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A novel 96-well plate cell culture assay for lineage-specific hematopoietic cell toxicity screening

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Introduction

The current gold standard for assessing potential toxicity of compounds on bone marrow (BM) and the hematopoietic system is the *in vitro* colony-forming unit (CFU) assay, in which hematopoietic stem and progenitor cells (HSPCs) cultured in either methylcellulose or collagen-based media differentiate into mature cells of different lineages. However, the CFU assay is time-consuming and not amenable to a high-throughput format, thus limiting the number of compounds that can be efficiently screened. In addition, the effects of some drugs on colony size or morphology are difficult to quantify. We have developed a new 96-well liquid media assay for HSPC toxicity screening. CD34⁺ cells enriched from human cord blood (CB) or BM were cultured in the presence of varying concentrations of small molecule compounds from three classes with known hematopoietic toxicity [Topotecan, Irinotecan, Sunitinib, Imatinib, 5-Fluorouracil (5-FU), Cisplatin] in HemaTox™ Erythroid Medium or HemaTox™ Megakaryocyte Medium supplemented selectively promote either erythroid or megakaryocyte lineage cell outgrowth. The cells were cultured for 7 or 10 days in the erythroid and megakaryocyte-specific cultures, respectively. Lineage specificity, assay reproducibility, and comparison with performance on the CFU assay were evaluated.

Materials & Methods

Cells: Normal human CB $CD34^+$ cells (each sample pooled from at least 3 donors) and BM $CD34^+$ cells (from 7 donors) were stored at -152°C until required for the assay. Cells were thawed rapidly at 37°C, diluted in 50 mL of PBS, and washed by centrifugation (1,200 rpm for 10 min, room temperature). The cell pellet was resuspended in a known volume of IMDM + 2% FBS (STEMCELL Technologies Inc.) and a cell count (3% glacial acetic acid) and viability assessment (trypan blue exclusion) were performed.

96-Well Plate Assay: Cells were seeded in HemaTox™ Erythroid Medium containing HemaTox™ Erythroid Supplement or HemaTox™ Megakaryocyte Medium containing HemaTox™ Megakaryocyte Supplement. Cells were cultured for 7 or 10 days and then counted and phenotyped in the culture plate using the Guava EasyCyte™ flow cytometer. Cultured erythroid cells were stained with CD71-PE (Clone OKT9, eBiosciences) and GpA-FITC (Clone 2B7, STEMCELL Technologies Inc.) antibodies. The expression levels of these two markers change as erythroid progenitors mature, therefore, all three populations of erythroid progenitors ($CD71^+GpA^-$, $CD71^+GpA^+$, $CD71^-GpA^+$) were counted in this assay. Megakaryocytes were identified by expression of the CD41a marker (CD41a-FITC, clone HIP8, BioLegend).

Colony-forming Unit (CFU) Assay: $CD34^+$ BM cells were cultured in MethoCult™ H84434 and MegaCult™ for 14 days and 11 days, respectively, followed by the enumeration of BFU-E and CFU-Mk arising from erythroid and megakaryocytic progenitors, respectively.

Compounds: The assay was tested on a panel of hematotoxic compounds consisting of topoisomerase inhibitors (Topotecan, Irinotecan), tyrosine kinase inhibitors (Sunitinib, Imatinib), and antiproliferatives (5-FU, Cisplatin). A cisplatin solution in dH2O was prepared fresh on each day of use; all other compounds were solubilized in DMSO and added to the cultures to ensure that the final concentration of DMSO did not exceed 0.2%. Topotecan, Irinotecan, 5-FU, and Cisplatin were purchased from Sigma; Sunitinib and Imatinib were purchased from Cayman Chemicals.

