Poster #:1584-529

MANIPULATION OF THE STANDARD CFU-GM ASSAY: TARGETED SCREENING ON HEMATOPOIETIC MYELOID PROGENITORS

Elaine Lau[1], Jackie Damen[1], and Allen Eaves[1,2]

[1] STEMCELL Technologies Inc., Vancouver, BC, Canada [2] Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada

Introduction

A key feature of hematopoietic stem cells and their progeny is that their proliferation and differentiation can be exquisitely regulated by external stimulation from various cytokines. Cytokines differentially affect intracellular signaling pathways to direct lineage commitment and differentiation in hematopoietic progenitors that give rise to mature cell types such as granulocytes, monocyte/macrophages and erythrocytes. The development of new chemical entities, such as kinase inhibitors that specifically target key components of these hematopoietic signaling pathways, has increased dramatically. A crucial tool for the continued discovery and characterization of kinase inhibitors is the use and modification of the colony-forming unit – granulocyte monocyte (CFU-GM) assay, which simultaneously gives rise to isolated colonies of mature granulocytes (CFU-G), monocytes (CFU-M) or combinations (CFU-GM). Importantly, this *in vitro* assay has been shown to yield clinically predictive information allowing for better planning and overall reduction in *in vivo* studies [Pessina et al. Toxicol Sci. 2003. 75(2): 355-367]. By manipulating the type and concentration of cytokines, this assay can be customized to support the growth of specific myeloid progenitors, allowing the differential assessment of compounds on the progenitor cells of interest. To standardize this approach we evaluated various media formulations to establish the ideal growth conditions for detecting inhibition of myeloid progenitors from human bone marrow, as well as testing 2 tyrosine kinase inhibitors (Sunitinib and Imatinib) and the anti-proliferative compound 5-Fluoruracil to determine the effects on each progenitor population.

Materials & Methods

CELLS

Frozen normal human bone marrow mononuclear cells from three donors were stored at -152°C until required for the assay. Cells from each sample 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 (STEMCELL Technologies Inc.) and a cell count (3% glacial acetic acid) and viability assessment (trypan blue exclusion test) were performed.

MEDIA

MethoCult™ GF H84534 (STEMCELL Technologies Inc.) is a medium formulated for optimal growth of myeloid colonies. MethoCult™ H4230 (STEMCELL Technologies Inc.) is a cytokine-free medium supplemented with rhGM-CSF, rhG-CSF or rhM-CSF for optimal growth of myeloid colonies.

COMPOUNDS

Imatinib, Sunitinib (Cayman Chemical; Ann Arbor, MI) and 5-Fluorouracil (Sigma; St Louis, MO) 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 (CFC) ASSAY

Each compound stock was added to the appropriate MethoCult™ 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 from each donor 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 14 days, which is required for proper colony development. Colonies were enumerated by trained personnel.

IC_{50} VALUE DETERMINATION

To calculate the concentration of 50% inhibition of colony growth (IC_{50}) 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^((LogIC50-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.

CYTOLOGICAL ANALYSIS OF MYELOID COLONIES

Individual colonies were picked from dishes containing M-CSF or G-CSF and transferred to a tube containing 100 µL of PBS + 2% FBS. The entire volume was then placed into a cytospin reservoir and cells were centrifuged onto glass slides at 1000 rpm for 5 minutes. Slides were air dried before fixation with methanol. Standard cytology staining was then performed using May-Grunwald followed by Giemsa stain (1:10 ratio of Methanol:Giemsa). Cells from CFU-M colonies are large with an oval to round shape and appear to have vacuoles. Cells from CFU-G colonies are round, bright and much smaller and more uniform in size colonies and a typical lobular nucleus can be visualized.

Results

FIGURE 1: Regulation of Hematopoiesis by Myeloid Cytokines

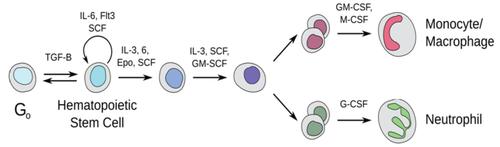

TABLE 1: Myeloid Cytokines Direct Lineage Commitment to CFU-G and CFU-M Progenitors in Customized CFC Assay

MethoCult® 4230 + Myeloid Cytokines (ng/mL)		CFU-G	CFU-GM	CFU-M	Total Myeloid
GM-CSF	10	23±5	3±1	35±3	61±6
	1	20±2	3±2	34±6	57±7
	0.1	25±4	6±3	35±4	66±3
	0.03	9±3	4±1	35±6	48±8
	0.01	5±3	2±1	30±4	37±5
	0.001	2±1	2±1	16±2	19±2
G-CSF	100	40±7	6±3	16±3	62±7
	30	45±4	9±3	13±5	67±4
	10	27±4	6±2	16±3	49±5
	1	17±5	3±1	21±2	41±5
	0.1	7±1	5±1	23±2	35±4
	0.03	7±1	1±1	21±1	29±2
	0.01	4±1	1±1	18±3	23±2
M-CSF	30	3±1	4±4	44±5	52±7
	10	2±1	2±2	40±5	44±3
	1	2±1	1±1	25±3	28±5
	0.1	3±1	0±1	20±2	23±3
	0.03	2±0	0±0	17±5	19±5
	0.01	3±3	1±1	16±3	20±5
	0.003	2±1	0±0	9±3	11±3

