Chapter 4

Development of in vitro murine and human bone marrow derived CFU-GM assays
4.1 Introduction

Hematopoiesis is the process by which all lineages of blood cells are generated in a hierarchical and stepwise manner from immature cells present in the bone marrow (BM) and subsequently released into circulating blood and peripheral organs for further maturation steps and/or effector function.

Immunotoxicity is defined as the toxicological adverse effects of xenobiotics including pharmaceuticals on the normal functioning of the immune system. Immunotoxicity can be induced in either direct or indirect ways (Trizio D et al., 1988). Direct immunotoxicity is caused by the effects of chemicals on the immune system. This subsequently leads to immunosuppression and reduced resistance to infectious diseases or certain forms of non-genotoxic carcinogenicity. Hemaotoxicity is reported as one of the most severe form of immunotoxicity associated with anticancer agents.

4.1.1 Anticancer agents induce hematotoxicity

Even with the recent advancement in modern drug biology, new techniques and development of molecular targeting based therapeutic approaches, majority of the anticancer-agents are still known to be accompanied with hematotoxicity as their common side effect. Amongst hepatotoxicity, cardiotoxicity, neurotoxicity, renal toxicity and others, toxicity to the blood-forming system (hematotoxicity) has been considered as one of the accepted toxicities, especially for anti-cancer drugs (Maxwell MB, Maher KE, 1992). In the process of destroying abnormally proliferating cancerous cells, chemotherapy also causes considerable adverse toxicity to other normal and healthy rapidly dividing cells, including hematopoietic cells in the bone marrow. Conventional antineoplastics agents, radiation, high-dose therapies and drug combinations induce severe bone marrow suppression with the risk for infection and hemorrhagic complications (Hoagland HC, Gastineau DA, 1996). The therapeutic index for virtually all the anti-cancer treatments is essentially defined by the differential effect of the drug on cancer cells and normal cells.
Potential toxicity caused to hematopoietic tissue must be evaluated early in the development of such compounds. Hematotoxicity is an important part of the general toxicology studies performed during pre-clinical animal studies, which includes analyzing blood parameters, tissue and organ pathology (Gribaldo L et al., 1996). Unfortunately, animal hematotoxicity is rarely predictive and most often cannot be extrapolated to the human situation. If any in vitro studies are performed, transformed cell lines are usually used and have little bearing on the in vivo human situation because of the correlation (Negro GD et al., 2001).

4.1.2 In vitro hematotoxicity - role in drug development

It is noted over the years, that results obtained from routinely used cancer models in animals (i.e., murine or human tumor xenografts in nude mice) have provided little predictive value for clinical pharmacodynamics and pharmacokinetics in humans. Consequently, it’s a common feature during drug development process that many promising anticancer drugs fail during Phase I clinical trials on account of severe myelosuppression as side effect in patients (Rich IN, 2003). Thus, selection of the starting dose for a Phase I trial, which is typically based on the maximum-tolerated dose (MTD) of the most sensitive species, is very critical. The goal is to decrease the risk of a lethal overdose in the first cohort of patients, whereas effectively determining the MTD to achieve the maximum therapeutic effect of an anticancer drug. To achieve this, it is always desirable to predict the human MTD for safe and efficient clinical dose escalation studies, while at the same time reducing the number of patients treated with an ineffective dose. For almost all of the cytotoxic agents, clinical testing starts at a dose that kills 10% of the tested animals (LD10). The dose is then gradually increased in modified Fibonacci steps (Eisenhauer EA et al., 2000).

When combined with efficacy data, preclinical evaluation of hematopoietic toxicity may identify the least toxic analogue with the best therapeutic index in humans. Such reports highlight the role of in vitro hematotoxicity studies in drug development. In vitro models of hematopoiesis are now increasingly being applied as screens and investigative tools.
in the clinical setting, bone marrow transplantation and other research purposes such as screening of new entities with hematotoxic potential during preclinical development (Deldar A et al., 1995). These assays are very helpful in investigating myelotoxic effects specific to target cell subpopulations and mechanism of toxic effects (Masubuchi N et al., 2004). In vitro hematotoxicity assessment using colony-forming assays, act as a surrogate marker of myelosuppression and could play a key role in linking animal toxicology studies to clinical investigations. However, the predictive bone marrow MTD for the neutrophil lineage obtained in CFU assay will correlate with the actual human MTD only when the myelopoietic tissue of bone marrow is a primary target of toxicity for the drug or in other words, when neutropenia is the dose-limiting toxicity.

In adult bone marrow, a small number of hematopoietic stem cells (HSCs) lead to production of heterogeneous populations of actively dividing hematopoietic progenitors (Colony Forming Units/CFU). These CFUs proliferate and differentiate resulting in the generation of millions of mature blood cells daily. In vitro assay systems have been developed to quantify multi-potential progenitors and lineage-restricted progenitors of the erythroid, granulocytic, monocyte-macrophage and megakaryocyte-myelopoietic subsets. When cultured in a suitable semi-solid matrix, individual progenitors called colony-forming cells (CFCs) proliferate to form discrete cell clusters or colonies (CFU). CFC assays are performed by placing cell suspension into a semi-solid medium, such as methylcellulose, supplemented with nutrients and cytokines followed by incubation at 37°C for periods ranging from a few days to several weeks. Effects of test agent can be assessed by incubating cells in methylcellulose with growth factor. The CFCs are classified and enumerated based on the morphological recognition of one or more types of hematopoietic lineage cells within the colony. Various gelling agents including agar, agarose, methylcellulose, collagen and fibrin clots have been used for CFC assays. Methylcellulose is preferred generally, since it is relatively inert polymer that forms a stable gel with good optical clarity and permits counting of colonies easily. It is commonly used at a final concentration of 0.9 - 1.2% in culture medium supplemented with nutrients and growth factors.
In vitro specific myelotoxicity assays, such as the granulocyte-macrophage colony-forming unit (CFU-GM) clonogenic assay, are emerging as useful tools for predicting the adverse effects of new compounds on the blood-forming system (Teicher BA, 2009). The in vitro CFU-GM assay was validated by ECVAM in 2006 as a substitute to using a second species, such as dog, for regulatory testing (ESAC Statement, March 21, 2006). ECVAM has approved CFU-GM assay as the alternative method to predict acute neutropenia and MTD in humans in 2006. For antitumoral drug, Yondelis (Ecteinascidin; ET-743), this model was applied to predict human MTD, which was within fourfold of the actual MTD (Gomez SG et al., 2003). Similar studies were performed with anticancer agents such as, PNU-159548 (4-demethoxy-3'-deamino-3'-aziridinyl-4'-methylsulphonyl-daunorubicin) (Moneta D et al., 2003) and XK469 (NSC 656889) (Lo Russo PM et al., 1998). The IC90 ratio of drug in human versus murine CFU-GM assay allows predicting the MTD.

In Chapter 4, we describe the establishment of in vitro murine and human CFU-GM assays from bone marrow cells as precursor populations. The assay conditions for generation of these CFU-GM assays, morphological characterization of colonies and validation with standard anticancer drugs (5-FU and Paclitaxel) is discussed. Figure 4.1 outlines the experimental design of murine/human CFU-GM assay.
Figure 4.1 – Experimental design of CFU-GM assay (Adapted from Pessina A et al., Toxicol Sci, 2003)
4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Animals

For in vitro generation of murine CFU-GM assays, C57BL/6 mice were employed in the studies. Details of the animals are as below:

a) Source

Specific pathogen-free male C57BL/6 mice (20 to 25 gm, 8–10 weeks) were obtained from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, India.

b) Housing conditions

- Animals were kept in the in-house animal facility maintained at 22±3°C and 55±15% relative humidity with 12 h light-dark cycle and 12 air changes per h.
- Animals were maintained in a breeding colony kept in groups of four in plastic mouse cages with conventionally available rodent food purchased from a commercial supplier (autoclaved pelleted feed) and filtered drinking water ad libitum.

c) Animal ethics approval

All experiments employing the mice were performed under the protocols approved by the Institutional Animal Ethics Committee (IAEC) of Dabur Research Foundation. The test facility is registered for breeding and experiment of animals with the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India.

