Toxicity on Bone Marrow Progenitors from Different Species: An *in vitro* test to predict Myelosuppression and Neutropenia

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Introduction

The use of hematopoietic stem and progenitor cells in colony-forming cell (CFC) assays to test the effects of environmental toxins, chemotherapeutics, and other drug classes has been well documented. These assays have been used to screen compounds for toxicity before the initiation of costly clinical trials and as a tool to help determine maximum tolerated doses (MTDs). While several assays exist for studying different progenitor types, a great deal of research has focused on use of the colony-forming unit-granulocyte/macrophage (CFU-GM) assay as a measure of the progenitors of the granulocytic/monocytic lineage. In fact, this assay has been optimized and validated for use as an *in vitro* predictor of acute-onset neutropenia by potential hematotoxicants. Choice of species is an important consideration when testing a new drug. Mouse CFC assays can be an important tool for determining toxic doses before committing to an *in vivo* study. Previous studies by Pessina *et al.* (Tox Sci. 2003. 75:355-367) indicate that for many drugs, human MTDs can be predicted by adjusting mouse-derived MTDs. However, some studies indicate that significant differences exist among human, canine, rat, and mouse hematosensitivities to certain pharmaceuticals/toxins, with human cells showing higher sensitivity to the toxic effects of the compounds studied. Consequently, information from comparative CFC assays may be of great importance before entering clinical trials. Recent studies were conducted using chemotherapeutics and an environmental toxin to evaluate their effects on myeloid (CFU-GM) progenitors acquired from human, canine, rat, and mouse bone marrow. The resulting data show that while all tested compounds displayed a dose-dependent toxic effect on colony growth, each compound demonstrated species specificity. While conditions in the human body cannot be completely reproduced *in vitro*, CFC assays can be used to help bridge the gap between high throughput screening technologies and *in vivo* studies.

Materials & Methods

CELLS

Frozen normal human and canine bone marrow mononuclear cells were stored at –152°C until required for the assay. Cells were thawed rapidly at 37°C, diluted in 10 mL of Iscove's Modified Dulbecco's medium containing 2% fetal bovine serum (IMDM + 2% FBS), and washed by centrifugation (1200 rpm for 10 minutes, room temperature). The cell pellet was resuspended in a known volume of IMDM + 2% FBS and a cell count (3% glacial acetic acid) and viability assessment (trypan blue exclusion test) were performed. C57BL/6 mice and Sprague-Dawley rat were housed and sacrificed at the British Columbia Cancer Agency (BCCA, Vancouver, BC, Canada). Whole bone marrow cells were harvested from femurs of C57/BL6J mice and Sprague-Dawley rat by standard protocols. A cell count (3% glacial acetic acid) and viability assessment (trypan blue exclusion test) was performed.

MEDIA

MethoCult™ GF H84534 (Human CFU-GM), MethoCult™ GF M3534 (Mouse CFU-GM), MethoCult™ GF R3774 (Rat CFU-GM), and Canine CFU-GM (proprietary formulation; STEMCELL Technologies; Vancouver, Canada) are medias formulated for optimal growth of myeloid colonies in each species.

COMPOUNDS

Topotecan, Irinotecan, Camptothecin, Doxorubicin, Cisplatin, 5-Fluorouracil, and Lead Nitrate (Sigma; St Louis, MO), Sunitinib Malate, Imatinib Mesylate (Cayman Chemical; Ann Arbor, MI) and Erlotinib HCl (LC Laboratories; Woburn, MA) were dissolved in DMSO at 1000x the highest test concentration desired and subsequently diluted in DMSO to make 1000-fold stock solutions of each test concentration.

COLONY-FORMING CELL ASSAY

Each compound stock was added to the appropriate MethoCult™ formulation to give the desired final concentrations (1x in 0.1% DMSO). Standard control cultures (containing no compound or solvent) and solvent control cultures (containing 0.1% DMSO but no compound) were also initiated. Bone marrow cells were added to the media formulations to obtain the appropriate final plating concentrations. For each test concentration, cultures were plated in triplicate dishes and incubated at 37°C, 5% CO_2 for 8-14 days (depending on the species), which is required for proper colony development. Colonies were enumerated by trained personnel.

IC_{50} VALUE DETERMINATION

To calculate the concentration at which 50% inhibition (IC_{50}) of colony growth is observed for each compound, a dose response graph was generated by plotting the log of the compound concentration versus the percentage of control colony growth using GraphPad (Prism®) software. To generate a curve fitting these data points, a log $_{(inhibitor)}$ vs. normalized response (variable slope) equation was used. An IC_{50} value was determined from this curve fit by the following equation: y=100/[1+10^((LogIC$_{50}$-x)*HillSlope))]. In some cases, the IC_{50} value reported by GraphPad is a value beyond the range of the concentrations that were actually tested; therefore, greater than the highest concentrations tested is reported.