Percent Coefficient of Variation (%CV) Determination: %CV was calculated by dividing the sample standard deviation by the mean of replicates and multiplying by 100. $\%CV = \sigma / \bar{X}$, where:

σ = Standard Deviation = $\sqrt{[\sum (X_i - \bar{X})^2 / (n - 1)]}$

$\bar{X}$ = Average = $\sum X_i / n$

IC_{50} Determination: The concentration of 50% inhibition of progenitor cell / colony growth (IC_{50}) for each compound was calculated for each compound by plotting the log of the compound concentration against the percentage of maximum progenitor cell / colony growth using GraphPad Prism® 5. To generate a curve fitting these data points, the following equations were used:

log(inhibitor) vs. normalized response – variable slope:

$Y = 100/[1 + 10^{((Log IC_{50} - X) * HillSlope)}]$

log(inhibitor) vs. response (3 parameters):

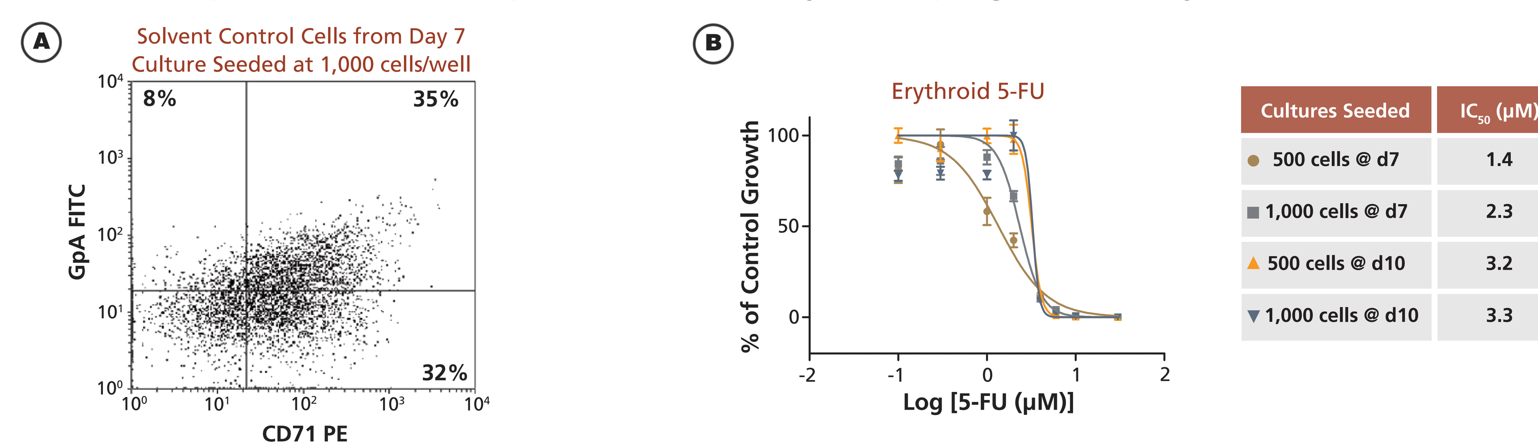
$Y = bottom + (top - bottom) / [1 + 10^{(X - Log IC_{50})}]$

log(inhibitor) vs. response – variable slope (4 parameters):

$Y = bottom + (top - bottom) / [1 + 10^{((LogIC_{50} - X) * HillSlope)}]$

Results

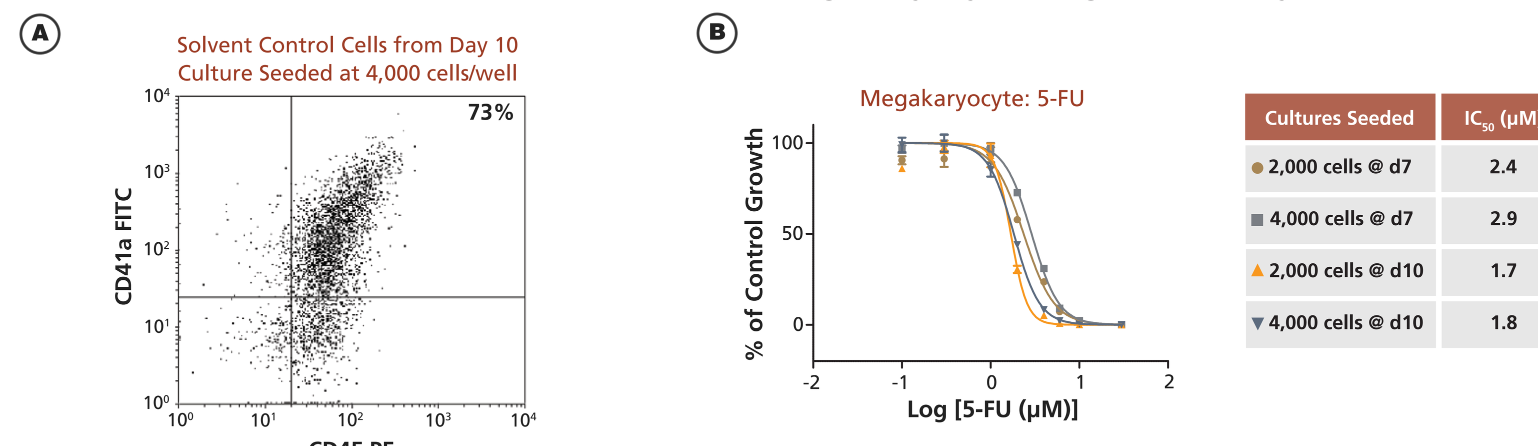
FIGURE 1: Optimization of the protocol for the erythroid progenitor assay



