After 15 minutes of incubation at room temperature in the dark, a wash step was performed using BB solution. The pellet was resuspended in 20 mL of BB for analysis in Vivia’s ExviTech platform.

ExviTech Platform. This novel flow cytometry based system incorporates a CyAn ADP cytometer (Beckman Coulter, Brea, CA)
and Vivia’s novel End Point Sampler (EPS) plate handler (Saryna Technologies, San Diego, CA). The EPS aspirated the contents of each well of the assay plate, and it delivered the contents to the flow cell of the cytometer. Each 96-well assay plate was collected as a single .fcs file from the CyAn cytometer. The EPS was run from the same computer as the cytometer, recording a second file for each plate. This timing file was integrated with the .fcs file for data analysis by our proprietary software program, FCS Analyzer (Saryna Technologies). This program was designed to separate the acquired data from the cytometer into specific groups and assign well numbers to each group. Each 96-well plate was then analyzed as a single file, and each well could be examined individually as needed.

**Data Analysis.** Summit software (Beckman Coulter) was used for the initial analysis. Identification of pathological cells was performed using a gating strategy based on FSC/SSC and expression or lack of expression of the different MoAb markers. We did not measure apoptosis; we measured depletion. Depletion was measured as the difference in the number of live cells in a well with drugs vs. the control wells without drugs. Once, the pathological cell subset was identified, we use annexin-V to exclude dying cells and measure only the number of live cells, in the drug wells and in the control wells. Those cells without annexin-V staining and appropriate FSC/SSC were considered live cells.21 As the results of our analysis relied on the number of live cells remaining at the end of the assay, it was sufficient to identify this population using FSC/SSC and lack of staining with annexin-V–FITC. There was no need to distinguish between apoptosis and necrosis, and necrotic leukocytes change in both FSC and SSC, and thus the use of propidium iodide was not deemed necessary. Using the above parameters, FCS Analyzer was used to determine the effect of each of the individual drugs. Data was transferred to ActivityBase (IDBS, Guilford, UK), our data base program for final analysis.

**Population Pharmacodynamic Modeling.** In this evaluation, drug response was measured using the absolute number of live malignant cells. For each monotherapy treatment, the response vs. concentration relationship was described following the population approach with NONMEM 7.2.22 The estimation method used for the current pharmacokinetic evaluation was the First Order Conditional Estimation (FOCE) with the INTERACTION option. Interpatient variability (IPV) was associated with each of the previously defined pharmacodynamic parameters. Interpatient differences were also allowed for the variance accounting for the residual variability. In this analysis, the covariate effects of the patient characteristics were not explored. The pharmacodynamic interactions among 2 or 3 drugs in combination treatments was characterized by computing the standard combination index (CI) as defined by Chou & Talalay,23 which is a parameter representing synergism.

**Results**

**Whole Sample vs. Isolated Leukocytes**

We have pioneered using a whole sample rather than isolated leukocytes to incubate patient primary cells with the drugs.24 Leukocytes were isolated only after incubation, when drug-induced cell death had already occurred in the whole-sample environment. Figure 2 shows the significant differences in the pharmacological behavior for both methods. The top panel shows the dose responses of IDA and CYT on 1 patient sample in terms of their survival index, the percentage of live cells relative to control wells without drug, which decreases as drug concentration increases toward the right. Dose responses using whole sample are colored blue, and those using isolated leukocytes are colored red. Whereas IDA shows a significant shift in its potency or EC_{50} of nearly 10-fold, CYT shows no difference. The lower panels show the same comparison for each drug in terms of their EC_{50} (potency) values for 8 patient samples. The middle panel shows differences in potency for CYT ordered from largest to smallest; the first 3 samples show large differences, whereas the last 6 samples show no significant differences. The bottom panel shows the potency differences for the same 9 samples for IDA: Differences in potency for IDA are not related to differences for CYT, as the top-panel example shows. In most cases, isolated leukocytes have higher potency and lower EC_{50}, compared with those of the whole sample.

