A Flexible 96-Well Plate Assay for Screening Toxicity to Granulocyte Production

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Introduction

An important step in chemotherapeutic drug development is to test candidate therapeutic agents for the potential to cause neutropenia *in vivo*. Neutrophils, which are the most abundant type of granulocytes, are derived from hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM) and play an essential role in the innate immune system. *In vitro* assays on primary cells that can predict neutropenia early in the drug development process have obvious advantages for the selection of successful candidates. The current gold standard for assessing drug toxicity to granulocytes is the *in vitro* colony-forming unit (CFU-GM) assay, in which HSPCs are plated in methylcellulose-based media and granulocyte and macrophage (GM) colonies are counted after 14 days of culture. However, the CFU-GM assay is not amenable to a high-throughput format, thus limiting the number of drugs that can be efficiently screened. We have developed a microwell plate-based hematotoxicity assay, in which the number, viability, and phenotype of cells generated by expansion and granulocyte-specific differentiation of HSPCs is quantified after 7 days of liquid culture. CD34$^+$ cells enriched from human cord blood (CB) or BM were cultured in the presence of increasing concentrations of small molecule drugs known to cause neutropenia (Topotecan, Irinotecan, Sunitinib, Imatinib, 5-Fluorouracil (5-FU), Cisplatin). The robustness of this 96-well plate assay and its correlation to the results of the CFU-GM assay were evaluated.

Materials and Methods

Cells

Normal human CB and BM CD34$^+$ cells were isolated using the EasySep™ Human Cord Blood CD34 Positive Selection Kit II (STEMCELL Technologies Inc.) and frozen until required for the assay. Cells were thawed rapidly at 37°C, diluted in 50 mL of PBS, and washed by centrifugation (1200 rpm for 10 min, room temperature). The cell pellet was resuspended in IMDM + 2% FBS and the nucleated cell count (3% acetic acid) and viability (trypan blue exclusion) were determined.

HemaTox™ Myeloid Assay

CD34$^+$ cells were seeded at 1000 cells/well in 96-well flat bottom tissue culture plates in 200 µL of complete HemaTox™ Medium (medium and supplement) per well. Cells were cultured for 7 days, stained in the culture plate with labeled antibodies against myeloid markers (CD13, CD14, CD15) and PI (to stain dead cells), and then counted using a Guava EasyCyte™ 8HT flow cytometer.

Colony-Forming Unit (CFU) Assays

CFU-GM assays were performed by plating CD34$^+$ or mononuclear BM cells in either MethoCult™ H84534 or H84434 and counting granulocyte and macrophage colonies after 14 days of culture. Colony formation by erythroid (BFU-E) and megakaryocyte (CFU-Mk) progenitors was measured in Methocult™ H84435 and MegaCult™-C medium, respectively.

Drugs

The assay was tested on a panel of drugs known to cause neutropenia in vivo (Topotecan, Irinotecan, Sunitinib, Imatinib, 5-FU, Cisplatin). A fresh cisplatin solution in dH_2O was prepared on each day of use; all other drugs were dissolved and diluted in DMSO and added to cultures to ensure that the final concentration of DMSO did not exceed 0.2% and was the same in all cultures. Topotecan, Irinotecan, 5-FU, and Cisplatin were purchased from Sigma. Sunitinib and Imatinib were purchased from Cayman Chemicals. Each drug was tested at 7 - 10 serial dilutions in 3 - 5 replicate cultures for each dilution and control.

Determination of IC_{50}

The concentration of each drug that inhibited 50% of progenitor cell or colony growth (IC_{50}) was calculated from the average numbers of cells per well (HemaTox™ assay) and colony numbers (CFU assay) by plotting the log of the drug concentration against the percentage of maximum cell or colony growth using GraphPad Prism 5 software. To generate a curve fitting the data points, the following equation was used: log[inhibitor] vs. normalized response - variable slope: $Y = 100/(1+10^{((LogIC50-X)*HillSlope)})$

Determination of Percent Coefficient of Variation (%CV)

%CV was calculated by dividing the sample standard deviation by the mean of replicates and multiplying by 100.