4.2.1.2 Human bone marrow mononuclear cells

Frozen stocks of 15 million human bone marrow mononuclear cells (MNC) were procured (ABM008F, Stem cell Technologies) in 1.8 ml solution of 50% IMDM, 40% FBS, and 10% DMSO.
### 4.2.1.3 Chemicals, reagents and kits

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<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Catalog No.</th>
</tr>
</thead>
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<tr>
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<td>Sigma (USA)</td>
<td>F6627</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma (USA)</td>
<td>A9418</td>
</tr>
<tr>
<td>DMSO</td>
<td>Sigma (USA)</td>
<td>D8418</td>
</tr>
<tr>
<td>DNAse I Deoxyribonuclease I from bovine pancreas, Type II, lyophilized powder, Protein $\geq 80%$, $\geq 2,000$ units/mg protein</td>
<td>Sigma (USA)</td>
<td>D4527</td>
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<td>Ethanol</td>
<td>Merck (India)</td>
<td>100967</td>
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<tr>
<td>FBS</td>
<td>Life Technologies (India)</td>
<td>10437028</td>
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<tr>
<td>HCI</td>
<td>Merck (India)</td>
<td>100317</td>
</tr>
<tr>
<td>IMDM</td>
<td>Gibco (India)</td>
<td>12200-036</td>
</tr>
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<td>Methyl cellulose (MCM)</td>
<td>R&amp;D systems</td>
<td>HSC001</td>
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<tr>
<td>NaCl</td>
<td>Qualigens (India)</td>
<td>27605</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Sigma (USA)</td>
<td>S6014</td>
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<tr>
<td>Paclitaxel</td>
<td>Sigma (USA)</td>
<td>T7402</td>
</tr>
<tr>
<td>Penicillin-streptomycin</td>
<td>Himedia (India)</td>
<td>A018</td>
</tr>
<tr>
<td>rhGMCSF (carrier free)</td>
<td>R&amp;D systems, Minneapolis (USA)</td>
<td>215-GM-010/CF</td>
</tr>
<tr>
<td>rmGMCSF (carrier free)</td>
<td>R&amp;D systems, Minneapolis (USA)</td>
<td>415-ML-050/CF</td>
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<tr>
<td>Trypan Blue</td>
<td>Sigma (USA)</td>
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4.2.1.4 Cell culture ware

Table 4.2

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<td>546021</td>
</tr>
<tr>
<td>50 ml centrifuge tube</td>
<td>Tarsons (India)</td>
<td>546041</td>
</tr>
<tr>
<td>Cryovial (1.8 ml)</td>
<td>Nunc (USA)</td>
<td>375418</td>
</tr>
<tr>
<td>Disposable syringe (Insulin)</td>
<td>Dispovan (India)</td>
<td>U-40</td>
</tr>
<tr>
<td>Filter 0.22 μm</td>
<td>Sterivex, Millipore (India)</td>
<td>SVG01050</td>
</tr>
<tr>
<td>Kleenex tissues</td>
<td>Kimberley (India)</td>
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<td>Dispovan (India)</td>
<td>HN18G38</td>
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<td>Petridish (Non-TC grade)</td>
<td>BD Falcon (India)</td>
<td>NIMS 130</td>
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<td>Petridish 35 mm (TC grade)</td>
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<tr>
<td>Syringe 5 ml</td>
<td>Dispovan (India)</td>
<td>HMD</td>
</tr>
<tr>
<td>Syringe 2 ml</td>
<td>Dispovan (India)</td>
<td>HMD</td>
</tr>
<tr>
<td>200 μl tips</td>
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<td>521010</td>
</tr>
<tr>
<td>1 ml tips</td>
<td>Tarsons (India)</td>
<td>521020</td>
</tr>
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4.2.1.5 Equipments

Table 4.3

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<td>Centrifuge</td>
<td>Heraeus (Stratos)</td>
</tr>
<tr>
<td>CO₂ Incubator</td>
<td>Shell labs (3517-2)</td>
</tr>
<tr>
<td>Deep freezer</td>
<td>Revco</td>
</tr>
<tr>
<td>Hemocytometer</td>
<td>Sigma (Z37,535-7)</td>
</tr>
</tbody>
</table>
4.2.2 Methods

4.2.2.1 Preparation of buffers, reagents and drugs

4.2.2.1.1 Preparation of IMDM

a) In a clean flask, sterile distilled water (5% less than desired total volume of medium) was measured out.

b) Powdered IMDM from the package/bottle was added as per requirement to distilled water with gentle stirring. Rinsed out inside of package/bottle to remove all traces of powder and added to the medium.

c) NaHCO₃ was weighed (according to the manufacturer’s instructions) and added to the medium followed by stirring using a magnetic stirrer until dissolved.

d) The pH of medium was adjusted to 7.2-7.4 using 10 N NaOH or 1N HCl (added slowly with stirring).

e) Distilled water was added to make a final volume of 1 litre.

f) Medium was sterilized through a 0.22 μm filter by using a filtration unit (Millipore) in laminar air flow.

g) Filtered medium was collected in sterile container/flask. The container was sealed with parafilm.

h) Before use, medium was incubated at 37°C for 24 h for sterility check.

i) When required for use in culture work, medium was supplemented with heat inactivated Fetal Bovine Serum (FBS).
j) The medium was kept in CO₂ incubator (Shell labs) for immediate use.

k) The medium was stored at 4°C.

Antibiotics were not added to growth medium prepared for CFU-GM assays since they might have adverse effects on development of colonies from primary BM precursors.

4.2.2.1.2 FBS heat inactivation

FBS was heat inactivated following the procedure as mentioned previously in section 2.2.2.1.2.

4.2.2.1.3 Methylcellulose (MCM) stock

a) Stock methylcellulose (2.8 %) in IMDM was thawed at 4°C overnight.

b) After complete thawing, bottle was shaken vigorously to thoroughly mix the contents. The air bubbles were allowed to escape by placing the bottle either at RT for 1 h.

c) Aliquots of methylcellulose were prepared by dispensing 1.42 ml into pre-labeled sterile 15 ml centrifuge tubes using sterile 5 ml syringe and 18 G needle inside a laminar air flow.

d) Aliquots were stored at -20°C and individual tubes were thawed and used as needed.

4.2.2.1.4 Preparation of rmGMCSF/rhGMCSF aliquots

a) Lyophilized rmGM-CSF/rhGMCSF powder was dissolved in sterile IMDM-10 (IMDM + 10% FBS) at concentration of 1000 ng/ml inside a laminar air flow.

b) Aliquots of rmGM-CSF/rhGMCSF were prepared inside a laminar air flow by dispensing 1 ml each into pre-labeled cryovials (concentration=1000 ng/ml).

c) The aliquots were stored at -80°C till used.

d) Before use, rmGMCSF aliquots were thawed at RT.

4.2.2.1.5 Preparation of 4% Bovine Serum Albumin (BSA)

a) 2 gm of BSA was weighed and transferred into a clean beaker.

b) 50 ml of IMDM was added to above contents and dissolved by gentle stirring.

c) 4% BSA was sterilized through a 0.22 μm membrane filter inside a laminar air flow.

d) BSA solution was stored in sterile flasks at 4°C till used.
4.2.2.1.6 Preparation of Trypan Blue (0.4%)
Trypan blue solution for cell counting was prepared following the procedure as mentioned previously in section 2.2.2.1.6.