**Pharmacological Profiles Ex Vivo of Single Drugs**

The pharmacological properties potency, estimated as the EC_{50} (effective concentration inducing 50% cell death), and efficacy, estimated as the E_{MAX} (effective maximum response), were determined for each drug in each sample. The pharmacodynamic parameters for the 8 tested drugs are shown in Table 1, and their estimate the response of any drug at a given concentration by the number of malignant cells left alive after 48 hours of incubation. The efficacy is quantified by the parameter E_{MAX} and is the maximum decrease fraction in response with respect to baseline that the drug is able to induce. The potency is quantified by the parameter EC_{50} and is the value of drug concentration eliciting half of E_{MAX}, and γ is the parameter controlling the steepness of the response vs. concentration curve. During analysis, both the typical and participant-specific estimates of E_{MAX} were constrained between 0 and 1 using the logistic transformation. For presentation of data, the survival index was computed, with the number of live leukemic cells in control wells that were not exposed to any drugs being set as 100%. The number of live cells in each drug-treated well was compared with this control value, and the survival index for each drug at each concentration was determined as the percentage of live pathologic cells at every tested concentration.

Interpatient variability (IPV) was associated with each of the previously defined pharmacodynamic parameters. Interpatient differences were also allowed for the variance accounting for the residual variability. In this analysis, the covariate effects of the patient characteristics were not explored. The pharmacodynamic interactions among 2 or 3 drugs in combination treatments was characterized by computing the standard combination index (CI) as defined by Chou & Talalay,23 which is a parameter representing synergism.
Whole Sample vs. Isolated Leukocytes. (A) Dose-Response Curves for Idarubicin (IDA) and Cytarabine (CYT) in Isolated Leukocytes and Whole Sample. Data, From Sample 6 Below, Display a Logarithmic Difference in the Potencies (EC_{50}s) for IDA but Equal Results for CYT. (B) The EC_{50} (y-Axis) of the Whole Sample and the Isolated Leukocyte Fraction From 9 Patient Samples With CYT. (C) The EC_{50} of the Same Samples With Idarubicin.
average dose-response curves, obtained by simulating the typical parameter estimates (Table 1), are shown in Figure 3A. (Note that population models refer to typical rather than average values, with an equivalent meaning.) The survival index is displayed on the y-axis, starting at 100% and decreasing with increasing drug concentration. All drugs tested show a significant measurable effect at depleting leukemic AML blast cells ex vivo, in a dose-dependent manner, with significant differences among drugs. Curves to the left show the death of AML blasts at lower concentrations and are thus more potent than right-shifted curves. There was good correspondence between median potency of the drugs and their generally perceived clinical efficacy. Starting from the left, active at lower doses, we see the cluster of the 3 potent anthracyclines IDA, MIT, and DAU (blue). These are followed by the group of nucleoside analogues (red), starting from the left, with CLO, FLU, CYT, and THIO. CLO stands out because its average dose-response curve shows submaximal efficacy (E\(^{\text{MAX}}\); it cannot kill all AML blasts. CYT and FLU have a similar behavior, better than CLO but on average unable to induce complete leukemic cell death. THIO was the least potent drug tested, and its E\(^{\text{MAX}}\) was fixed to 0 to improve the fitting (IPV not estimated in Table 1). This is shown in Table 1 by the 0 or near 0 values of their typical E\(^{\text{MAX}}\) values and the minimal IPV of their E\(^{\text{MAX}}\). For IDA and DAU, the E\(^{\text{MAX}}\) was fixed to (complete response) rather than calculated, improving the accuracy of the population-model fitting, and their IPV was hence not estimated.