%CV = σ/$\bar{X}$, where:

$$\bar{X} = \text{Average} = \frac{\sum X_i}{n}$$

$$\sigma = \text{Standard Deviation} = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \bar{X})^2}{n-1}}$$

Assay Reproducibility and Z'-Factor Assessment

Assay reproducibility was determined by testing one drug (5-FU) in 3 - 6 replicate culture wells in twelve separate experiments using three different CD34$^+$ cells and calculating IC_{50} values for each experiment (Table 1). A Z' factor (Zhang et al. J Biomol Screen 4: 68-73, 1999) was calculated using pooled data from the solvent control and the highest 5-FU concentration (30 µM) as positive and negative control, respectively. Individual data were normalized against the mean for the solvent control in each experiment to calculate the overall mean and standard deviation for all experiments. Z' was then calculated as follows, where σ is the sample standard deviation and µ is the sample mean of the positive (p) and negative (n) controls:

$$\text{Estimated Z-factor} = 1 - \frac{3(\hat{\sigma}_p + \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}$$

Results

FIGURE 1: Dose response curves generated by the HemaTox™ Myeloid assay for different test drugs

By the end of the 7-day culture period, a majority (>85%) of cells were positive for the CD13 early myeloid marker, with the expression of the CD15 granulocyte marker varying between 39 - 72% in different donors (n = 4 CB and BM). A representative FACS plot (from CB CD34$^+$ cells) is shown in (A). Dose response curves were generated for six test drugs to determine IC_{50} values. Results from one representative experiment performed with each drug on BM CD34$^+$ cells are shown in (B). Each data point shows the average cell number of 3 replicate wells as a percentage of the maximal response obtained with each drug. IC_{50} values were obtained in multiple experiments using CD34$^+$ cells from multiple BM and CB samples (n= 6 for CB; n=4 for BM) (C). The error bars in (B) and (C) indicate standard error of the mean.

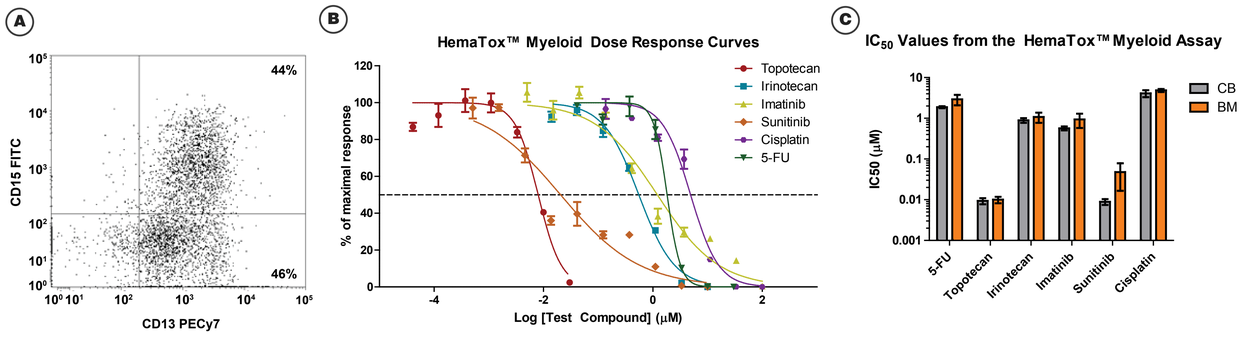


FIGURE 2: Mean IC_{50} values determined using the HemaTox™ Myeloid assay correlate well with the mean IC_{50} values determined using the CFU-GM assay

The results of the HemaTox™ Myeloid Assay correlate well with the CFU-GM assay (correlation coefficient $R^2 = 0.91$) and the ranking of drugs from most to least toxic is very similar regardless of the assay used. HemaTox™ Myeloid assays were performed using BM CD34$^+$ cells from 4 donors while the CFU-GM assay was performed with BM mononuclear cells from one donor.

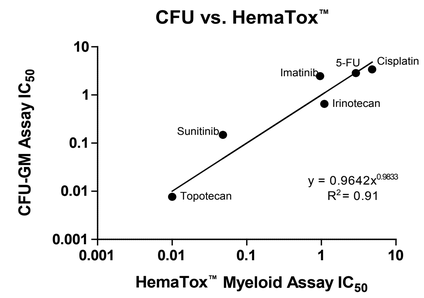
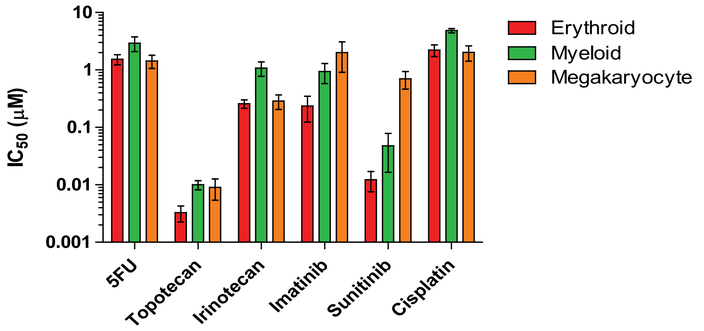


TABLE 1: IC_{50} values show low inter-assay and between-experiment variability between replicates performed with cells from different donors