Cultures Seeded	IC_{50} (µM)
500 cells @ d7	1.4
1,000 cells @ d7	2.3
500 cells @ d10	3.2
1,000 cells @ d10	3.3

Different culture periods (7 and 10 days) and initial seeding densities (500 and 1,000 cells/well) were assessed using 5-FU as a model toxic compound. Erythroid ($CD71^+GpA^-$, $CD71^+GpA^+$, $CD71^-GpA^+$) cell purity **(A)** at day 7 was comparable between the cultures seeded at 500 and 1,000 cells/well (median = 80%). In addition, IC_{50} values for 5-FU from dose response curves were very similar for day 7 and 10 cultures at the different seeding densities **(B)**. However, the variability between the six technical replicates tested was lower in cultures initially seeded at 1,000 cells/well [median %CV = 11 (1,000 cells/well) vs. 22 (500 cells/well)]. Also, a lower viability of the solvent control cultures at day 10 (~60%) as compared to day 7 (~80%) at both seeding concentrations suggested that the cultures were overgrown at this later time point. Therefore, the optimal conditions for the erythroid assay were determined to be a 7-day culture with an initial seeding density of 1,000 cells/well. Under these conditions, typical fold expansion in the solvent control cultures ranged from 50- to 100-fold in different donors.

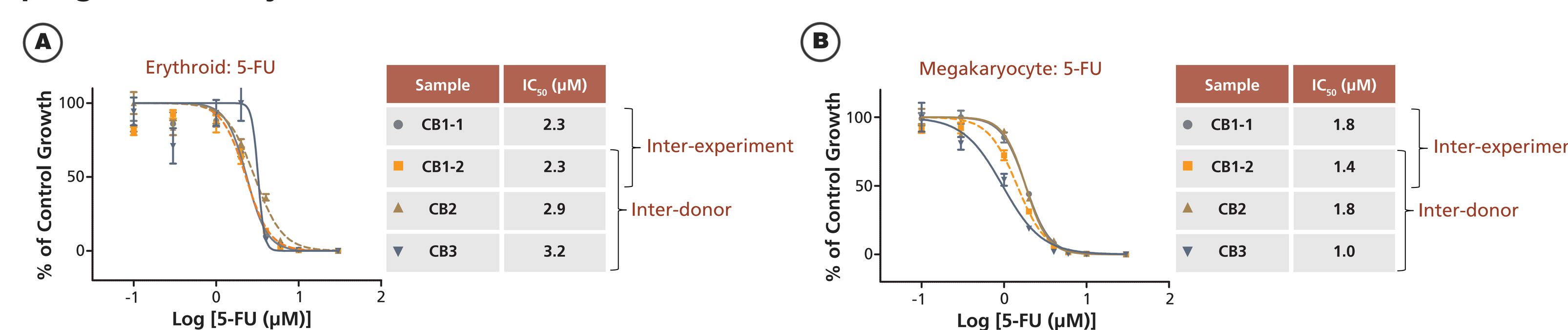
FIGURE 2: Optimization of the protocol for the megakaryocyte progenitor assay



