Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) Assay
INVITTOX n° 101

Haematotoxicity

The haematotoxic potential of xenobiotics is determined by the evaluation of the inhibition of CFU-GM growth.

Objective

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potency, hazard/risk assessment
PURPOSE OF TESTING	:	general classification and labelling

The CFU-GM assay is used in a screening mode to predict chemical induced myelosuppression, one of the haematological parameters to be considered for the assessment of the haematotoxic potential of chemical compounds of various kinds. The assay shall find its application in addition to general screening purposes, first of all, during pre-clinical drug developments of antineoplastics and clinical therapy settings.

After the conclusion of the ECVAM validation study, the SOP herewith enclosed has been proposed by the Management Team of the study to be included as an integral part of the test batteries of toxicological methods required by regulators for the approval of pharmaceuticals, industrial chemicals and food additives (Anon., 2000; Gribaldo*et al.,* 1996; Pessina *et al.*, 2001 and 2003).

The ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the CFU-GM assay for predicting acute neutropenia in humans as a substitute to using a second species, such as the dog, for this purpose (24^{h} meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 2006) (Pessina et al. 2001, 2003).

Rationale

Colony forming assays are employed to investigate the direct adverse effects of xenobiotics on the blood-forming system.
Multipotential stem cells of this system, also called colony-forming units (CFU), give rise to progenitors already committed to differentiate into different lineages of mature blood cells depending on humoral growth factors and local cytokines in their particular microenvironment.

Conditions have been developed that support the formation also *in vitro* of colonies of red cells, granulocytes, macrophages etc. by the clonal growth and maturation of progenitor cells. Which type of haematopoietic colonies is formed depends on the provision of appropriate nutrients and growth factors added to the culture medium.

The procedure of the attached SOP describes the use of semi-solid media, which allow the clonal progeny of the precursors of granulocytes and/or macrophages: CFU-GM assay (Colony Forming Unit of Granulocytes and/or Macrophages).
This CFU-GM *in vitro* test detects the direct adverse effects of xenobiotics on the proliferative capacities of the progenitors and should be able to predict the exposure level of xenobiotics that would cause clinical neutropenia after onset acute exposure (Gribaldo *et al.,* 1996; Pessina *et al.*, 2000).

Experimental Description

Endpoint and Endpoint Measurement:

CELL PROLIFERATION: Inhibition of cell proliferation by colony scoring

Endpoint Value:

IC_{90}: Inhibition of cell proliferation; 90% inhibitory concentration values

Experimental System:

BONE MARROW (murine): Murine bone marrow Mono Nuclear Cells (MNC)
BLOOD (human): Human Cord Blood Mono Nuclear Cells (Hu-CBMNC)

Murine bone marrow cells are collected from femura isolated from sacrificed mice and, as human tissue, cord blood cells are used which are known to contain high numbers of primitive haemopoietic progenitor cells. Thus, cord blood mononuclear cells (CBMNC) are assumed to be a good alternative to human bone marrow haemopoietic stem cells for both research and clinical applications. The culture method is enclosed in the attached SOP.

CFU-GM Assay Procedure
The methylcellulose culture media (MCM) has to be prepared and dispensed in round bottom tubes.
The test compounds, the MCM and the cells have to be mixed prior to plating the culture dishes. For each experiment there are: three linearity control dishes, two vehicle controls, and six for the drug-curve. The volumes of vehicle and drug stock have to be added to the methylcellulose tubes (see tables of the SOP). Then the mouse MNC or Hu-CBC cell suspensions have to be added to each tube and the cell-medium mixture to be distributed into each of three Petri dishes.
The cell cultures will be exposed to the test compounds for 7 days at 37°C in air + 5% CO_2 under saturated humidity. Colonies are counted at the end of the 7 days incubation time (murine cells) and 14 days (human cells). CFU-GM colonies are scored by scanning the whole Petri dish by using an inverted microscope, following the criteria for colony counting.

Data Analysis/Prediction Model

The Prediction Model (PM) focuses on one clinical parameter of neutropenia: the depth of nadir (severity). Animal data have shown that there is a clear relationship between the reduction in CFU-GM and the decrease of the absolute neutrophil count (ANC). The prediction of the acute xenobiotic exposure levels that would cause these maximum tolerated decreases in ANC is the goal of *in vitro* haematotoxicology (Erickson-Miller *et al.*, 1997; Parchment *et al.*, 1994).

For the purpose of the ECVAM's study "*In Vitro* tests for haematotoxicity: prevalidation and validation of Colony Forming Unit Granulocyte/Macrophage (CFU-GM) assays for predicting acute neutropenia" (1997 – 2000) the IC90 (90% inhibitory concentration values) determined for each drug in human and murine were used to predict a human Maximum Tolerated Dose (MTD). Accurate prediction is defined as the prediction of human MTD that lies with 4-fold of the actual human value.

Example:

Xenobiotic	**IC_{90} ratio (Human:murine)**	**Murine MTD** (mg/m^2 /cycle)	**Predicted Human MTD**	**Actual Human MTD** (mg/ m^2/cycle)	**Successful Prediction** (Yes/No)
Flavopiridol	0.41	100	41	50*	yes

Pessina *et al.*, 2000.

