STUDY # STI001

Evaluation of the Toxicity of Test Compounds on Human Myeloid and Erythroid Progenitors Using Methylcellulose-based *In Vitro* Colony Assays

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STEMCELL TECHNOLOGIES

CONFIDENTIAL

Study name: Evaluation of the Toxicity of Test Compounds on Human Myeloid and Erythroid Progenitors Using Methylcellulose-based *In Vitro* Colony Assays

Study Number: STI001

Sponsor: STEMCELL Technologies, 400 – 570 West 7th Ave., Vancouver BC, Canada, V5Z 1B3

Summary: The effects of compounds X, Y and Z on human erythroid and myeloid progenitor proliferation in MethoCult® 84434 were examined in this study. Compound Y (IC_{50} = 5.22 µM) displayed the greatest potency against erythroid progenitor proliferation followed by Compound X (IC_{50} = 20.01 µM) and Compound Z (IC_{50} = 35.51 µM). Compound X (IC_{50} = 16.45 µM) displayed the greatest potency against myeloid progenitor proliferation, followed by Compound Y (IC_{50} = 26.92 µM) and Compound Z (IC_{50} = 42.86 µM). Colony morphology (size and/or density) for erythroid and myeloid progenitors in the presence of test compounds were compromised where toxicity was observed.

Study Director: Jackie Damen PhD

Testing Laboratory: StemCell Technologies Inc., 570 West Seventh Ave, Suite 400, Vancouver, BC, Canada V5Z 1B3

Date of Study: July – August 2009

Definitions:

CFU-E:	Colony-Forming Unit – Erythroid. This is the most mature erythroid colony forming cell. The small erythroid colony derived from this cell contains one to 2 clusters with a total number of 8-200 erythroblasts.
BFU-E:	Burst-Forming Unit – Erythroid. This is a more primitive cell. The larger erythroid colony derived from this cell contains greater than 200 erythroblasts.
CFU-GM:	Colony-Forming Unit – Granulocyte, Macrophage. This colony forming cell is capable of producing colonies with 40 or more granulocyte–monocyte and/or macrophage cells.
CFU-GEMM:	Colony-Forming Unit – Granulocyte, Erythroid, Macrophage, Megakaryocyte. This is the most primitive colony forming cell. The colony derived from this cell contains cells from more than one lineage, including erythroid cells as well as 20 or more granulocytes, macrophages and megakaryocytes.

Cytokines:

Epo	Erythropoietin
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte/Monocyte-Colony Stimulating Factor
IL-3	Interleukin-3
SCF	Stem Cell Factor

Purpose:

The aim of the study was to evaluate the potential effects of Compounds X, Y and Z at 6 concentrations on human bone marrow derived erythroid and myeloid progenitor proliferation in MethoCult® media.

Tests performed:

Human bone marrow derived hematopoietic progenitor cells were incubated with test compounds in MethoCult® 84434, a methylcellulose-based media containing Epo, G-CSF, GM-CSF, IL-3 and SCF.

Cells:

Normal human bone marrow light density cells (Poietics Lot 07B21195) were stored at -152^{o}C until required for the assay. On the day of the experiment, the cells were thawed rapidly at 37^{o}C, the contents of the vial were diluted in 10 ml of Iscove's modified Dulbecco's medium containing 2% fetal bovine serum (IMDM + 2% FBS) and washed by centrifugation (1200 r.p.m. for 10 minutes, room temperature). The supernatant was discarded and the cell pellet resuspended in a known volume of IMDM + 2% FBS. A nucleated cell count (3% glacial acetic acid) and viability assessment (trypan blue exclusive test) was performed.

Compounds:

STEMCELL Technologies provided 3 compounds (Compound X, Y and Z) as powders. All compounds were dissolved in DMSO as 50 mM stocks. The 50 mM stocks were diluted in DMSO to make 1000 fold working stock solutions at 15, 5, 1.5, 0.5, and 0.15 mM. These working stock solutions were subsequently added to MethoCult® media to provide the appropriate final test concentrations required: 50, 15, 5, 1.5, 0.5, and 0.15 µM.

Method Summary:

Test compounds were added to MethoCult® 84434 to give final concentrations of 50, 15, 5, 1.5, 0.5, and 0.15 µM. Standard control cultures (containing no compound) and solvent control cultures (containing no compound but equivalent concentrations of DMSO) were also initiated. Thawed human bone marrow cells (as described above) were then added to the media formulation containing compounds. Hematopoietic cultures were set up in triplicate with 1.00 x 10^4 cells per culture at each compound concentration tested. Following 14 days in culture, hematopoietic colonies were assessed and scored by trained personnel.