Cultures Seeded	IC_{50} (µM)
2,000 cells @ d7	2.4
4,000 cells @ d7	2.9
2,000 cells @ d10	1.7
4,000 cells @ d10	1.8

Different culture periods (7 and 10 days) and initial seeding densities (2,000 and 4,000 cells/well) were assessed using 5-FU as a model toxic compound. Megakaryocyte ($CD41a^+$) cell purity **(A)** was higher in day 10 cultures than in the day 7 cultures (median purity at 10 and 7 days = 65% and 58%, respectively). In addition, IC_{50} values for 5-FU from dose response curves were very similar for day 7 and 10 cultures at the different seeding densities **(B)**. The viability of the solvent control cultures at day 10 was comparable to that from day 7 (~70%), indicating that the longer culture period did not lead to culture overgrowth. Therefore, the optimal conditions for the megakaryocyte assay were determined to be a 10-day culture with initial seeding density of 4,000 cells/well. Under these conditions, typical fold expansion in the solvent control cultures ranged from 2- to 15-fold in different donors.

FIGURE 3: Inter-experiment and inter-donor variability in the erythroid and megakaryocyte progenitor assays



(A) Erythroid: 5-FU

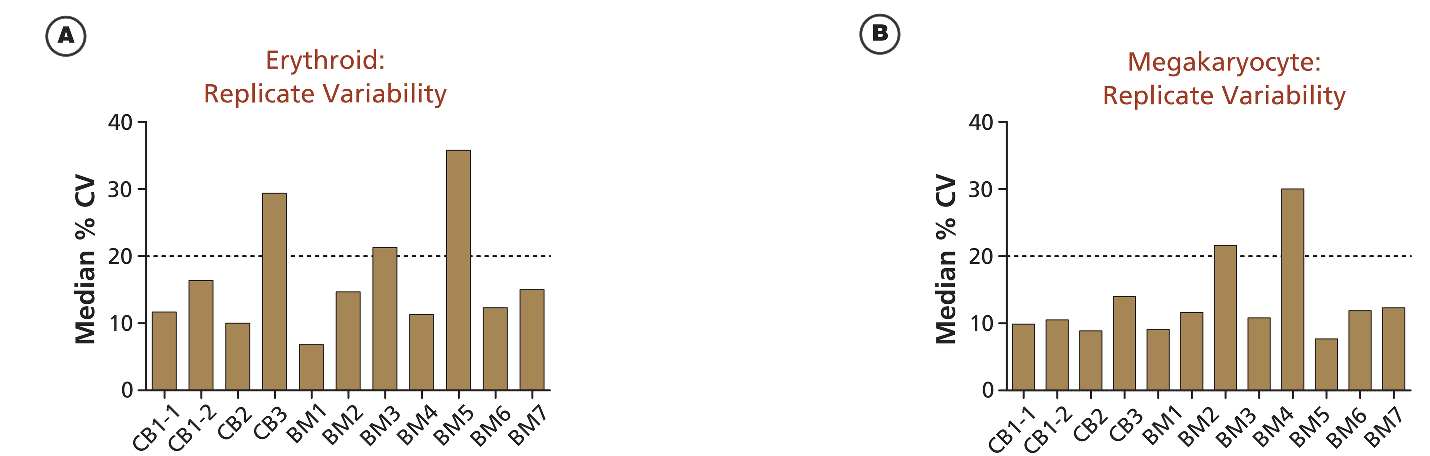
Sample	IC_{50} (µM)
CB1-1	2.3
CB1-2	2.3
CB2	2.9
CB3	3.2