4.2.2.1.7 Preparation of DNAse I
a) Lyophilized DNAse I was dissolved in sterile 0.15 M NaCl at 5 mg/ml.
b) Aliquots of DNAse were prepared by dispensing 50 μl into prelabeled sterile 0.5 ml centrifuge tubes inside a laminar air flow.
c) Aliquots were stored at -20 °C and individual tubes were thawed and used as needed.

4.2.2.1.8 Preparation of 5-FU (M.W. 130.08)
a) 1.3 mg of 5-FU was dissolved in 1 ml of DMSO to prepare main stock solution of 10 mM.
b) This stock solution was further diluted serially with serum-free medium (SFM) to obtain 200X stock solutions in the concentration range of 10 μg/ml to 200 μg/ml.

For the murine CFU-GM assay:

\[
\begin{align*}
76.9 \mu l &+ 400 \mu l + 600 \mu l + 750 \mu l + 250 \mu l + 666 \mu l + 333 \mu l + 500 \mu l + 500 \mu l \\
923.1 \mu l &+ SFM + SFM + SFM + SFM + SFM + SFM
\end{align*}
\]

1.3 mg/ml → 100 μg/ml → 40 μg/ml → 30 μg/ml → 20 μg/ml → 10 μg/ml

For the human CFU-GM assay:

\[
\begin{align*}
154 \mu l &+ 846 \mu l + 500 \mu l + 500 \mu l + 500 \mu l + 500 \mu l + 400 \mu l + 600 \mu l + 500 \mu l + 500 \mu l \\
1.3 \mu g/ml &+ SFM + SFM + SFM + SFM + SFM + SFM + SFM + SFM + SFM
\end{align*}
\]

1.3 mg/ml → 200 μg/ml → 100 μg/ml → 50 μg/ml → 20 μg/ml → 10 μg/ml

4.2.2.1.9 Preparation of Paclitaxel (Mol. Wt. 853.9)
a) 8.539 mg of Paclitaxel was dissolved in 1 ml of DMSO to prepare main stock solution of 10 mM.
b) This stock solution was further diluted serially with serum-free medium (SFM) to obtain 200X stock solutions in the concentration range of 0.2 μg/ml to 2 mg/ml.
For the murine CFU-GM assay:

\[
\begin{array}{cccccc}
118 \mu l + 882 \mu l & 100 \mu l + 900 \mu l & 100 \mu l + 900 \mu l & 160 \mu l + 840 \mu l & 750 \mu l + 250 \mu l \\
\text{SFM} & \text{SFM} & \text{SFM} & \text{SFM} & \text{SFM} \\
8.539 \text{ mg/ml} & \rightarrow & 1 \text{ mg/ml} & \rightarrow & 100 \mu \text{g/ml} & \rightarrow & 10 \mu \text{g/ml} & \rightarrow & 1.6 \mu \text{g/ml} & \rightarrow \\
\end{array}
\]

\[
\begin{array}{cccc}
666 \mu l + 333 \mu l & 250 \mu l + 750 \mu l \\
\text{SFM} & \text{SFM} \\
1.2 \mu \text{g/ml} & \rightarrow & 0.8 \mu \text{g/ml} & \rightarrow & 0.2 \mu \text{g/ml} \\
\end{array}
\]

For the human CFU-GM assay:

\[
\begin{array}{cccccc}
235 \mu l + 765 \mu l & 100 \mu l + 900 \mu l & 500 \mu l + 500 \mu l & 500 \mu l + 500 \mu l & 400 \mu l + 600 \mu l \\
\text{SFM} & \text{SFM} & \text{SFM} & \text{SFM} & \text{SFM} \\
8.539 \text{ mg/ml} & \rightarrow & 2 \text{ mg/ml} & \rightarrow & 2 \mu \text{g/ml} & \rightarrow & 1 \mu \text{g/ml} & \rightarrow & 500 \text{ ng/ml} & \rightarrow \\
\end{array}
\]

\[
\begin{array}{c}
500 \mu l + 500 \mu l \\
\text{SFM} \\
200 \text{ ng/ml} & \rightarrow & 100 \text{ ng/ml} \\
\end{array}
\]

4.2.2.1.10 Determination of Evaporation rate for saturated humidity

To ensure proper moisture conditions during incubation of CFU-GM cultures, the evaporation rate (ER) of CO\(_2\) incubator was determined.

a) Three 60-mm petri dishes (area 28.27 cm\(^2\)) were filled with 10 ml (\(V_0\)) of distilled water and put in the incubator in the centre of the middle shelf.

b) After 72 h, the volume of water (\(V_{72}\)) in each dish was measured and the ER of each one was determined as follows:

\[
\text{ER (\(\mu l \times h^{-1} \times cm^2\))} = \frac{(V_0 - V_{72})}{2.035}, \text{ expressed on the mean±S.D. of the three dishes.}
\]

c) ER values ranging from 1.1 to 2.1 were considered good; from 2.1 to 2.7, acceptable. Values over 2.7 were considered unacceptable.
4.2.2.2 Murine CFU-GM assay

4.2.2.2.1 Isolation of bone marrow suspension

a) Intact femur bones were isolated from C57BL/6 mice (male, 8-10 weeks) following the same procedure as described stepwise in section 2.2.2.2.1 and Figure 2.3 A.

b) Bones were transferred into a sterile petridish containing 10 ml of IMDM supplemented with antibiotics (penicillin 100 U/ml streptomycin 100 μg/ml) and left over for 5 min at 4°C on ice.

c) Petridishes containing femur bones were transferred to laminar air flow in TC facility for further processing.

d) Bones were transferred to HBSS in sterile petridish for rinsing off ethanol.

e) After rinsing, bones were transferred to IMDM medium in sterile petridish.

f) Both the ends of femur bones were trimmed carefully using a sterile, sharp scissors to expose the interior marrow shaft, as specified earlier in Figure 2.3 B.

g) The contents of marrow were flushed with 2 ml of IMDM without antibiotics using an insulin syringe fitted with a 30 G needle. The contents were collected into a sterile 50-ml centrifuge tube.

h) Whole marrow was collected with the syringe by repeated flushing. The bones should appear white once all the marrow has been expelled.

4.2.2.2 Preparation of cell suspension

a) The BM cell suspension collected above was diluted with IMDM to a final volume of 20 ml. Any clusters within the bone marrow suspension were disintegrated by vigorous pipeting.

b) The cell suspension was centrifuged at 400 g for 6 min.

c) The supernatant was removed and cell pellet was resuspended in 20 ml of IMDM.

d) Cells were centrifuged at 400 g x 6 min (wash 1).

e) Cells were washed again by following above steps (wash 2).

f) SN was removed and cell pellet was resuspended in 20 ml of IMDM supplemented with 30% FBS, without antibiotics to prepare a homogenous suspension.

g) An aliquot (20 μl) of cell-suspension was taken out in 1.5 ml centrifuge tube for cell counting.
h) Total number of cells was counted using a hemacytometer and trypan blue staining method for cell viability as described in section 2.2.2.2.6.

i) Cells were centrifuged at 400 g x 6 min.

j) According to the cell count obtained, cell pellet was resuspended in culture medium (IMDM + 30% FBS) to achieve a final cell density:

   For linearity curve - 1.5 X 10^6 cells/ml.
   For CFU-GM assays - 0.6 X 10^6 cells/ml.

k) Cells were stored at 4°C till used.

4.2.2.2.3 Generation of Linearity curve

Figure 4.2 outlines the steps involved in setting up CFU-GM assays. The linearity curve (number of cells seeded/number of colony formation) was performed as follows:

a) MCM (section 4.2.2.1.3) and rmGMCSF stock tubes (section 4.2.2.1.4) were thawed at RT.

b) 5 tubes of MCM master mix (4 ml) were prepared as described in Table 4.4.