Statistical Analyses of CFC numbers:

The mean colony number (+/- 1 standard deviation) was calculated for triplicate cultures at each condition. Standard t-tests were performed to compare solvent control to each compound. Due to the potential subjectivity of colony enumeration, a p value of less than 0.01 is deemed significant.

IC_{50} and IC_{90} Determination:

To calculate the concentration of 50% and 90% inhibition of colony growth (IC_{50} and IC_{90}) for each compound, a dose response curve was generated plotting the log of the compound concentration versus the percentage of control colony growth using Microcal™ Origin™. A Sigmoidal curve was then fit to the graph and from this curve the inhibitory concentration (µM) was then calculated using the Boltzman equations:

$\log(x_{50}) = x_0 + dx * LN((A1-A2)/(50-A2)-1)$

$\log(x_{90}) = x_0 + dx * LN((A1-A2)/(10-A2)-1)$

where A1 = the initial value (baseline response), A2 = final value (maximum response), x_0 = center (drug concentration that provokes a response halfway between A1 and A2) and dx = width (of the rapidly varying part of the curve) as determined by Microcal™ Origin™.

Morphological Assessment of Colonies:

Photographs were taken of representative erythroid and myeloid derived colonies from various compound concentrations illustrating normal colonies and colonies where the growth was perturbed due to the activity of the compounds.

Results:

Hematopoietic Progenitor Proliferation

The average colony counts per dish (+/-1 SD) obtained from cells after continuous exposure to test compounds are presented in Table 1. There were no statistically significant differences between the solvent control cultures (DMSO) and the standard control cultures (containing no compound and no DMSO). Statistical analysis was also performed to compare the number of colonies in compound treated cultures to the solvent control culture.

Table 1. Average Colony Counts per Dish For Cultures After Incubation with Test Compounds in MethoCult® 84434.

	CFU-E	BFU-E	Total Erythroid	CFU-GM	CFU-GEMM	Total CFC
Standard	14+/-3	35+/-6	48+/-6	45+/-3	4+/-2	97+/-8
Solvent Control	13+/-2	36+/-3	49+/-2	49+/-10	4+/-1	102+/-10
Compound X						
50 µM	ND**	ND##	ND##	ND#	ND*	ND##
15 µM	7+/-3	23+/-1*	30+/-2**	43+/-8	2+/-1	75+/-7
5 µM	9+/-4	30+/-5	40+/-7	50+/-8	1+/-1	90+/-4
1.5 µM	12+/-3	36+/-8	48+/-11	47+/-1	3+/-2	98+/-9
0.5 µM	14+/-2	35+/-5	49+/-4	46+/-2	3+/-2	98+/-6
0.15 µM	13+/-6	36+/-8	49+/-10	48+/-6	4+/-1	101+/-16
Compound Y						
50 µM	1+/-0 *	ND##	5+/-6**	14+/-6$	ND*	19+/-8**
15 µM	15+/-2	2+/-2##	17+/-3##	32+/-6	1+/-1	50+/-8*
5 µM	14+/-2	9+/-4#	23+/-5#	41+/-2	1+/-1$	65+/-6*
1.5 µM	11+/-6	24+/-3$	35+/-9	45+/-6	1+/-2	81+/-7
0.5 µM	12+/-3	33+/-8	44+/-11	50+/-9	2+/-2	96+/-18
0.15 µM	10+/-1	38+/-3	48+/-2	50+/-10	3+/-1	101+/-9
Compound Z						
50 µM	9+/-4	4+/-1##	13+/-4##	20+/-5$	1+/-1$	34+/-8#
15 µM	10+/-2	28+/-7	38+/-6	40+/-2	2+/-1	79+/-7
5 µM	12+/-1	28+/-8	40+/-8	51+/-5	4+/-2	95+/-2
1.5 µM	11+/-2	33+/-6	45+/-6	49+/-8	2+/-2	95+/-4
0.5 µM	11+/-4	29+/-5	40+/-8	48+/-7	4+/-2	92+/-5
0.15 µM	13+/-2	30+/-5	44+/-5	50+/-3	4+/-3	97+/-3

ND = none detected
$ $p < 0.01$ compared to solvent control
* $p < 0.005$ compared to solvent control
# $p < 0.001$ compared to sovlent control
** $p < 0.0005$ compared to solvent control
$p < 0.0001$ compared to solvent control

Dose Response Curves and IC_{50} & IC_{90} Concentrations

Dose response curves plotting percent of control colony growth versus log of compound concentration are shown in Figures 1-3. IC_{50} and IC_{90} values were derived from these curves, and a summary of the predicted IC_{50} and IC_{90} values are contained in Table 2.