(B) Megakaryocyte: 5-FU

Sample	IC_{50} (µM)
CB1-1	1.8
CB1-2	1.4
CB2	1.8
CB3	1.0

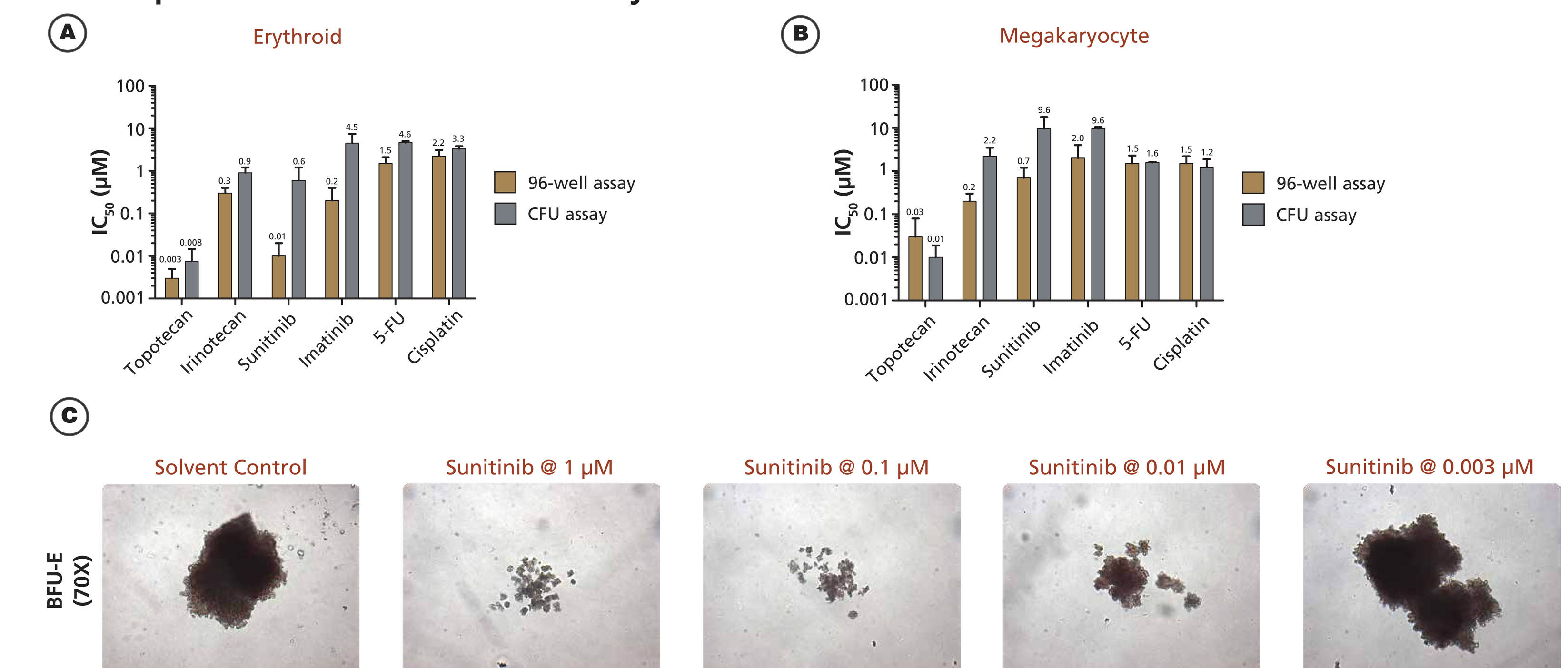
Cord blood $CD34^+$ cells were plated at 1,000 or 4,000 cells/well in 200 µL of appropriate HemaTox™ medium supplemented with either the HemaTox™ Erythroid Expansion Supplement **(A)** or HemaTox™ Megakaryocyte Expansion Supplement **(B)**, respectively. Cells were treated with various concentrations of 5-FU and cultured for 7 days (erythroid) or 10 days (megakaryocytes) and then enumerated and phenotyped to generate dose response curves.