Table 4.4 - Preparation of MCM master mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock</th>
<th>Final conc. required</th>
<th>Volume taken (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylcellulose</td>
<td>2.8%</td>
<td>1%</td>
<td>1.42</td>
</tr>
<tr>
<td>FBS</td>
<td>100 %</td>
<td>30%</td>
<td>1.20</td>
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<tr>
<td>BSA</td>
<td>4.0%</td>
<td>1%</td>
<td>1.00</td>
</tr>
<tr>
<td>rmGMCSF/rhGMCSF</td>
<td>1000 ng/ml</td>
<td>10 ng/ml</td>
<td>0.04</td>
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<td>IMDM</td>
<td></td>
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<td>0.44</td>
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<tr>
<td></td>
<td></td>
<td><strong>Total Volume</strong></td>
<td><strong>4.00</strong></td>
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c) Each tube was vortexed for 2 X 5 sec.

d) To generate the linearity curve, murine BM cells were seeded at final density of 2500, 5000, 10,000, 20,000, 40,000 murine BM cells/dish. 1 ml stock cell suspension was prepared for each cell density as described in Table 4.5.
Table 4.5 – Preparation of linearity curve (murine CFU-GM assay)

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Final cell No./dish</th>
<th>Volume (µl) from 1.5 \times 10^6 \text{ cells/ml} (section 4.2.2.2.2-j)</th>
<th>IMDM+30% FBS (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2500</td>
<td>25</td>
<td>975</td>
</tr>
<tr>
<td>2</td>
<td>5000</td>
<td>50</td>
<td>950</td>
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<tr>
<td>3</td>
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<tr>
<td>5</td>
<td>40000</td>
<td>400</td>
<td>600</td>
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</tbody>
</table>

e) 35-mm culture petriplates were labeled on an incubator tray, according to the experimental design.

f) 0.30 ml of murine bone marrow cells suspension (from tube 1-5, Table 4.5) was added to each of the MCM master mix tube above and vortexcved for 3 X 8 sec.

g) After thorough mixing, 1 ml of MCM master mix + cells from each tube was distributed using a 5 ml sterile syringe fitted with 18 G needle into three prelabeled, sterile 35-mm culture dishes.

h) The final cell number was 2500-40,000 cells/ml/petridish. Each petridish was gently rotated to spread the mixture evenly by allowing the meniscus to attach to the dish wall.

i) As the humidity level during incubation is critical, six 35-mm culture dishes with one 60-mm dish (without lid) containing sterile water/medium were placed inside a 150-mm petridish covered with a lid to maintain the humidity.
Figure 4.2 - Procedural steps in CFU-GM assay. (1) Labeled sterile tubes and 30-mm petridishes (2) The Master mix was prepared (MCM mix+cells or MCM mix+cells+Drug) (3-4) 1 ml of master mix was added into 30-mm petridishes (5-7) Homogenous spread of master mix by rotating the petridishes (8-9) Six 35-mm petridishes were kept in 150-mm petridish along with one 60-mm petridish containing sterile water.
j) Cells were incubated at 37°C in air with 5% CO₂ under saturated humidity for 7 days.

k) The culture dishes were placed inside a 100 mm gridded tissue culture dish (prepared using scoring grid - Figure 4.3).

l) CFU-GM colonies (≥50 cells) were scored by scanning the whole petridish by using an inverted microscope. Photomicrographs were recorded at magnification of 40X.

Figure 4.3 – Scoring grid for CFU-GM colonies. The diagram can be used as a template for scoring CFU-GM colonies and can be marked on a 100 mm petridish by tracing the grid with a marker pen.

4.2.2.4 Validation of murine CFU-GM assay with 5-FU and Paclitaxel

a) Drug stocks and dilutions (200X) were prepared immediately prior to use as described in section 4.2.2.1.8 and 4.2.2.1.9.

b) MCM (section 4.2.2.1.3) and rmGMCSF stock tubes (section 4.2.2.1.4) were thawed at RT.

c) Tubes of MCM master mix (4 ml) were prepared as described in Table 4.4.

d) Each tube was vortexed for 2 X 5 sec.

e) 35-mm culture petriplates were labeled on an incubator tray, according to the experimental design.

f) 100 µl of IMDM was added to labeled MCM tubes of control.

g) 78 µl of IMDM was added to all the drug curve tubes of 5-FU and Paclitaxel.
h) 22 µl of 5-FU or Paclitaxel from the respective 200X stock (section 4.2.2.1.8 and 4.2.2.1.9) was added to each drug curve tube to achieve final concentrations (Table 4.6 and 4.7) and vortexed for 2 X 5 seconds.

i) 0.30 ml of murine bone marrow cells suspension (from 0.6 x 10^6 cells/ml suspension; section 4.2.2.2.2-j) was added to each of the MCM master mix tube above and vortexed for 3 X 8 sec.

Table 4.6 – Curve dilutions for 5-FU (murine CFU-GM assay)

<table>
<thead>
<tr>
<th>200X Stock conc. of 5-FU (µg/ml)</th>
<th>Volume of 5-FU to be taken</th>
<th>Volume of IMDM to be taken</th>
<th>Volume of MCM master mix</th>
<th>Volume of cell suspension (from 0.6 x 10^6 cells/ml suspension)</th>
<th>Final conc. of 5-FU (µg/ml) (in 4.4 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt</td>
<td>-</td>
<td>100 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.05</td>
</tr>
<tr>
<td>20</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.10</td>
</tr>
<tr>
<td>30</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.15</td>
</tr>
<tr>
<td>40</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Table 4.7 – Curve dilutions for Paclitaxel (murine CFU-GM assay)

<table>
<thead>
<tr>
<th>200X Stock conc. of Paclitaxel (µg/ml)</th>
<th>Volume of Paclitaxel to be taken</th>
<th>Volume of IMDM to be taken</th>
<th>Volume of MCM master mix</th>
<th>Volume of cell suspension (from 0.6 x 10^6 cells/ml suspension)</th>
<th>Final conc. of Paclitaxel (µg/ml) (in 4.4 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt</td>
<td>-</td>
<td>100 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.2</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.001</td>
</tr>
<tr>
<td>0.8</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.004</td>
</tr>
<tr>
<td>1.2</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.006</td>
</tr>
<tr>
<td>1.6</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.008</td>
</tr>
</tbody>
</table>

j) After thorough mixing, 1 ml of MCM master mix + drug + cells from each tube was distributed into three prelabeled, sterile 35-mm culture dishes using a 5 ml syringe fitted with 18 G needle.
k) The final cell number is 40,000 cells/ml/petridish. Each petridish was gently rotated to spread the mixture evenly by allowing the meniscus to attach to the dish wall.

l) As the humidity level during incubation is critical, six 35-mm culture dishes with one 60-mm dish (without lid) containing sterile water/medium were placed inside a 150-mm petri dish covered with a lid to maintain the humidity.

m) Cells were incubated at 37°C in air with 5% CO₂ under saturated humidity for 7 days.

n) The culture dishes were placed inside a 100 mm gridded tissue culture dish (prepared using scoring grid - Figure 4.3) and CFU-GM colonies (>50 cells) were scored by scanning the whole petridish by using an inverted microscope.