Table 2. IC_{50} and IC_{90} Values for Test Compounds on Human Erythroid and Myeloid Progenitor Proliferation in MethoCult® 84434.

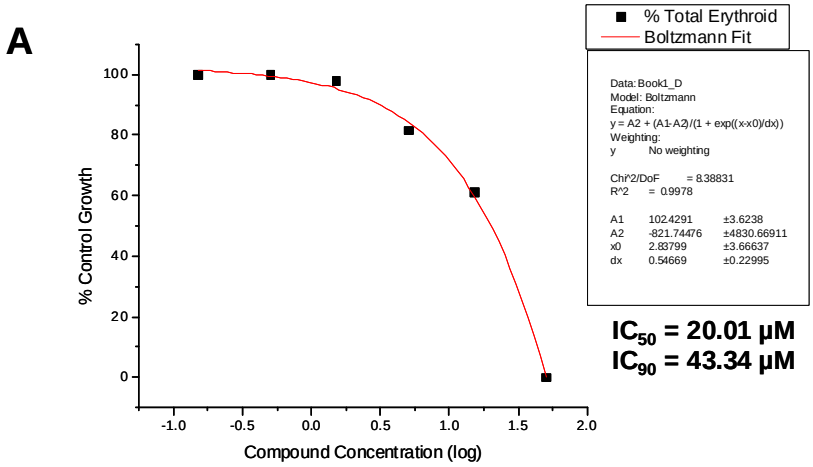
	Total Erythroid		Myeloid	
Compound	**IC_{50} (µM)**	**IC_{90} (µM)**	**IC_{50} (µM)**	**IC_{90} (µM)**
X	20.01	43.34	16.45	18.10
Y	5.22	55.08^{ex}	26.92	76.52^{ex}
Z	35.51	59.53^{ex}	42.86	75.34^{ex}

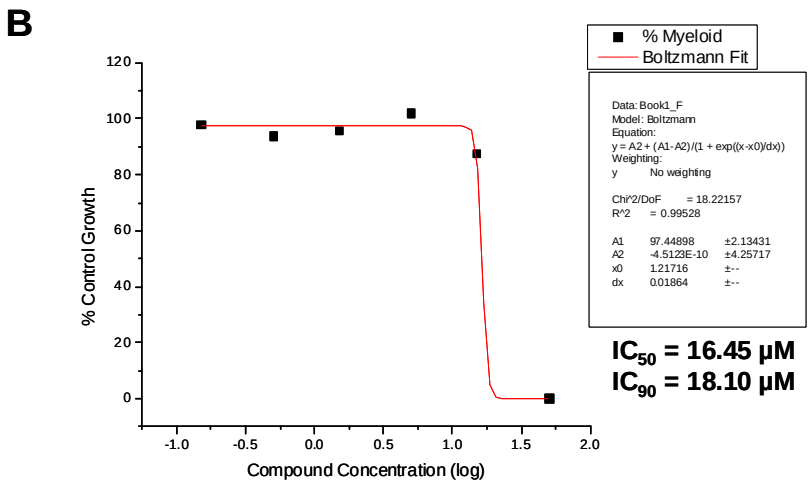
^{ex} = extrapolated by the Boltzmann equation

Compound X

IC_{50} and IC_{90} values for erythroid growth were determined to be 20.01 µM and 43.34 µM, respectively (Figure 1A). IC_{50} and IC_{90} values for myeloid growth were determined to be 16.45 µM and 18.10 µM, respectively (Figure 1B). The morphology (size and/or density) of erythroid and myeloid colonies was compromised at the highest test concentration where colonies were present, 15 µM, and returned to solvent control size at 5 µM (see Photographs 3 - 6 in Appendix).

Figure 1 - Dose Response Curves for the Effect of Compound X on Human (A) Erythroid and (B) Myeloid Progenitor Proliferation in MethoCult® 84434.