In order to more fully evaluate the range of patient-specific responses, we have estimated the IPV in drug responses. The range of the IPV can be seen in Figure 3B. The individual dose-response curves for FLU (gray) are displayed in the background of the average dose responses shown in Figure 3A. The individual dose responses of FLU cover a wide range, some reaching left into the area of the potent anthracyclines, and for other patients, to the right into the range of the weakest drugs. Thus, ex vivo interpatient variability is a factor to be taken into consideration and is as important as the commonly used relative strength of the average dose-response curves of these drugs.

Figure 4 shows the complete set of each individual patient dose response (gray) for the 8 drugs tested. The red line is the average response, as displayed in Figure 3. Among the anthracyclines, sensitivity represented by their potencies is more variable for MIT (IPV = 221) than IDA (IPV = 157) or DAU (IPV = 135), as can be observed in their dose-response curves in Figure 4. ETO

<table>
<thead>
<tr>
<th>DRUG</th>
<th>Median SE</th>
<th>Median SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYT-IDA</td>
<td>0.486</td>
<td>0.14</td>
</tr>
<tr>
<td>CYT-FLU</td>
<td>0.548</td>
<td>0.24</td>
</tr>
<tr>
<td>CYT-IDA-FLU</td>
<td>0.441</td>
<td>0.13</td>
</tr>
<tr>
<td>CYT-DAU</td>
<td>0.743</td>
<td>0.26</td>
</tr>
<tr>
<td>CYT-MIT</td>
<td>0.533</td>
<td>0.20</td>
</tr>
<tr>
<td>CYT-DAU-FLU</td>
<td>0.677</td>
<td>0.27</td>
</tr>
<tr>
<td>CYT-MIT-FLU</td>
<td>0.317</td>
<td>0.09</td>
</tr>
<tr>
<td>CYT-IDA-ETO</td>
<td>0.535</td>
<td>0.16</td>
</tr>
<tr>
<td>CYT-DAU-ETO</td>
<td>0.551</td>
<td>0.23</td>
</tr>
<tr>
<td>CYT-MIT-ETO</td>
<td>0.421</td>
<td>0.32</td>
</tr>
<tr>
<td>CYT-THIO</td>
<td>0.617</td>
<td>0.35</td>
</tr>
<tr>
<td>CYT-CLO</td>
<td>0.552</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Abbreviations: CLO = clofarabine; CYT = cytarabine; DAU = daunorubicin; ETO = etoposide; FLU = fludarabine; IDA = idarubicin; MIT = mitoxantrone; ne = not estimated; THIO = 6-thioguanine.

*Estimate is not significantly different from 0.

---

**Table 1** Pharmacological Population Parameters. Individual Drug Typical and Random (Variability and Residual Error (RE)) Values for Efficacy (E\(^{\text{MAX}}\)) and Potency (EC\(^{50}\)) are Shown on the Left Side. Estimates of Intertreatment Variability (IPV) are Expressed as Coefficient of Variation (%); Synergism (Right Side) is Computed Using the Combination Index (CI), Showing Median and Standard Error
behaves like the anthracyclines, albeit with a significantly lower potency (higher EC50). Maximal efficacy or EMAX, i.e., when all blasts are eliminated at the highest doses, occurs for essentially all samples for the 3 anthracyclines, IDA, DAU, and MIT, and ETO. Among nucleosides, the magnitude of their potency IPV for CYT, FLU, and CLO are roughly similar, with CLO showing a slightly higher value, varying more among patient samples. However, when considering the variability in their efficacies or EMAX, only the nucleosides display variability for this parameter. The efficacy IPVs of CYT, FLU, and CLO are significant (25-36%) and similar. This variability, in combination with the submaximal efficacy of these drugs, shows that for a significant number of patient samples, these drugs are not capable of inducing 100% cell death in the leukemic population. THI is the weakest nucleoside, and for most of the samples, it is neither potent nor effective. As 100 μM was the highest drug concentration we could achieve for physical solubility limit, for most samples a full dose-response curve was not obtained. For analysis of this data, we have set the EMAX value to 0 to enable the population models to calculate an EC50 value. Because of this, the IPV of the EMAX was not estimated. However, a very small subset of samples were shown to be highly sensitive (left shift), in which all blasts were eliminated (EMAX = 0), indicating that for these very few patients, the drug or combination could potentially be an effective treatment option.