*The plate with highest concentration of drug was scored first, to determine the minimal acceptable aggregate considered to be a colony. At this highest drug concentration, the colonies will be the smallest and the most difficult to define because of the toxicity.

o) Photomicrographs were recorded at magnification of 40X.

p) % CFU-GM inhibition was calculated as:

\[
\left\{ \frac{\text{No. of CFU-GM colonies in control sample} - \text{No. of CFU-GM colonies in drug treated sample}}{\text{No. of CFU-GM colonies in control sample}} \right\} \times 100
\]

4.2.2.3 Human CFU-GM assay

4.2.2.3.1 Processing of human BM cells

a) Human bone marrow mononuclear cells (MNC) were procured frozen in a 1.8 ml solution of 50% IMDM, 40% FBS, and 10% DMSO (0.2 μm filtered).

b) Cells were thawed at 37°C in water bath.

c) Aliquots of cells were prepared by quickly dispensing 200 μl of cell suspension into sterile prelabeled cryovials and were stored in liquid nitrogen (-135°C) till used.

d) IMDM containing 10% FBS was warmed in a 37°C water bath.

e) The frozen vial of cells was wiped with 70% alcohol before thawing.

f) The vial was thawed in a 37°C water bath. Wiped the outside of the vial with 70% ethanol.

g) The vial was gently inverted to mix cells.

h) The cell suspension was then transferred inside a laminar air flow to a 15 ml conical tube containing DNase I (0.1 mg/ml) to prevent cell clumping.
i) The inside of cryovial was rinsed with 1 ml IMDM+10% FBS to recover remaining cells and this medium was slowly added drop-wise to the cells in the 15 ml tube while gently swirling the tube.

j) IMDM+10% FBS was slowly added to the cells to make the final volume of 10 ml. Gently inverted the tube to mix the contents.

k) Cells were centrifuged at 200 x g at RT for 15 min.

l) The supernatant was removed carefully, leaving a small amount of medium behind to ensure that cell pellet is not disturbed. The cell pellet was gently resuspended by tapping the edge of the tube.

m) The tube was filled with 10 ml of IMDM and gently inverted to mix the contents.

n) The cell suspension was centrifuged at 200 x g at RT for 15 min (wash 1).

o) The supernatant was removed carefully, leaving a small amount of medium behind to ensure that the cell pellet is not disturbed.

p) The tube was gently flicked to resuspend the cell pellet and 2 ml of IMDM+10% FBS was added to cells.

q) An aliquot (20 μl) of cell-suspension was taken out in 1.5 ml centrifuge tube for cell count. Total number of cells was counted using a hemacytometer and trypan blue staining method for cell viability as described in section 2.2.2.2.6.

r) Cell suspension was prepared at a final density of
   For linearity curve - 2 X 10^6 cells/ml.
   For CFU-GM assays – 1.1 X 10^6 cells/ml.

4.2.2.3.2 Generation of linearity curve
The linearity study (number of cells seeded/number of colony formation) was performed as follows:

a) MCM (section 4.2.2.1.3) and rhGMCSF stock tubes (section 4.2.2.1.4) were thawed at RT.

b) 5 tubes of MCM master mix (4 ml) were prepared as described in Table 4.4.

c) Each tube was vortexed for 2 X 5 sec.
d) To generate the linearity curve, human BM cells were seeded at final density of 5000, 10000, 25000, 50000, 75000 and 100000 human BM precursor cells/dish. 1 ml stock cell suspension was prepared for each cell density as described in Table 4.8.

Table 4.8– Preparation of linearity curve (human CFU-GM assay)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Cell No./dish</th>
<th>Volume (μl) from 2.0 X 10^6 cells/ml (Section 4.2.2.3.1.r)</th>
<th>IMDM+30% FBS (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5000</td>
<td>35.8</td>
<td>964.2</td>
</tr>
<tr>
<td>2</td>
<td>10000</td>
<td>71.6</td>
<td>928.4</td>
</tr>
<tr>
<td>3</td>
<td>25000</td>
<td>179.0</td>
<td>821.0</td>
</tr>
<tr>
<td>4</td>
<td>50000</td>
<td>358.0</td>
<td>642.0</td>
</tr>
<tr>
<td>5</td>
<td>75000</td>
<td>537.5</td>
<td>462.5</td>
</tr>
<tr>
<td>6</td>
<td>100000</td>
<td>716.0</td>
<td>284.0</td>
</tr>
</tbody>
</table>

e) 35-mm petriplates were labeled on an incubator tray, according to the experimental design.

f) 0.30 ml of human bone marrow cells suspension (from tube 1-5, Table 4.8) was added to each of the MCM master mix tube above and vortexed for 3 X 8 sec.

g) After thorough mixing, 1 ml of MCM master mix + cells from each tube was distributed using a sterile 5 ml syringe fitted with 18 G needle into three prelabeled, sterile 35-mm culture dishes.

h) The final cell number was 5000-100,000 cells/ml/dish. Each petridish was gently rotated to spread the mixture evenly by allowing the meniscus to attach to the dish wall.

i) As the humidity level during incubation is critical, six 35-mm culture dishes with one 60-mm dish (without lid) containing sterile water/medium were placed inside a 150-mm petri dish covered with a lid to maintain the humidity.

j) Cells were incubated at 37°C in air with 5% CO₂ under saturated humidity for 14 days.
The culture dishes were placed inside a 100 mm gridded tissue culture dish (prepared using scoring grid - Figure 4.3).

CFU-GM colonies (\( \geq 30 \) cells) were scored by scanning the whole petridish by using an inverted microscope.

Photomicrographs were recorded at magnification of 40X.

### 4.2.2.3.3 Validation of human CFU-GM assay with 5-FU and Paclitaxel

a) Drug stocks and dilutions (200X) were prepared immediately prior to use as described in section 4.2.2.1.8 and 4.2.2.1.9.

b) MCM (section 4.2.2.1.3) and rhGMCSF stock tubes (section 4.2.2.1.4) were thawed at RT.

c) Tubes of MCM master mix (4 ml) were prepared as described in Table 4.4.

d) Each tube was vortexed for 2 X 5 sec.

e) 35-mm petriplates were labeled on an incubator tray, according to the experimental design.

f) 100 \( \mu l \) of IMDM was added to labeled MCM tubes of Control.

g) 78 \( \mu l \) of IMDM was added to drug curve tubes of 5-FU and Paclitaxel.

h) 22 \( \mu l \) of 5-FU or Paclitaxel from the respective 200X stock (section 4.2.2.1.8 and 4.2.2.1.9) was added to each drug curve tube to achieve final concentrations (Table 4.9 and 4.10) and vortexed for 2 X 5 seconds.

i) 0.30 ml of human bone marrow cells suspension (from 1.1 x 10^6 cells/ml suspension; section 4.2.2.3.1-r) was added to each of the MCM master mix tube above and vortexed for 3 X 8 sec.

j) After thorough mixing, 1 ml of MCM master mix + drug + cells from each tube was distributed into three prelabeled, sterile 35-mm culture dishes using a 5 ml sterile syringe fitted with 18 G needle.

k) The final cell number was 75,000 cells/ml/dish. Each petridish was gently rotated to spread the mixture evenly by allowing the meniscus to attach to the dish wall.
As the humidity level during incubation is critical, six 35-mm culture dishes with one 60-mm dish (without lid) containing sterile water/medium were placed inside a 150-mm petri dish covered with a lid to maintain the humidity.

Table 4.9 - Curve dilutions for 5-FU (Human CFU-GM assay)

<table>
<thead>
<tr>
<th>200X Stock conc. of 5FU (µg/ml)</th>
<th>Volume of 5-FU to be taken</th>
<th>Volume of IMDM to be taken</th>
<th>Volume of MCM master mix</th>
<th>Volume of cell suspension (from 1.1 x 10⁶ cells/ml suspension)</th>
<th>Final conc. of 5-FU (µg/ml) (in 4.4 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt</td>
<td>-</td>
<td>100 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.05</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>0.10</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 4.10 - Curve dilutions for Paclitaxel (Human CFU-GM assay)

<table>
<thead>
<tr>
<th>200X Stock conc. of Paclitaxel (ng/ml)</th>
<th>Volume of Paclitaxel to be taken</th>
<th>Volume of IMDM to be taken</th>
<th>Volume of MCM master mix</th>
<th>Volume of cell suspension (from 1.1 x 10⁶ cells/ml suspension)</th>
<th>Final conc. of Paclitaxel (ng/ml) (in 4.4 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt</td>
<td>-</td>
<td>100 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>500</td>
<td>22 µl</td>
<td>78 µl</td>
<td>4 ml</td>
<td>0.30 ml</td>
<td>2.5</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Cells were incubated at 37°C in air with 5% CO₂ under saturated humidity for 14 days.