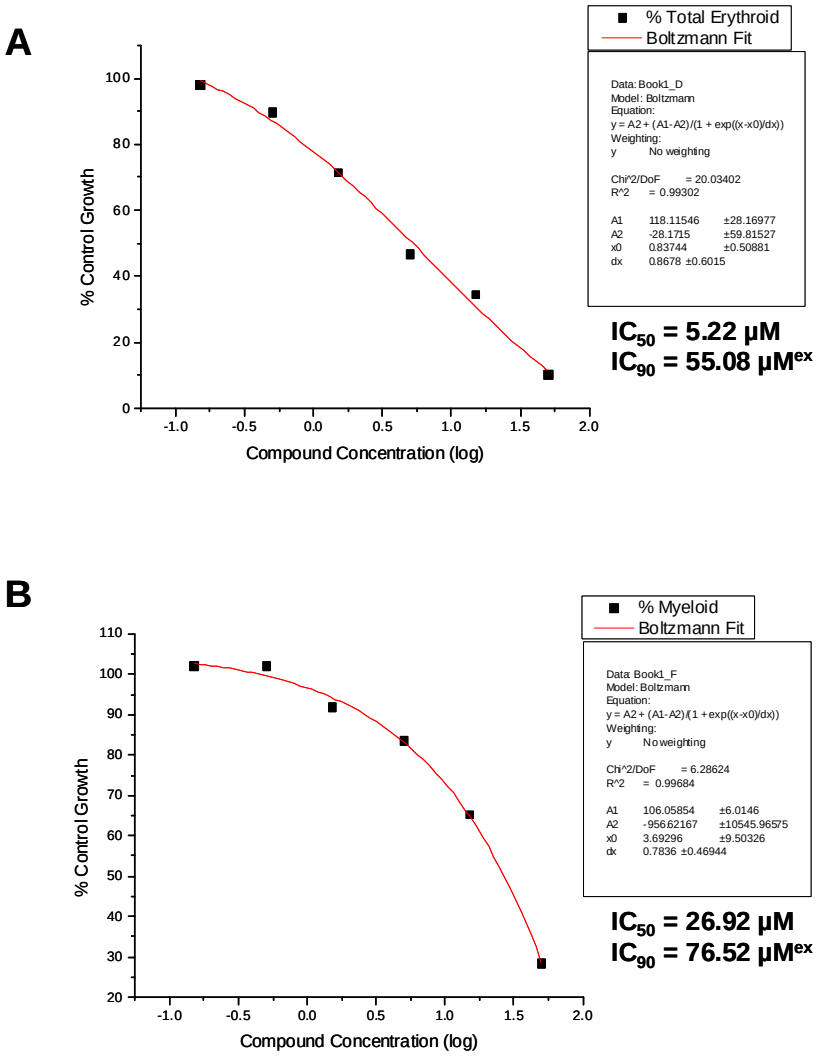




Compound Y

IC_{50} value for erythroid growth was determined to be 5.22 µM; an IC_{90} value of 55.08 µM was extrapolated by Boltzmann equation (Figure 2A). IC_{50} value for myeloid growth was determined to be 26.92 µM; an IC_{90} value of 96.52 µM was extrapolated by Boltzmann equation (Figure 2B). The morphology (size and/or density) of erythroid colonies was compromised at concentrations between 15 and 1.5 µM and returned to solvent control size at 0.5 µM. Myeloid colonies were compromised at concentrations between 50 and 5 µM and returned to solvent control size at 1.5 µM (see Photographs 7 - 14 in Appendix).