FIGURE 4: Replicate reproducibility in 5-FU assays



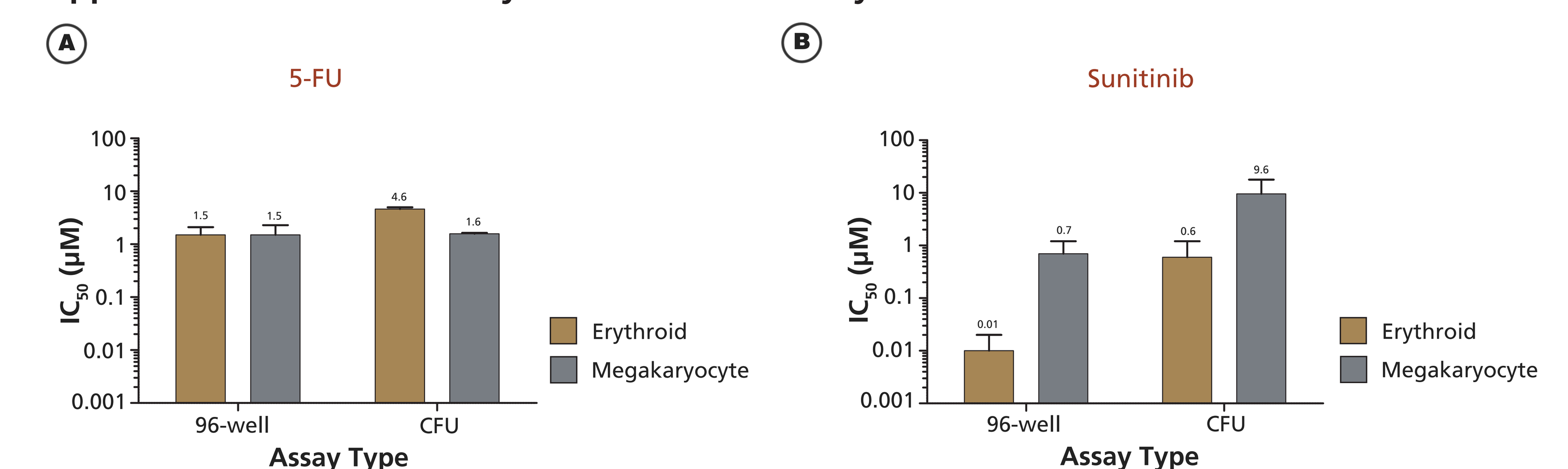
Enriched cord blood (CB) and bone marrow (BM) $CD34^+$ cells were plated in the erythroid **(A)** and megakaryocyte **(B)** 96-well plate assays at 6 replicates per compound dose. The percent coefficient of variation (%CV) of replicates per dose was calculated, and the median %CV per experiment is shown. The median %CV was under 20% in most experiments.

FIGURE 5: The 96-well assay may provide a more quantitative measure of the effects of compounds on cell proliferation than the CFU assay



For most drugs IC_{50} values obtained in the 96-well and CFU assays were similar on both erythroid **(A)** and megakaryocyte lineages **(B)** [96-well data show mean ± 95% C.I., n = 7 - 8; colony data show mean ± range, n = 2]. For two drugs, Imatinib and Sunitinib, IC_{50} values were much lower in the 96-well assay (see also figure 6). The CFU assay measures progenitor frequencies (colony numbers) and can reveal drug effects on colony size and morphology (see panel C for representative images of BFU-E colonies in the presence of various concentrations of Sunitinib). These effects on cell numbers are difficult to quantify in the CFU assay, but can be measured in the 96-well assay.

FIGURE 6: Lineage-specific differences in sensitivity to hematotoxic compounds may be more apparent in the 96-well assay than in the CFU assay



For some compounds tested, e.g. 5-FU, similar IC_{50} values were obtained in the erythroid and megakaryocyte assays using both the 96-well and CFU assays **(A)**. For other compounds, e.g. Sunitinib, the IC_{50} values for the erythroid lineage was lower than that for the megakaryocyte lineage in both the 96-well and CFU assays with the 96-well away showing a 100-fold difference in IC_{50} values between the two lineages and the CFU assay showing only a 10-fold difference **(B)**.

Conclusions

- Highly enriched erythroid (median frequency of erythroid cells = 81%) and megakaryocyte (median frequency of megakaryocyte cells = 61%) progenitor cultures were obtained
- Replicate reproducibility was very good (median %CV generally under 20% in both erythroid and megakaryocyte cultures in most experiments)
- Inter-experiment and inter-donor variability between calculated IC_{50} values was low
- The ranking of hematotoxic compounds was similar between the 96-well plate assay and the CFU assay
- The liquid assay may provide a more quantitative measure of the effects of compounds on cell proliferation than the CFU assay