The culture dishes were placed inside a 100 mm gridded tissue culture dish (prepared using scoring grid - Figure 4.3).

CFU-GM colonies (≥30 cells) were scored by scanning the whole petridish by using an inverted microscope.

*The plate with highest concentration of drug was scored first, to determine the minimal acceptable aggregate considered to be a colony. At this highest drug
At the lowest concentration, the colonies will be the smallest and the most difficult to define because of the toxicity.

p) Photomicrographs were recorded at magnification of 40X.

q) % CFU-GM inhibition was calculated as:

\[
\frac{\text{No. of CFU-GM colonies in control sample} - \text{No. of CFU-GM colonies in drug treated sample}}{\text{No. of CFU-GM colonies in control sample}} \times 100
\]
4.3 Results

4.3.1 Murine CFU-GM assay

4.3.1.1 Establishment of culture conditions for murine CFU-GM assay

In vitro CFU-GM cultures were generated from bone marrow progenitor cells of C57BL/6 mice and used in setting up subsequent assays for assessing hematotoxicity of new compounds. Various experimental conditions involved in generation and characterization of CFU-GM cultures were standardized in-house.

The steps involved in setting up CFU-GM assays from murine bone marrow cells required strict adherence to culture conditions and assay parameters. These critical parameters could have a huge impact on the quality and characteristics of murine CFU-GM colonies. Hence it was ensured to follow the steps which helped in maintaining the homogeneity of cultures generated in batch-wise manner. This minimized variations in multiple sets of assays and also helped in attaining the reproducible BM cell yield at day-0 and number of colonies at day-7. These factors are enlisted in detail in Table 4.11.

4.3.1.2 Optimization of cell No. for murine CFU-GM assays

Before setting up CFU-GM assays, it was required to determine the correct seeding density of murine BM precursor cells. To determine the optimum seeding cell density, CFU-GM assay was performed with different cell density (2500-40000 cells/petri dish) and number of colonies obtained at day-7 were enumerated. A linearity curve was generated showing proportional increase in number of CFU-GM colonies with increase in number of starting cell densities ($r^2 = 0.9944$) (Figure 4.4). From these experiments, a cell density of 40,000 murine bone marrow precursors/dish was selected for initiating subsequent experiments, which resulted in at least 100 CFU-GM colonies within multiple cultures ($100 \pm 5.8$; $n=10$).

Figure 4.4 shows the linearity curve generated by plotting number of murine CFU-GM colonies obtained on day-7 vs. number of BM precursor cells seeded/plate.

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Table 4.11 – Critical parameters which may influence the generation of murine CFU-GM cultures from hematopoietic precursors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy C57BL/6 mice, procured from same source, same age group (8-10 weeks), same sex (male), housing conditions were maintained</td>
</tr>
<tr>
<td>Procedural steps</td>
<td>• Uniform aliquoting of methyl cellulose</td>
</tr>
<tr>
<td></td>
<td>• Similar composition of MCM master mix</td>
</tr>
<tr>
<td></td>
<td>(10 ng/ml GMCSF, 30 % FBS, 1% BSA, 1% MCM)</td>
</tr>
<tr>
<td></td>
<td>• Maintenance of integrity of bone during excision for effective ethanol disinfection</td>
</tr>
<tr>
<td></td>
<td>• BM harvesting was done while maintaining uniformity of procedure</td>
</tr>
<tr>
<td></td>
<td>• Starting cell density of BM cells was kept constant</td>
</tr>
<tr>
<td></td>
<td>• Shear forces were minimized by gently mixing MCM + cells</td>
</tr>
<tr>
<td>Growth factors and reagents</td>
<td>Same source of procurement for IMDM, FBS, FBS, antibiotics, GMCSF, MCM, culture petridishes</td>
</tr>
<tr>
<td>Humidity</td>
<td>Saturated humidity was maintained during incubation of CFU-GM cultures</td>
</tr>
<tr>
<td>Colony counting</td>
<td>Performed by at least 2 observers simultaneously</td>
</tr>
</tbody>
</table>
Figure 4.4 – Linearity curve of murine CFU-GM assay. Bone marrow cells were harvested from C57BL/6 mice and seeded at different densities ranging from 2500 - 40,000 cells/dish. Cells were incubated in presence of rmGMCSF for 7 days and CFU-GM colonies were counted. Aggregates containing 50 or more cells were counted as CFU-GM colonies. (n=3).

4.3.1.3 Morphological characterization of murine CFU-GM colonies

Culture of murine bone marrow precursors in the presence of rmGMCSF (10 ng/ml) for 7-days resulted in the formation of 3 types of CFU-GM colonies with variable morphology. These colonies were observed throughout in multiple cultures and represented overall health and typical characteristics of CFU-GM colonies. In addition, morphology of CFU-GM colonies is also subjected to change upon treatment with hematotoxic agents; hence this is an integral parameter for comparison of results post-treatment with test compounds.

The CFU-GM classification includes CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), and CFU-granulocyte-macrophage (CFU-GM). The colonies contain 50 to thousands of granulocytes (CFU-G), macrophages (CFU-M) or both cell types (CFU-GM). CFU-GM colonies often contain multiple cell clusters (dense core surrounded by cells). The "monocytic" lineage cells are large cells with an oval to round shape and appear to have a grainy or grey centre. The "granulocytic" lineage cells are round, bright, and are much smaller and more uniform in size than macrophage cells.
Figure 4.5 shows 3 different types of colonies obtained in murine CFU-GM assays with variable morphology.

a) **Compact Colonies**: A central dense nucleus (N) and a peripheral halo (H). These colonies are easy to score.

b) **Diffuse and spread colonies**: Without apparent nucleus and presence of diffused halo.

c) **Multicentric colonies**: Colonies with two or more dense nuclei nearby, with a common peripheral halo growing at the same depth in plate. These are considered as single colony.

![Figure 4.5 - Murine CFU-GM colonies with different morphology on day 7. Murine BM cells were cultured in methylcellulose in presence of rmGMCSF (10 ng/ml) for 7 days. (A) Compact colonies (B) diffuse and spread colonies and (C) multi-centric colonies. Colonies were counted using inverted microscope at magnification of 40X. Aggregates containing 50 or more cells were counted as CFU-GM colonies. Colony scoring was performed according to CFU-atlas ECVAM INVITTOX no. 101.](image-url)
4.3.1.4 Effects of 5-FU and Paclitaxel on murine CFU-GM colonies

To validate the murine CFU-GM assay in-house, anticancer drugs 5-FU and Paclitaxel were included. These 2 drugs are well-reported to be associated with neutropenia as a dose-limiting toxicity. Toxic effect on bone marrow was studied by culturing murine BM precursor cells with 5-FU (0.05 μg/ml – 0.2 μg/ml) and Paclitaxel (0.001 μg/ml – 0.008 μg/ml) in the presence of rmGMCSF (10 ng/ml) for 7 days. Colonies were counted after 7 days and resultant inhibition of CFU-GM was determined.

- Continuous *in vitro* exposure of murine BM cells to 5-FU and Paclitaxel resulted in inhibition of CFU-GM colonies. There was a dose dependent inhibition of murine CFU-GM with reference to increasing concentrations of 5-FU (Figure 4.6) and Paclitaxel (Figure 4.7).

- IC\(_{50}\) values for 5-FU and Paclitaxel in murine CFU-GM assay were determined as 0.137 μg/ml and 3.5 ng/ml respectively, showing a good correlation between the reported values (5-FU- 0.140 μg/ml and Paclitaxel-3.9 ng/ml; Pessina A *et al.*, 2003).