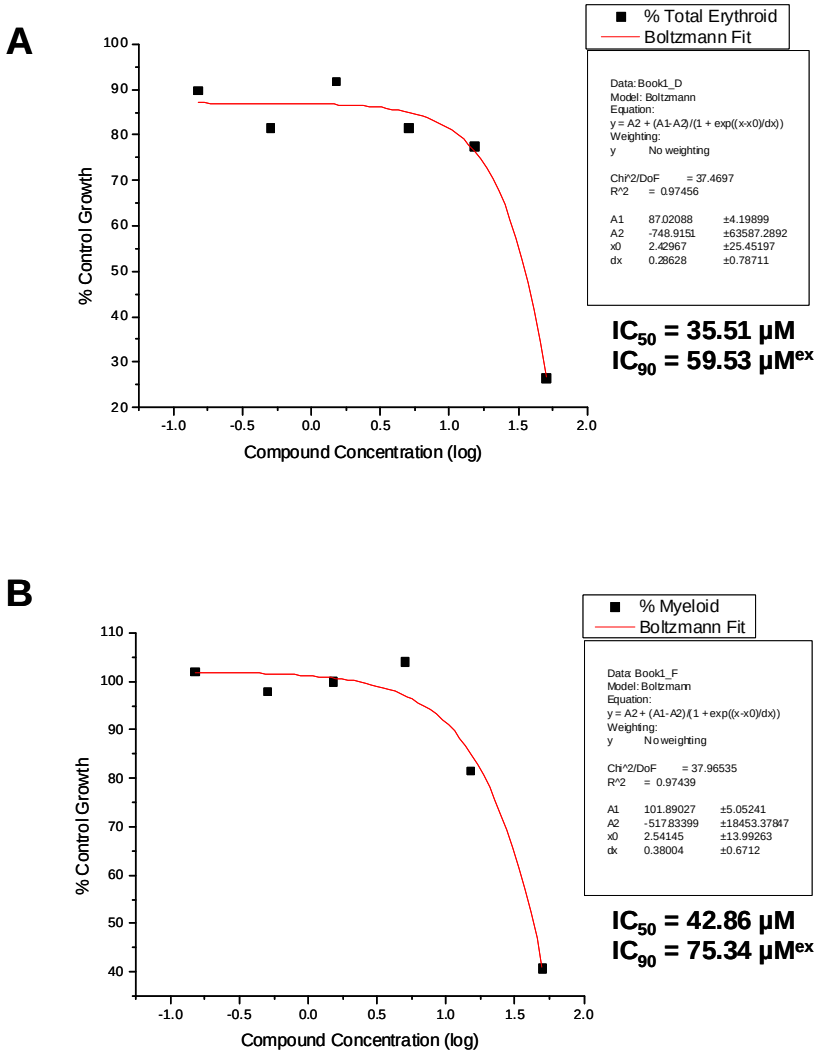
Figure 2 - Dose Response Curves for the Effect of Compound Y on Human (A) Erythroid and (B) Myeloid Progenitor Proliferation in MethoCult® 84434.
^{ex} – extrapolated by Boltzmann equation



Compound Z

IC_{50} value for erythroid growth was determined to be 35.51 µM; an IC_{90} value of 59.53 µM was extrapolated by Boltzmann equation (Figure 3A). IC_{50} value for myeloid growth was determined to be 42.86 µM; an IC_{90} value of 75.34 µM was extrapolated by Boltzmann equation (Figure 3B). The morphology (size and/or density) of erythroid and myeloid colonies incubated was compromised at 50 and 15 µM and returned to solvent control size at 5 µM (see Photographs 15 - 20 in Appendix).

Figure 3 - Dose Response Curves for the Effect of Compound Z on Human (A) Erythroid and (B) Myeloid Progenitor Proliferation in MethoCult® 84434.
^{ex} – extrapolated by Boltzmann equation



Conclusion:

The effects of compounds X, Y and Z on human erythroid and myeloid progenitor proliferation in MethoCult® 84434 were examined in this study. Compound Y (IC_{50} = 5.22 µM) displayed the greatest potency against erythroid progenitor proliferation followed by Compound X (IC_{50} = 20.01 µM) and Compound Z (IC_{50} = 35.51 µM). Compound X (IC_{50} = 16.45 µM) displayed the greatest potency against myeloid progenitor proliferation, followed by Compound Y (IC_{50} = 26.92 µM) and Compound Z (IC_{50} = 42.86 µM). Colony morphology (size and/or density) for erythroid and myeloid progenitors in the presence of test compounds were compromised where toxicity was observed.

Sponsor and Testing Facility:
The testing described herein was performed for STEMCELL Technologies by:

StemCell Technologies Inc.
570 West Seventh Ave, Suite 400
Vancouver, BC, Canada V5Z 1B3

Final Report Approved by:

Study Director	Date
Report Auditor	Date

Appendix

Controls

Photograph 1: CFU-GM from solvent control in MethoCult® 84434 (photographed at 30X)

Photograph 2: BFU-E from solvent control in MethoCult® 84434 (photographed at 70X)

Compound X

Photograph 3: Small CFU-GM with compound X at 15 μM in MethoCult® 84434 (photographed at 30X)

Photograph 4: CFU-GM with compound X at 5 μM in MethoCult® 84434 (photographed at 30X)

Photograph 5: Small BFU-E with compound X at 15 μM in MethoCult® 84434 (photographed at 70X)

Photograph 6: BFU-E with compound X at 5 μM in MethoCult® 84434 (photographed at 70X)

Compound Y

Photograph 7: Small CFU-GM with compound Y at 50 μM in MethoCult® 84434 (photographed at 30X)