**Pharmacological Profiles Ex Vivo of Drug-Combination Treatments**

Synergism for the 12 main drug-combination protocols used across Europe was evaluated by calculating the CI from Chou.
Figure 4: Average and Individual Dose Responses ex vivo for AML Drugs. Dose-Response Analysis was Completed for 8 Individual Drugs in Bone Marrow Samples From 86 to 125 Patients With Acute Myeloid Leukemia. The Survival Index (y-Axis) Ranges From 100% to 0%, Displaying the Selective Acute Myeloid Leukemia Cell Depletion Calculated With Population Models. The Gray Lines Display Each Individual Response, With the Median Response Shown in red. Panels are Shown for (A) Idarubicin, (B) Mitoxantrone, (C) Daunorubicin, (D) Etoposide, (E) Cytarabine, (F) Clofarabine, (G) Fludarabine, and (H) 6-Thioguanine.
and Talalay. In this analysis, a value of 1 is an additive response, a value < 1 indicates a synergistic response, and a value > 1 reflects an antagonistic response. The CIs at 4 different levels of response (20%, 40%, 60%, 80%) were calculated and the average reported in Table 1. The CI is graphically displayed in Figure 5, in terms of percentiles across the population, using a box plot. This representation enables us to compare the synergism for all 12 drug-combination treatments. All treatments are
synergistic on average, as their medians are < 1. The differences shown in Figure 5 among the median synergism (CI) for all treatments are not substantial given their standard error (horizontal dotted lines).

More remarkable are the different levels of IPV, shown by the horizontal stretch for each treatment. Variability was highest among the CYT-DAU, CYT-MIT-ETO, and CYT-CLO combinations. Interestingly, for a subset of patient samples, these 3 combinations induced an antagonistic response (bar extending to the left of 1). This finding was particularly so for the CYT-DAU combination, which had a CI of 0.743 (Table 1), a score closer to an additive interaction than any of those tested. In comparison, CYT-MIT-ETO had a CI of 0.421, among the lowest CIs recorded, thus one of the most synergistic. Thus, these synergistic indexes provide 2 different inferences for any individual patient sample; first, the relative position of a given sample on this synergy map identifies its ranking among the population of patient samples, whether an individual patient sample may be especially synergistic or not compared with the population of patient samples. Second, the absolute value indicates whether there is synergism or not for a given treatment for that sample.

The responses to CYT-DAU and CYT-CLO were quite variable among patient samples, compared with that of CYT-IDA and even that of CYT-MIT. Their patterns are significant in that CYT-DAU stretches more toward additive-antagonism whereas CYT-IDA and CYT-MIT have a longer arm toward synergism. This means there are patients for whom clinicians would like to avoid the combination CYT-DAU, as it is antagonistic. Similarly, for the CYT-CLO combination, a subset of samples displays a highly synergistic value, whereas for another subset, the value is additive-antagonistic. The combinations of 3 drugs also displayed large differences in IPV. For instance, CYT-MIT-ETO shows a much higher IPV than CYT-IDA-ETO or CYT-DAU-ETO, equivalent combinations exchanging the anthracycline MIT, IDA, or DAU. However, the same combinations with ETO removed show different IPV profiles, with CYT-DAU showing a higher IPV than that of CYT-MIT and that showing higher than that of CYT-IDA. Hence adding ETO to CYT-IDA, CYT-DAU, or CYT-MIT changes substantially the relative IPV of these cytarabine-anthracycline combinations.