- IC\(_{90}\) values for 5-FU and Paclitaxel in murine CFU-GM assay were determined as 0.366 μg/ml and 9 ng/ml respectively, which correlated well with the reported values (5-FU-0.240 μg/ml and Paclitaxel-6.1 ng/ml; Pessina A *et al.*, 2003).
Figure 4.6 – Effects of 5-FU on murine CFU-GM colony formation. Murine BM cells were cultured in presence of rmGMCSF (10 ng/ml) with continuous exposure to 5-FU (0.05 μg/ml – 0.2 μg/ml) for 7 days. Aggregates containing 50 or more cells were counted as CFU-GM colonies. (A) Decrease in number of CFU-GM colonies, (B) Percent inhibition of CFU-GM colonies formation by 5-FU was determined in comparison with control cells (n=3).
Figure 4.7 — Effects of Paclitaxel on murine CFU-GM colony formation. Murine BM cells were cultured in presence of rmGMCSF (10 ng/ml) with continuous exposure to Paclitaxel (0.001 μg/ml – 0.008 μg/ml) for 7 days. Aggregates containing 50 or more cells were counted as CFU-GM colonies. (A) Decrease in number of CFU-GM colonies, (B) Percent inhibition of CFU-GM colonies formation by Paclitaxel was determined in comparison with control cells (n=3).
4.3.1.5 Effects of 5-FU and Paclitaxel on morphology of murine CFU-GM colonies

In addition to reduction in number of murine CFU-GM colonies formation, changes were also induced by 5-FU and Paclitaxel in the size and overall morphology of CFU-GM colonies. As compared to untreated cells, size of CFU-GM colonies was found to be reduced (Figure 4.8). Moreover, there was a noticeable change in morphology of CFU-GM colonies under the influence of 5-FU and Paclitaxel. A shift towards adherent, monocytic lineage with grey centre can be seen upon drug treatment as compared to control cells.

![Control, 5-FU, Paclitaxel](image)

**Figure 4.8 – Effects of 5-FU and Paclitaxel on size and morphology of murine CFU-GM colonies**

4.3.2 Human CFU-GM assay

4.3.2.1 Establishment of culture conditions for human CFU-GM assay

*In vitro* human CFU-GM assays were developed using human bone marrow cells as precursors (commercially procured) and employed in setting up assays for evaluating hematotoxic potential of test compounds. Critical steps and parameters involved in these assays were standardized to ensure maximum uniformity among multiple sets of cultures. This helped in attaining reproducible number of human CFU-GM colonies counted on day-14 (Table 4.12).
Table 4.12 – Critical parameters which may influence the generation of human CFU-GM cultures from hematopoietic precursors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comments</th>
</tr>
</thead>
</table>
| Human BM cells                   | • Human BM cells procured from same source were used  
• Uniform aliquoting of cells  
• Avoided repeated freeze thaw of cells |
| Procedural steps                 | • Uniform aliquoting of methyl cellulose and GMCSF  
• Similar composition of MCM master mix  
(10 ng/ml GMCSF, 30% FBS, 1% BSA, 1% MCM)  
• Starting cell density was kept constant  
• Shear forces were minimized by gently mixing MCM + cells  
• DNAse treatment was followed by immediate addition of medium |
| Growth factors and reagents      | Same source of procurement for IMDM, FBS, FBS, antibiotics, GMCSF, MCM, culture petridishes                                                                                                                   |
| Humidity                         | Saturated humidity was maintained during incubation of CFU-GM cultures                                                                                                                                 |
| Colony counting                  | Performed by at least 2 observers simultaneously                                                                                                                                                        |

4.3.2.2 Optimization of cell No. for human CFU-GM assays

The optimum number of human BM precursors to initiate CFU-GM assays was identified before employing these assays for screening purposes. To determine appropriate seeding cell density, human CFU-GM assays were performed with different starting cell numbers (5000-100,000 cells/petridish). At day-14, numbers of human CFU-GM colonies obtained were counted.

Figure 4.9 demonstrates the linearity curve generated with number of human CFU-GM colonies counted on day-14 vs. number of human BM precursor cells seeded/plate. There
was a proportional increase in number of CFU-GM colonies with starting cell densities \( r^2 = 0.9910 \). The cell density of 75,000 was selected from these studies as optimum for setting up subsequent experiments, which resulted in at least 50 CFU-GM colonies within multiple cultures (50 ± 7.9, n=8).

**Figure 4.9** — Linearity curve of human CFU-GM assay. Human bone marrow cells were seeded at different densities ranging from 5000 – 100,000 cells/dish. Cells were incubated for 14 days in presence of rhGMCSF and resulting CFU-GM colonies were counted. Aggregates containing 30 or more cells were counted as CFU-GM colonies. (n=3).

### 4.3.2.3 Morphological characterization of human CFU-GM colonies

Colonies obtained on day-14 when human BM precursor cells were cultured in presence of rhGMCSF represented typical CFU-GM morphology. These colonies were observed throughout in multiple human CFU-GM cultures and represented the overall health and typical characteristics of CFU-GM colonies.

Figure 4.10 shows 3 different types of colonies obtained in human CFU-GM assays with variable morphology:
a) **Compact Colonies**: A central dense nucleus (N) and a peripheral halo (H). These colonies are easy to score.

b) **Diffuse and spread colonies**: Without apparent nucleus and presence of diffused halo

c) **Multicentric colonies**: Colonies with two or more dense nuclei nearby, with a common peripheral halo growing at the same depth in plate. These are considered as one colony.

Figure 4.10 - Human CFU-GM colonies with different morphology on day 14. Human BM cells were cultured in methylcellulose in presence of rhGMCSF (10 ng/ml) for 14 days. (A) Compact colonies (B) diffuse and spread colonies and (C) multi-centric colonies. Colonies were counted using inverted microscope at magnification of 40X. Aggregates containing 30 or more cells were counted as CFU-GM colonies. Colony scoring was performed according to CFU-atlas ECVAM INVITTOX no. 101.
4.3.2.4 Effects of 5-FU and Paclitaxel on human CFU-GM colonies

For the validation of human CFU-GM assays, anticancer drugs 5-FU and Paclitaxel were included which are well reported to be associated with neutropenia as dose-limiting toxicity. Toxic effect on human bone marrow was studied by culturing human BM precursor cells with 5-FU (0.05 μg/ml – 1 μg/ml) and Paclitaxel (0.5 μg/ml – 10 μg/ml) in the presence of rhGMCSF (10 ng/ml) and counting the colonies after 14 days.

- Continuous in vitro exposure of human BM cells to 5-FU and Paclitaxel resulted in inhibition of CFU-GM colonies in dose dependent fashion. There was a proportional inhibition of CFU-GM with increasing concentrations of 5-FU (Figure 4.11) and Paclitaxel (Figure 4.12).

- For 5-FU and Paclitaxel, IC$_{50}$ values of CFU-GM inhibition in human assay were determined to be as 0.552 μg/ml and 4.5 ng/ml respectively, which correlated well with the reported values (0.71 μg/ml and 4 ng/ml respectively; Pessina A et al., 2003).

- IC$_{90}$ values of 5-FU and Paclitaxel were obtained as 1.93 μg/ml and 9.8 ng/ml respectively. The reported IC$_{90}$ values for 5-FU and Paclitaxel were 1.46 μg/ml and 7 ng/ml (Pessina A et al., 2003).
Figure 4.11 – Effects of 5-FU on human CFU-GM colony formation. Human BM cells were cultured in presence of rhGMCSF (10 ng/ml) with continuous exposure to 5-FU (0.05 μg/ml – 1 μg/ml) for 14 days. Aggregates containing 30 or more cells were counted as CFU-GM colonies. (A) Decrease in number of CFU-GM colonies, (B) Percent inhibition of CFU-GM colonies formation by 5-FU was determined in comparison with control cells (n=3).
Figure 4.12 - Effects of Paclitaxel on human CFU-GM colony formation. Human BM cells were cultured in presence of rhGMCSF (10 ng/ml) with continuous exposure to Paclitaxel (0.5 ng/ml - 10 ng/ml) for 14 days. Aggregates containing 30 or more cells were counted as CFU-GM colonies. (A) Decrease in number of CFU-GM colonies, (B) Percent inhibition of CFU-GM colonies formation by Paclitaxel was determined in comparison with control cells (n=3).
4.3.2.5 Effects of 5-FU and Paclitaxel on morphology of human CFU-GM colonies

Alterations in the normal morphology and size of human CFU-GM colonies were induced when precursor cells were cultured in presence of anticancer drugs; 5-FU and Paclitaxel for 14 days (Figure 4.13). These changes were observed in addition to expected decrease in number of colonies formed. In contrast to control cells, there was a decrease in overall size and changes were observed in typical morphology of CFU-GM colonies. Treatment of BM precursor cells with drugs resulted in biasing the morphology of CFU-GM colonies more towards monocytic lineage than granulocytes.