Photograph 8: Small CFU-GM with compound Y at 15 μM in MethoCult® 84434 (photographed at 30X)

Photograph 9: Small CFU-GM with compound Y at 5 μM in MethoCult® 84434 (photographed at 30X)

Photograph 10: CFU-GM with compound Y at 1.5 μM in MethoCult® 84434 (photographed at 30X)

Photograph 11: Small BFU-E with compound Y at 15 μM in MethoCult® 84434 (photographed at 70X)

Photograph 12: Small BFU-E with compound Y at 5 μM in MethoCult® 84434 (photographed at 70X)

Photograph 13: Small BFU-E with compound Y at 1.5 μM in MethoCult® 84434 (photographed at 70X)

Photograph 14 BFU-E with compound Y at 0.5 μM in MethoCult® 84434 (photographed at 70X)

Compound Z

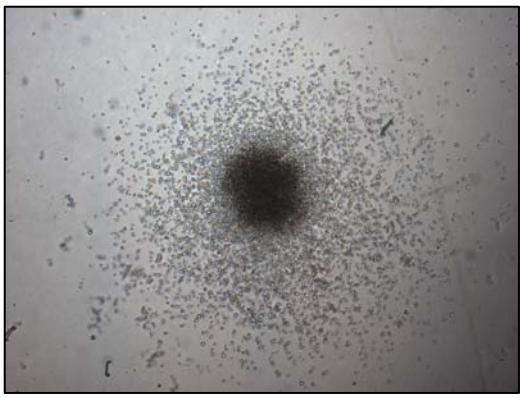
Photograph 15: Small CFU-GM with compound Z at 50 μM in MethoCult® 84434 (photographed at 30X)

Photograph 16: CFU-GM with compound Z at 15 μM in MethoCult® 84434 (photographed at 30X)

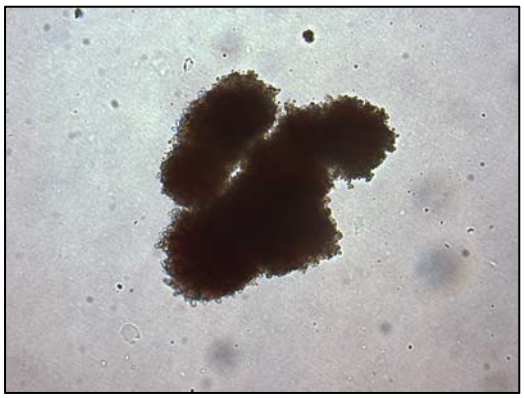
Photograph 17: CFU-GM with compound Z at 5 μM in MethoCult® 84434 (photographed at 30X)

Photograph 18:	Small BFU-E with compound Z at 50 μM in MethoCult® 84434 (photographed at 70X)
Photograph 19:	Small BFU-E with compound Z at 15 μM in MethoCult® 84434 (photographed at 70X)
Photograph 20:	BFU-E with compound Z at 5 μM in MethoCult® 84434 (photographed at 70X)

Appendix



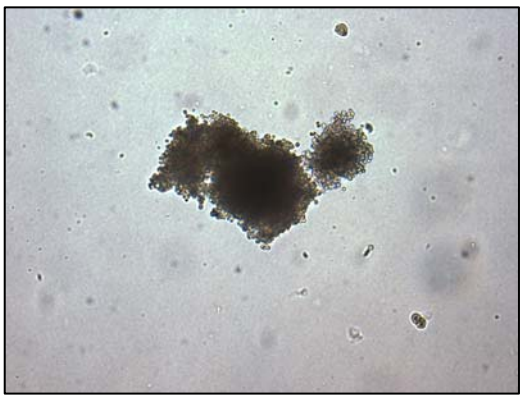
1. CFU-GM from solvent control in MethoCult® 84434 (30X)



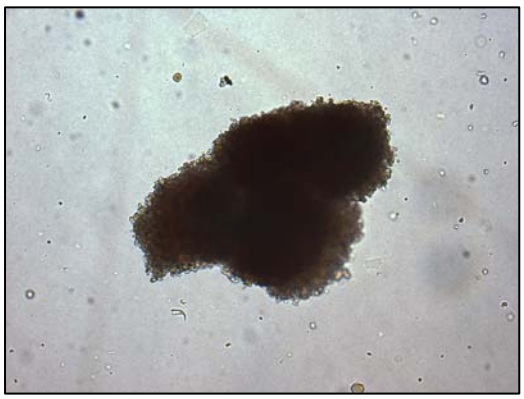
2. BFU-E from solvent control in MethoCult® 84434 (70X)