The most clinically relevant information that can be extracted from these series of dose-response curves on patient samples is the relative ranking of each individual, for each drug or combination treatment, within the entire population of patient samples. As an example, CYT, as seen in Figure 6, has a subset of samples whose curves are shifted left of the average, 1 of which is colored green. For this patient sample, CYT is more potent than average, thus a low dose may be sufficient to kill the leukemic blast cells. This finding means this patient’s ex vivo sample is especially sensitive to this drug. Alternatively, those curves shifted rightward mean that this drug needs high doses to kill blasts from these patients’ ex vivo samples; this finding means the patient ex vivo sample highlighted in green is especially resistant toward this drug (CYT). To capture this population ranking, we use the percentiles diagram below the dose-response curves of Figure 6; a single patient sample potency (EC50), shown in green, is ranked with a given percentage, such that the most sensitive patient for a drug, the one whose line is farthest to the left, is the one for whom the drug is most potent and is thus ranked 100%. Conversely, the most resistant patient sample, farthest to the right, is the one for which the drug is least potent and is thus ranked 1%. Note that potency ranks 1%-100% go in the direction opposite to that of drug concentration (minimum to maximum) because a potent drug (high percentage) would eliminate patient cells with a low EC50 concentration. Furthermore, a subset of a patient’s leukemic cells may be resistant to a specific drug, as seen in the Figure 6 dose responses whose lines at the highest doses do not reach zero. In the following scheme, the dose response to represent the population pharmacological profile, we report this as the percent survival of malignant cells (in red, to the right of the percentile-ranking scheme).

The individual pharmacological profiles for 2 patients are shown in Figure 7. The relative drug potency for 2 patient samples, ie their ranking within the population, is shown in the left panels of Figure 7. For display purposes, the ranking order is reversed from that shown in Figure 6. In the left column the relative potencies for the 8 drugs for that patient sample are shown from 1-100%, most potent to the right, opposite to the drug concentration direction shown in Figure 6. The right columns in Figure 7 show the synergism of the drug combinations for that patient sample. The CI for each combination is shown in green, relative to the population profile.
Figure 7A illustrates data of an individual resistant patient treated with CYT-IDA, on these population pharmacological profiles. The most remarkable extreme phenotypes are highlighted by colored boxes. This patient sample is especially sensitive to CYT and FLU with potencies > 90% (green boxes), and is not synergistic for the CYT-IDA and CYT-DAU combination treatments (red boxes). The best individual drugs, CYT and FLU, can be combined in a treatment with good synergism (linked by green arrows). However, FLU has 15% potentially resistant cells. This patient was treated with CYT-IDA (red arrows) and was resistant. The potency of IDA is average, which means it is acceptable because it is an efficacious drug, and for CYT, the potency is excellent—within the top 10% of most sensitive patients. However, there is a lack of synergism for the CYT-IDA treatment in the right-hand panel. The lack of clinical

Abbreviations: MIT = mitoxantrone; THIO = 6-thioguanine.
response would suggest the lack of synergism prevails over the good sensitivity of the individual drugs, highlighting synergism as a potential key predictor of clinical outcome. Interestingly, ETO eliminates only half of the blasts for this patient, and CLO is inadequate in both sensitivity and the presence of potentially resistant clones. Its synergy profile is interesting in that, although it lacks synergism for CYT-IDA, it shows good synergism for CYT-FLU and the 3-drug combination CYT-IDA-FLU.