Results

FIGURE 1: Representative photos of CFU-GM colonies from various species

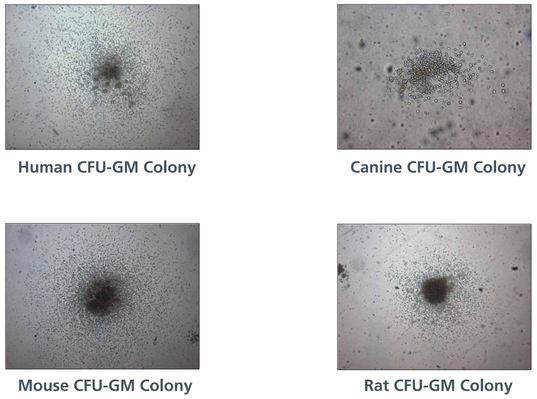

FIGURE 2: Effect of compounds on human CFU-GM colonies

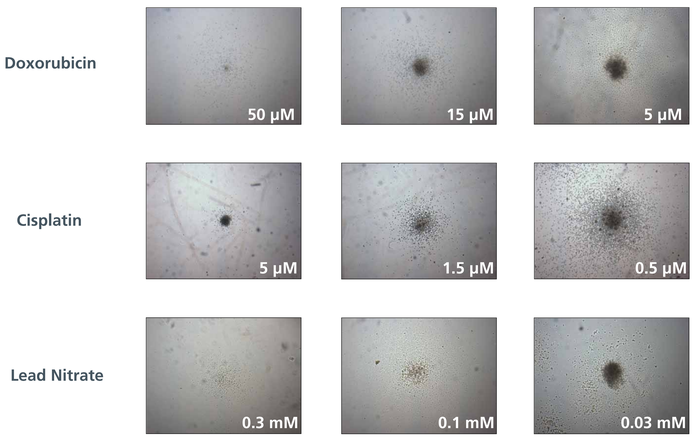

FIGURE 3: Example of comparing CFU-GM IC_{50} values from different species

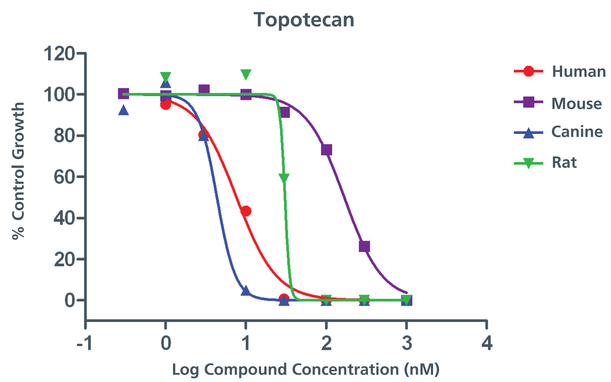

FIGURE 4: CFU-GM IC_{50} values for compounds in different species

Compound	Human	Mouse	Canine	Rat
Topoisomerase				
Topotecan	7.69 nM	168.80 nM	4.40 nM	30.96 nM
Irinotecan	288.10 nM	>1,000 nM	358.10 nM	>1,000 nM
Camptothecin	1.03 nM	6.16 nM	0.75 nM	9.16 nM
Anti-proliferative				
Doxorubicin	0.03 µM	0.01 µM	0.002 µM	0.006 µM
Cisplatin	4.21 µM	6.79 µM	0.97 µM	2.68 µM
5-Fluorouracil	3.84 µM	3.08 µM	0.23 µM	1.62 µM
Tyrosine Kinase Inhibitors				
Sunitinib	0.08 µM	1.10 µM	0.01 µM	0.22 µM
Imatinib	2.16 µM	>30 µM	1.99 µM	>30 µM
Erlotinib	15.27 µM	19.39 µM	10.36 µM	34.67 µM
Environmental Toxin				
Lead Nitrate	0.98 mM	2.05 mM	0.04 mM	1.20 mM

Conclusions

- In general, test compounds displayed a dose-dependent toxic effect on myeloid progenitor proliferation.
- For most compounds tested, canine myeloid progenitor proliferation was more sensitive than human, mouse, or rat myeloid progenitor proliferation.
- Different classes of chemotherapeutics as well as an environmental toxin showed species specificity:

  Topoisomerases & Tyrosine Kinase Inhibitors:
  Human and canine were most similar

  Anti-proliferative:
  Human and mouse were most similar

  Environmental toxin:
  Human and rat were most similar

Summary

The standardized CFU-GM assay has been previously validated by Pessina *et al.* [Toxicol Sci. 2003. 75(2): 355-367] to produce clinically relevant and predictive results, making this *in vitro* assay an indispensable tool for planning and reducing *in vivo* studies for assessing potential myelosuppressive effects. Studies were conducted using chemotherapeutics in different classes as well as an environmental toxin to evaluate their effects on myeloid (CFU-GM) progenitors from human, canine, rat, and mouse bone marrow. The resulting data showed that while all test compounds displayed dose-dependent toxic effect on colony growth, each compound class demonstrated species specificity.