FIGURE 2: Cytological Analysis and Confirmation of Progenitor Type

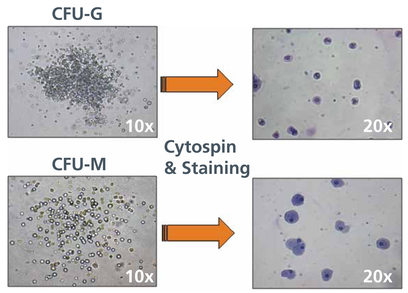

TABLE 2: Compounds Evaluated in Customized CFC Assay

Compound	Known Targets	Indication	Reference
Imatinib	ABL/PDGF/Kit	Chronic Myelogenous Leukemia (CML)	J Clin Oncol 22(1):77-85, 2004
Sunitinib	VEGFR1/KIT/PDGF/CSF-1R/Flt3	Renal Cell Carcinoma	J Clin Oncol 24(1):16-24, 2005
5-Fluorouracil	Thymidylate Synthase Inhibitor	Colorectal & Pancreatic Cancer	Tox Sci 75(2):355-367, 2003

TABLE 3: Imatinib and Sunitinib Display Selective Inhibition of CFU-M Progenitors in Customized CFC Assays

Imatinib

Media	Progenitor Type	IC_{50} (µM)	Published IC_{50} (µM)*
MethoCult® 84534	Total Myeloid	2.57	2.6
MethoCult®4230 + rhG-CSF	G-CSF	> 30	ND
MethoCult®4230 + rhM-CSF	M-CSF	2.98	ND

Sunitinib

Media	Type	IC_{50} (µM)	Published IC_{50} (µM)*
MethoCult® 84534	Total Myeloid	0.08	0.09
MethoCult®4230 + rhG-CSF	G-CSF	1.47	ND
MethoCult®4230 + rhM-CSF	M-CSF	0.06	ND

* See Table 2 for references
ND - not determined

TABLE 4: Selective Activity of Imatinib and Sunitinib on CFU-M Progenitors is Observed in Multiple Bone Marrow (BM) Samples

Imatinib		BM Lot 1	BM Lot 2	BM Lot 3	
Media	**Type**	**IC_{50} (µM)**	**IC_{50} (µM)**	**IC_{50} (µM)**	**Fold Difference**
MethoCult®4230 + rhG-CSF	CFU-G	> 30	>30	11.8	10 ± 5.5
MethoCult®4230 + rhM-CSF	CFU-M	2.21	2.35	3.15	

Sunitinib		BM Lot 1	BM Lot 2	BM Lot 3	
Media	**Type**	**IC_{50} (µM)**	**IC_{50} (µM)**	**IC_{50} (µM)**	**Fold Difference**
MethoCult®4230 + rhG-CSF	CFU-G	1.36	1.67	0.52	24.4 ± 18.9
MethoCult®4230 + rhM-CSF	CFU-M	0.05	0.04	0.12	

5-Fluorouracil		BM Lot 1	BM Lot 2	BM Lot 3	
Media	**Type**	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**	**Fold Difference**
MethoCult®4230 + rhG-CSF	CFU-G	0.37	0.37	0.22	1.2 ± 0.4
MethoCult®4230 + rhM-CSF	CFU-M	0.41	0.33	0.14	

Conclusions

- MethoCult™ medium can be customized with different cytokines to direct lineage commitment of myeloid CFU-G and CFU-M progenitors in a dose dependent manner.
- IC_{50} values for Sunitinib, Imatinib, and 5-Fluorouracil for myeloid (CFU-GM) inhibition were in agreement with values in published literature.
- Sunitinib and Imatinib displayed selective inhibition of CFU-M over CFU-G progenitors, while 5-Fluorouracil showed no differential activity.
- The selective inhibition of CFU-M progenitors by Sunitinib and Imatinib was seen in all bone marrow samples tested.

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. For example, tyrosine kinase inhibitors Sunitinib and Imatinib, which demonstrate *in vitro* IC_{50} values of 0.09 µM and 2.6 µM, respectively, in the CFU-GM assay, display similar *in vivo* potencies with clinical neutropenia grade I/II developing in 32% and 6% of patients, respectively [J Clin Oncol. 2006. 24(1): 16-24; J Clin Oncol. 2004. 22(1): 77-85]. We have further customized this assay to allow the assessment of compounds on specific myeloid progenitor populations, such as CFU-M and CFU-G. Using this customized assay, we have demonstrated that Sunitinib and Imatinib display selective inhibition of CFU-M progenitors. This selectivity is not surprising for Sunitinib, a known inhibitor of CSF-1R (macrophage colony-stimulating factor receptor). Although the mechanism behind Imatinib's selectivity is not clear, it is not unexpected as cellular signaling is the cumulative result of the function of multiple downstream kinases. In summary, this customized assay will be a powerful tool to allow for discrimination of compound effects on specific subpopulations of myeloid progenitors.