3. Small CFU-GM with compound X at 15 µM in MethoCult® 84434 (30X)

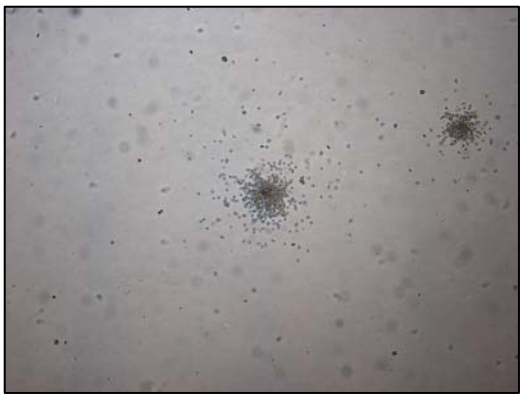
4. CFU-GM with compound X at 5 µM in MethoCult® 84434 (30X)



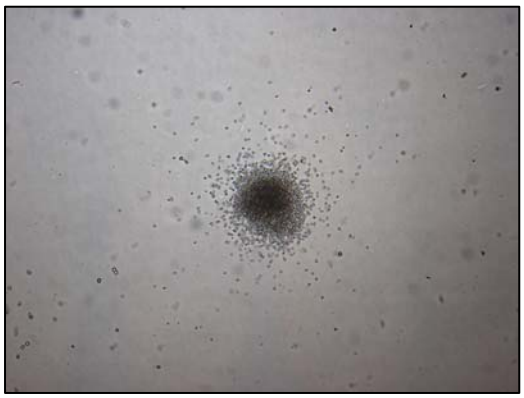
5. Small BFU-E with compound X at 15 µM in MethoCult® 84434 (70X)



6. BFU-E with compound X at 5 µM in MethoCult® 84434 (70X)

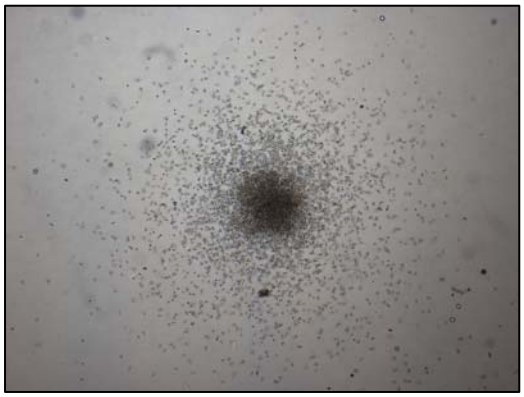


7. Small CFU-GM with compound Y at 50 µM in MethoCult® 84434 (30X)

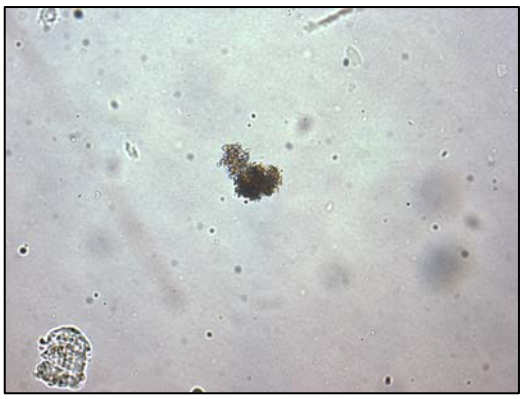


8. Small CFU-GM with compound Y at 15 µM in MethoCult® 84434 (30X)

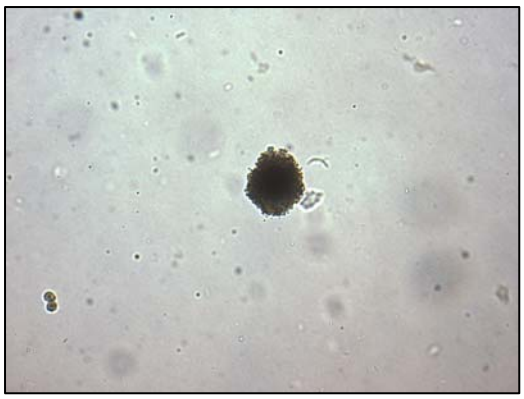
9. Small CFU-GM with compound Y at 5 µM in MethoCult® 84434 (30X)