Figure 4.13 – Effects of 5-FU and Paclitaxel on size and morphology of human CFU-GM colonies
4.4 Discussion

Bone marrow toxicity is one of the most severe side effects reported to be associated with administration of anticancer drugs in cancer patients (Dy GK, Adjei AA, 2013; Maxwell MB, Maher KE, 1992; Ye L et al., 2013). Neutropenia is identified as dose-limiting toxicity for a variety of anticancer agents (Crawford J et al., 2004; Lyman GH, 2006). Significant drop in neutrophil counts is frequently observed in patients being administered with chemotherapy (Freyer G et al., 2013). Growth factors are used to manage neutropenic conditions in patients on chemotherapy (Badalamenti G et al., 2013; Bennett CL et al., 2013).

CFU assays constitute an important arm of in vitro tests to assess and predict hematotoxicity of xenobiotic agents including anticancer compounds (Deldar A et al., 1993; Pamphilon D et al., 2013; Saxena A et al., 2013). Due to a high attrition rate of drugs in clinical trials on account of drug-induced neutropenia, limitations are faced in achieving the efficacious doses as human MTD. Hence, attempts in the direction of developing new methods which help us in achieving the human MTD of anticancer drugs in lesser span of time and with high prediction are necessary. CFU-GM assay is an in vitro preliminary method approved by ECVAM to predict human MTD for anticancer drugs with neutropenia as the dose limiting toxicity (Pessina A et al., 2003). CFU-GM assay is now included in the 2013 guidelines for alternatives to animal tests (ec.europa.eu, 2013).

The methodology of in vitro murine and human CFU-GM assays involves highly sophisticated procedures which require expertise in this area. Maintenance of homogeneity among various batches of CFU-GM cultures generated for screening of drugs is essential in order to preserve the integrity and basic characteristic features of CFU-GM colonies. Also, these factors are integral to utilize these assays in the assessment of in vitro hematotoxic effects of test items. Therefore, simple and standardized protocols defining stepwise method of murine as well as human CFU-GM
assays are helpful in establishment of these assays and employing them for predicting hematotoxic effects of test compounds. Realizing the importance of CFU-GM assays in in vitro hematotoxicology of anticancer drugs, we focused on developing these in vitro assays by standardizing and validating with standard anticancer drugs, so that we can employ these models to evaluate hematotoxic side effects of novel anticancer formulations.

In this section, we established murine and human CFU-GM assays to be used as platforms to assess hematotoxic effects of test compounds. Murine CFU-GM assays were generated from the bone marrow of C57BL/6 mice (male, 8-10 weeks) and morphologically characterized. BM cells were propagated in methylcellulose medium containing 10 ng/ml rmGMCSF for 7 days, which allowed differentiation of precursor cells into granulocyte-macrophage specific lineage and formation of CFU-GM colonies. Commercially procured human BM cells were used as the initiating precursor cell population for human CFU-GM assays. Human BM cells were cultured in MCM in presence of rhGMCSF for 14 days, resulting in the formation of human CFU-GM colonies, which were characterized morphologically.

All the parameters which could exert a direct or indirect influence on specific characteristics of CFU-GM were standardized. These key points included source of animal, procurement source of human BM cells, reagents, medium, FBS, growth factor, culture petridishes and procedural steps involved such as harvesting of murine BM, preparation of MCM master mix, minimizing sheer forces during mixing of cells with MCM, maintenance of saturated levels of humidity during incubation of cultures and uniformity in colonies counting. While all these steps were taken into consideration during assay development, we were able to standardize experimental conditions for murine and human CFU-GM assays and obtain reproducible number of colonies at day 7 and 14 of incubation respectively.

Murine and human CFU-GM assays in multiple batches displayed typical morphology of 3 types of colonies formed, which is characteristic feature of granulocyte-macrophages.
assays in semisolid medium as described in ECVAM atlas for CFU-GM assay (ECVAM DB-ALM Protocol n° 101). To employ these assays successfully for assessing inhibitory effects of test compounds on the development of CFU-GM, optimum number of starting cell population of precursor cells was first identified. A linearity curve was prepared by seeding different number of murine and human BM cells. This resulted in proportional increase in number of murine and human CFU-GM colonies and the seeding cell density was optimized for both the systems.

Subsequent to this step, murine and human CFU-GM assays were validated with reference anticancer drugs with neutropenia as dose limiting toxicity; 5-FU and Paclitaxel. Treatment with these agents resulted in a dose dependent inhibition of CFU-GM colonies. IC\textsubscript{50} and IC\textsubscript{90} values obtained experimentally for both murine and human system correlated well with the reported values in literature (Pessina A et al., 2003). Also, the colony size and morphology was altered when BM cells were continuously exposed to 5-FU and Paclitaxel in murine and human systems for 7 and 14 days respectively. These alterations in colony size and morphology as well inhibitory effect of anticancer drugs on colony-counts strongly supported the sensitivity of CFU-GM assays for the evaluation of hematotoxic effects of test compounds by inhibition of CFU-GM colonies developed from bone marrow. Hence, the CFU-GM assays developed in house can be suitably employed to screen neutropenic effects of test compounds subsequently.

Standardization of various experimental procedures and culture conditions involved in the generation of primary CFU-GM assays, morphological characterization of colonies and validation with standard anticancer drugs enabled us to employ these BM cells based assays in setting up subsequent screening for hematotoxic potential of test compounds.

CFU-GM based assays may certainly play promising role in the development of alternate strategies for assessing the hematotoxic side effects of test compounds and estimating a closer dose to human MTD. The conventional mode of determining human MTD for drugs involves multiple dose escalation studies in phase-I clinical trials, starting with
I/10\textsuperscript{th} of LD\textsubscript{10} in mice. This process also involves consumption of a large number of animals in preclinical hematology studies to reach human dosages.

Regulatory bodies like ECVAM are increasingly promoting and supporting alternative methods of toxicity assessment. Immunotoxicity associated with anticancer agents being one of the most important drug related toxicity, CFU-GM assays have secured a special place in alternative tests for hematotoxicity. \textit{In vitro} CFU-GM assays may help us in evaluating hematotoxic effects of test compounds by determining the ratio of inhibitory concentrations in human vs. murine CFU-GM assays (Figure 4.14). This method not only allows assessing the species-specific differential effect of drugs on murine and human CFU-GM development but also, this ratio is used to estimate the dosage for starting human MTD studies. This approach may help us in minimizing animal usage in various preclinical studies for determination of human MTD. In addition, this strategy will allow us to achieve the 3R’s principle (Reduce, Refine and Replace), which is need of the hour. In our in-house standardized procedures, we were able to obtain approximately 20 x 10\textsuperscript{6} BM precursor cells from one mouse, which is sufficient to screen hematotoxic potential of at least 5 compounds. Also, investigating the inhibitory effects of test compounds on development of CFU-GM \textit{in vitro} allows us to study possible effects on lineage and species specific differentiation at cellular level and helps us understanding their hematotoxic mechanism of action.
Figure 4.14 - Prediction of Human MTD using \textit{in vitro} CFU-GM assay vs. \textit{in vivo} method