One drug (5-FU) was tested in 3 - 6 replicate culture wells in thirteen separate experiments using three different CD34$^+$ cell pools. The IC_{50} values obtained in the separate experiments were used to calculate the variability between experiments with each of the three cell pools. The coefficient of variation (CV) ranged between 6.6 and 19.3% between replicate assays on each donor. Variability between donors was ~20%. Based on data from these experiments, the Z' factor was determined to be 0.64 for the HemaTox™ Myeloid assay performed in 96-well plates. A similar Z' factor, 0.67, was obtained for the standard CFU-GM assay in 35 mm culture dishes. These values indicate that both assays can detect a statistically significant response over biological noise generated by variability between replicates. Note that if the CFU-GM assay were scaled down to a 96-well format to increase throughput, the number of colonies in the positive controls would be insufficient to distinguish a response from noise, resulting in a Z' << 0.5.

IC_{50} values across multiple experiments and donors			
	Donor Pool 1 (µM)	Donor Pool 2 (µM)	Donor Pool 3 (µM)
Exp #1	1.5	2.2	2.5
Exp #2	1.7	2.5	1.6
Exp #3	1.8	2.1	1.2
Exp #4	n/a	2.2	1.3
Exp #5	n/a	2.4	1.7
Average	**1.7**	**2.2**	**1.4**
SD	0.1	0.1	0.3
%CV	8.8	6.6	19.3

FIGURE 3: Testing of drugs in the HemaTox™ Myeloid assay and HemaTox™ assays for Erythroid and Megakaryocyte lineages can identify differences in the drug sensitivity of erythroid, myeloid, and megakaryocyte progenitors



Previously, we have developed microwell plate-based assays for screening drug toxicity against Erythroid and Megakaryocyte cells. When used in conjunction with the Myeloid assay, five of the six drugs tested showed similar toxicity on all three hematopoietic lineages. For one drug, Sunitinib, myeloid cells were less sensitive (mean IC_{50} = 0.05 µM, n = 4) than erythroid cells (mean IC_{50} = 0.01 µM, n = 7), and more sensitive than megakaryocytes (mean IC_{50} = 0.7 µM, n = 8). Graph shows mean ± SEM.

TABLE 2: Lineage-specific difference in sensitivity to Sunitinib as determined by the different HemaTox™ Assays is predictive of differences seen also in the CFU assay

The IC_{50} value for the HemaTox™ Myeloid assay represents the mean of 4 experiments performed on different lots of BM CD34$^+$ cells while the IC_{50} value for the CFU-GM assay represent the mean of 5 experiments performed on different lots of BM mononuclear cells. Erythroid and Megakaryocyte HemaTox™ assays and respective CFU assays were performed on a single pool of BM CD34$^+$ cells.

HemaTox™ Assay	IC_{50} (µM)	CFU Assay	IC_{50} (µM)
Erythroid	0.002	BFU-E	0.04
Myeloid	0.05	CFU-GM	0.148
Megakaryocyte	0.62	CFU-Mk	>1

Summary

- The HemaTox™ Myeloid assay is a 7-day microwell assay for measuring drug-dependent inhibition of the proliferation and granulocyte-specific differentiation of CD34$^+$ HSPCs isolation from human BM or CB.
- The 50% inhibitory concentrations (IC_{50}) for six drugs in the 7-day HemaTox™ Myeloid assay correlate well with IC_{50} values measured in the standard 14-day CFU-GM assay ($R^2 = 0.91$) with similar ranking of toxicity in both assays (Fig. 2).
- High reproducibility between results of replicate assays on CD34$^+$ cells from different donors was observed, with the inter-assay CV ranging from 6.6% to 19.3% (Table 1).
- The HemaTox™ Myeloid assay performed in 96-well plates has a Z' factor of 0.64, similar to the CFU-GM assay performed in 35 mm dishes (Z' = 0.67).
- The HemaTox™ Myeloid assay can be used in combination with similar assays for Erythroid and Megakaryocyte progenitors to identify drugs with different toxicities on individual lineages, e.g. Sunitinib (Fig. 3, Table 2).
- The recommended readout method for the HemaTox™ assay is cell counting and phenotyping of the cultured cells by flow cytometry (Fig. 1). Other plate-reader based detection methods may also be used.
- The HemaTox™ assay format provides flexibility in assay design. In principle, drugs can be added at different culture time points to distinguish effects on primitive vs. more differentiated cells or to study drug interactions. Delayed addition experiments are not possible in the CFU-GM assay.
- HemaTox™ assays can be used in combination with conventional CFU assays to test hematotoxicity at various stages of drug development. The HemaTox™ Myeloid assay is most useful at the earlier stages of drug development when the number of candidate drugs is still large, while the CFU-GM is more suitable at later stages, when the number of test drugs is smaller.