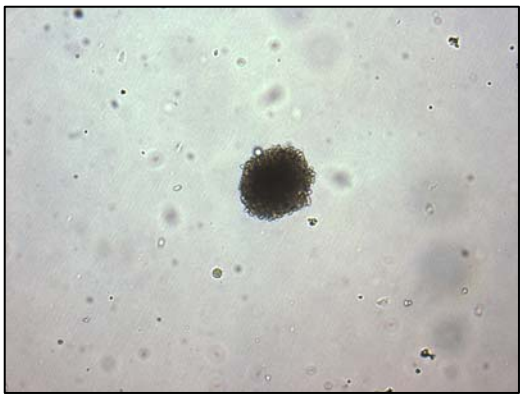
10. CFU-GM with compound Y at 1.5 µM in MethoCult® 84434 (30X)



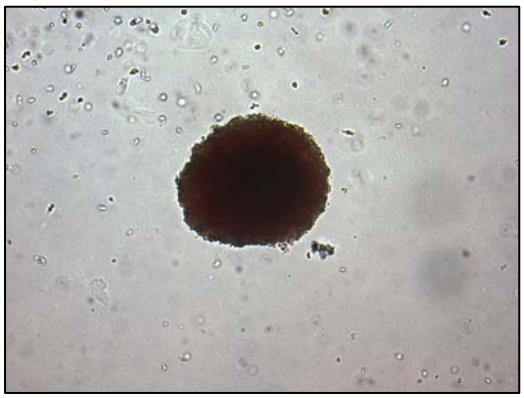
11. Small BFU-E with compound Y at 15 µM in MethoCult® 84434 (70X)



12. Small BFU-E with compound Y at 5 µM in MethoCult® 84434 (70X)



13. Small BFU-E with compound Y at 1.5 µM in MethoCult® 84434 (70X)

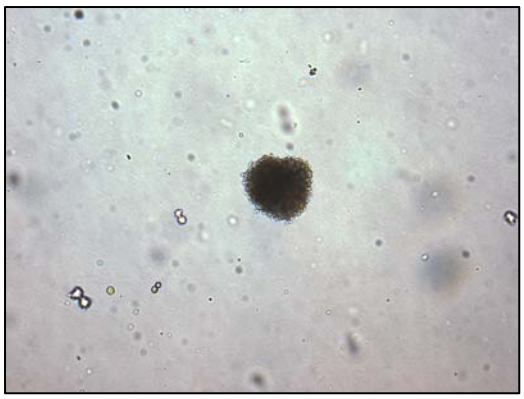


14. BFU-E with compound Y at 0.5 µM in MethoCult® 84434 (70X)

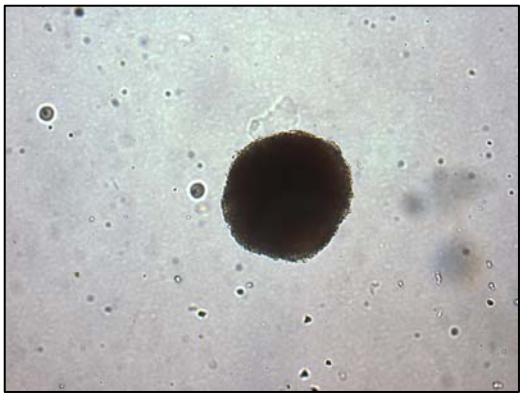
15. Small CFU-GM with compound Z at 50 µM in MethoCult® 84434 (30X)

16. CFU-GM with compound Z at 15 µM in MethoCult® 84434 (30X)

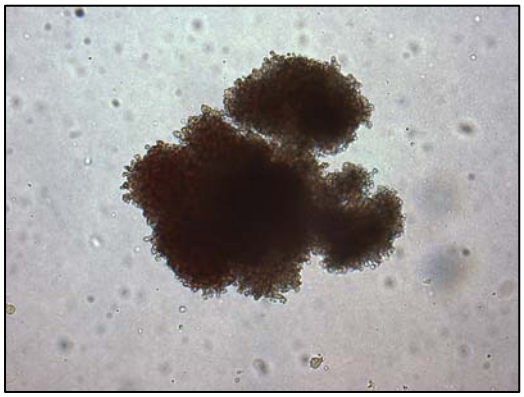
17. CFU-GM with compound Z at 5 µM in MethoCult® 84434 (30X)



18. Small BFU-E with compound Z at 50 µM in MethoCult® 84434 (70X)



19. Small BFU-E with compound Z at 15 µM in MethoCult® 84434 (70X)



20. BFU-E with compound Z at 5 µM in MethoCult® 84434 (70X)