Figure 7B shows a patient in whom the most extreme pharmacological profiles do not converge into the right treatment. The most remarkable features for this patient are that the sample is very sensitive to FLU and CLO (green boxes) and has strong lack of synergism for several combination treatments (marked by a red box): CYT-FLU, CYT-CLO, CYT-IDA-FLU, and CYT-IDAETO. These profiles are inconsistent with a good treatment linking individual drug potencies and synergistic treatments ex vivo, because the most sensitive drugs, FLU and CLO, can be combined best as CYT-FLU and CYT-CLO, but both treatments are non-synergistic. (This is shown for CYT-FLU as red arrows). The poor synergism of CYT-IDA-FLU may just be a reflection of the poor synergism of CYT-FLU, in spite of good, albeit not extreme, synergism for CYT-IDA. Actually, CYT-IDA has good drug potencies and synergism (shown by green arrows), even though none is an extreme best cases. In fact, this patient was treated with CYT-IDA, which achieved complete response. The potency of the individual drug IDA is very good, significantly right shifted, within the top 25% among the patient population. The potency for CYT is average at 50%, which is a good percentile because, on average, CYT is an efficacious drug. The right-hand panel shows that the synergy for CYT-IDA for this patient is quite good, within the top 25% among patients. These 3 good ex vivo pharmacological variables are consistent with the complete response achieved.

Discussion

We have developed a cell-based screening platform, called Exvi-Tech, which incorporates both automated sample preparation and automated evaluation by flow cytometry, in conjunction with proprietary analytical software and a database structure geared for rapid data acquisition, analysis, and reporting of results. By testing patient samples directly and in whole sample, we expect to bring the effectiveness of a given drug in vitro a step closer to what it is expected to do in vivo. The large level of variability among patient responses illustrates what is already known, that AML patients do not always respond well to treatments given according to standard protocols. There are 4 factors that distinguish the drug-sensitivity platform presented here. First is the use of the complete patient BM sample, retaining the erythrocyte population and serum proteins, and thus measuring pharmacological activity in a better approximation of their biological context. Second, using multiparametric flow cytometry to analyze the samples allows for the use of labeled monoclonal antibodies and annexin-V–FITC; this enables specific identification of the malignant cell population and determination of the number of live cells at the end of the assay. Third, a proprietary platform with extensive automation greatly expands the scope of analysis that can be done with each patient sample, enabling 8-point dose responses for drugs and combinations, and it increases the quality of the test by eliminating human error at several points in the process. Fourth, data is analyzed using population pharmacodynamic models, most often used to model clinical trial results, that fit pharmacological data for all patients simultaneously. This significantly enhances the accuracy of the individual patient pharmacological values, and diminishes considerably the associated error by filling in missed or error-prone data. All 4 points diminish the associated error and avoids artifacts resulting in significantly more reliable ex vivo pharmacological data for individual patient samples. The developments integrate previous efforts to promote ex vivo testing in AML, and they incorporate novelties that may enable the use of pharmacology data in patient samples for individualized patient treatment.

The translation from ex vivo sensitivity or resistance to patient responses is obviously not direct. Other data that may influence the interpretation includes pharmacokinetic, adsorption, distribution, metabolism, excretion, and other factors. These factors are expected to be less important in extreme cases of dose-response curves shifted maximally toward the left (sensitive) or toward the right (resistant), and more dominant in intermediate cases. Thus, the clinically more relevant data are the extremes, and for each patient we look among all drugs and all combination treatments for extreme sensitivities or extreme resistances, as shown for Figure 7. Additionally, this approach cannot account for the role of the marrow microenvironment. The microenvironment has been shown to increase chemoresistance, and thus it may affect ex vivo sensitivity but less so ex vivo resistance.

There are potential clinical inferences from this set of rather complete pharmacological data for AML drugs and treatments measured on freshly extracted patient samples. However, we should be very cautious of correlating ex vivo pharmacology with patient responses directly. The rank order of absolute median potencies ex vivo for each drug is consistent with clinical observations (Fig. 3).

