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 progenitor 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, Iriototecan, 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® H4434 or H4443 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® H4445 and MegaCult™-C medium, respectively.

Drugs

The assay was tested on a panel of drugs known to cause neutropenia in vivo (Topotecan, Iriototecan, Sunitinib, Imatinib, 5-FU, Cisplatin). A fresh cisplatin solution in dH2O was prepared on each day of use; all other drugs were dissolved and diluted in DMSO and added to cultures to ensure viability, and phenotype of cells generated by expansion and granulocyte-specific differentiation of HSPCs is the sample standard deviation and µ is the sample mean of the positive (p) and negative

Dose Response Curves

The concentration of each drug that inhibited 50% of progenitor cell or colony growth (IC50) 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 (Fig. 1). The results of the HemaTox™ Myeloid Assay correlate well with the CFU-GM assay (correlation coefficient R2 = 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.

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Figures

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

FIGURE 2: Mean IC50 values determined using the HemaTox™ Myeloid assay correlate well with the mean IC50 values determined using the CFU-GM assay

TABLE 1: IC50 values show low inter-assay and between-experiment variability between replicates performed with cells from different donors

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

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 isolated from human BM or CB.
- The 50% inhibitory concentrations (IC50) for six drugs in the 7-day HemaTox™ Myeloid assay correlate well with IC50 values measured in the standard 14-day CFU-GM assay (R² = 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 for the HemaTox™ Myeloid assay performed in 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.
- The HemaTox™ Myeloid assay is most useful at the earlier stages of drug development when the
- The HemaTox™ Myeloid assay can be used in combination with similar assays for Etrothyroid 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.