When the dose response of a drug for an individual patient shows that it is unable to eliminate all blasts at maximum doses, which can be referred as low efficacy or low E_{MAX}, it means there is subset of cells (possibly a clone) resistant or highly resistant to that drug. This occurs particularly for CLO, FLU, and CYT, in this order. However, in this experiment incubation was for 48 hours, whereas in clinical practice, drugs are given over multiple days in patterns of administration not represented in this pharmacological assay. If it is a truly resistant clone, then multiple days and doses would not eliminate it, and this single drug pharmacology assay is clinically relevant. We plan to evaluate whether synergism of combination treatments can eliminate this resistant population, or if increasing the incubation time to 72 or 96 hours is effective at eliminating these populations. Nonetheless, if the pharmacological profile shows that other drugs are more sensitive for this patient sample, it would seem wise to prevent administering treatments with a clear possibility of leaving resistant cells. We thus represent incomplete blast death as “potentially resistant clones” and as a warning sign pending further investigation. For the related nucleosides CYT, FLU, and CLO, there are a considerable percentage of samples (CYT 22%, FLU 32%, CLO 51%) in which the drug fails to eliminate all blasts, even at drug concentrations that are higher than that achieved in vivo. However, this lack of full efficacy stratifies quite well these
drugs, identifying 22%-51% of the population that may not be quite suitable to them. Excluding these patients may improve the response rate significantly, in clinical practice and in clinical trials. This approach may thus be suitable as a companion diagnostics in clinical trials, and to profile drug candidates in discovery and development.

We have characterized the pharmacological responsiveness of individual patient samples to available AML drug treatments by comparing the potency and efficacy of individual drugs with the synergism of the drug-combination treatment. Over time, clinical correlation data will indicate which of these variables is more important for clinical efficacy of a given treatment in an individual patient. Synergism represents a more integral component of the combination treatment, whereas single-drug strength represents the starting point for synergism. We hypothesize that the use of the individual patient ex vivo pharmacological profiles such as those shown in Figure 7 may help to guide a personalized treatment selection.26

Guidelines can be derived first from the extreme phenotypes, when an individual patient sample is highly sensitive or resistant to specific drugs, or very synergistic or antagonistic to specific combination treatments. However, in only a few cases do we identify the perfect case, highly sensitive drugs that are also highly synergistic in an approved combination treatment. More often, we need to evaluate choices that include submaximal efficacy and synergism, and thus this approach may represent valuable information to the hematologist who must interpret it based on her/his know how and the increasing number of prognostic factors.1-9

Conclusion
In conclusion, we have developed an improved methodology to measure the pharmacological activity of drugs and drug combinations in AML patient samples as well as to model their pharmacological behavior. This information may be useful in selecting the optimal treatment of the individual patient. By testing the drugs used in the treatment protocols for AML directly on patient samples, a pharmacologically based model has been developed to infer drug resistance or sensitivity, patient by patient. IDA, DAU, and MIT are commonly used in combination with CYT, and testing may be able to determine which would be better for each individual patient. To derive guidelines in interpreting these results, clinical correlation studies are ongoing with PETHEMA to validate these inferences for observational studies, to be followed by future interventional studies.

Clinical Practice Points
- The results are a large variability between patients of their dose response curves, opening a new dimension versus the consideration only of the average drug activity. The variability of Fludarabine covers the whole range of the average dose responses of the standard drugs in AML.
- Synergism between combined drugs can now be measured reliably. There is also a large interpatient variability in the synergism between drugs that form a given treatment, such as Cytarabine-Ldubricine.
- Different drugs and different treatments based on drug combinations show different degrees of interpatient variability in their single drug activity and their synergism. This means there is potential in leveraging these data to personalize treatment to individual patients.
- Clinical validation of this new data is still necessary before this new approach can deliver valuable information to the hematologists.

Acknowledgments
This study was funded with grants from the Spanish government Programa Reindustrialización 2011 MITYC (REI-040000-2011-777), Programa Torres Quevedo, and MICINN Programa Innorpora, and a grant from Castilla y Leon (Spain) ADE 2007 (04/06/SA/0009).

We would like to thank the patients for providing their samples and PETHEMA for their support and collaboration. We also thank our lab scientists for working the long 7-day weeks needed to process the samples as they arrive, always with high rigor and efficiency.

Disclosure
The authors have stated that they have no conflicts of interest.

References