*Maximum value must be: (41 x 4)

Test Compounds

Drugs (anti-neoplastics, anti-virals, anti-inflammatories, etc.); environmental contaminants (pesticides); food additives and contaminants (mycotoxins).

Discussion

Completely "predicting neutropenia" for a xenobiotic from*in vitro* testing requires an accurate prediction for each parameter (Parchment and Murphy 1997, Parchment 1998). Currently there are three clinical prediction models for the depth of the neutrophil nadir published in the literature (Parchment*et al.,* 1993; Parchment and Murphy 1997; Parchment 1998; Parchment *et al.,* 1998). They differ in complexity, the amount of pharmacological information required to make predictions, and the accuracy of those predictions. In general, the more data intensive models provide the most accurate predictions.

However, the most generally useful model is the one that requires the least amount of specialized pharmacological information, since parameters like plasma clearance rate are often unavailable at the time permissible exposure limits are set.

The prediction model selected for evaluating in Phase I of theECVAM study, " *In Vitro* tests for haematotoxicity: prevalidation and validation of Colony Forming Unit Granulocyte/Macrophage (CFU-GM) assays for predicting acuteneutropenia" (1997 – 2000), generates an estimate of human Maximum Tolerated Dose by adjusting an animal MTD from registration toxicology (for example rat) for the intrinsic difference in drug tolerance between the experimental species and humans, and also for the differential sensitivity between CFU-GM from the animal species and the human. This model can be used without knowledge of pharmacokinetic differences across species. Nevertheless, this model should be capable of predicting human exposure levels within 4-fold of the actual (the inter-species variation in tolerated dose due to differences in clearance rates), and this is sufficient accuracy to contribute to setting permissible exposure limits. This simple model is the one that was evaluated during the performance phase of this validation study.

It is important to note that available models predict the human exposure that produces a neutrophil nadir severe enough to be associated with significant clinical risk of infection. Therefore, the predicted value is the maximum tolerated dose of an acute exposure. To set safe exposure levels, the PEL would be set at some dose level below the predicted MTD and the scale of the reduction is dependent upon the intended use, if any, of the xenobiotic. (Anon., 1998).

The Rationale of the Prediction Model

The goal of*in vitro*haematotoxicology is the prediction of the acute xenobiotic exposure levels that cause these maximum tolerated decreases in absolute neutrophil count (ANC). The prediction model tested in Phase I of the ECVAM prevalidation study has evolved from recent studies that sought to correlate *in vitro* and *in vivo* data (Parchment *et al.,* 1994; Erickson-Miller*et al.* 1997; Parchment and Murphy 1997; Deldar and Parchment 1997; Parchment 1998). Initial experience with pyrazoloacridine (PZA) proved the feasibility of predicting drug exposure levels that cause grade 3-4 neutropenia in humans from*in vitro* data (Parchment *et al.,* 1994).
Grade 4 neutropenia is to be considered dose-limiting toxicity, and its severity represents about 90% decrease in ANC for patients with normal marrow function, while Grade 3 is to be considered tolerable and represents 75% reduction. The prediction model is therefore highly dependent upon the correct selection of the degree of inhibition of the dose-limiting neutrophil progenitor. From the PZA study in humans (Parchment *et al*., 1994), it was apparent that the drug exposure level that inhibits CFU-GM by 90%*in vitro* and decreases ANC by 90%*in vivo* were the same.

The importance of the IC90 value has been confirmed by different studies (Erickson-Miller *et al*., 1997; Parent-Massin and Parchment, 1998). All studies indicated that the normally used IC50 does not predict clinically important levels of drug exposure in patients with adequate marrow function (Parchment *et al,* 1994; Erickson-Miller*et al.,* 1997). In fact, the IC50 may represent a loss of CFU-GM that causes no more than mild neutropenia after acute exposure.

Some*in vitro* studies have emphasized IC70-IC75 values, reflecting the clinical view that it is more important to predict exposure levels that result in grade 3 neutropenia, rather than grade 4 (Ghielmini*et al.,* 1997). The IC70 may also be a useful endpoint in patients with mild neutropenia, because grade 4 neutropenia may result in these patients from a smaller decrease in ANC. However, determining the predictive IC endpoint for neutropenia might depend on the pretreatment ANC of the patient. A prospective or retrospective clinical trial will be required to determine the relative predictive value of the IC90 versus the IC70. For purposes of the ECVAM study, it was decided to use the IC90 in the prediction model because most exposed individuals would likely not be pre-exposed to marrow toxicants like anticancer drugs.

Determining the predictive IC test parameter for neutropenia might depend on the pretreatment ANC of the patient. A prospective or retrospective clinical trial will be required to determine the relative predictive value of the IC90 versus the IC70. For purposes of the ECVAM prevalidation study, the authors decided to use the IC90 in the prediction model because most exposed individuals would likely not be pre-exposed to marrow toxicants like anticancer drugs (Anon., 1998).

Status

The widespread use of this technique already in the past was documented by Gribaldo *et al.*, 1996.

The method has successfully been prevalidated and the conclusion of this study (January 1997 – July 1998) was that the assay provides adequate performance in terms of colony numbers, potential sensitivity and linearity. The prediction model accurately predicted the actual human MTD values for five of the six test substances.

On the basis of these first results, a further phase concluding the formal validation study was conducted and completed in 2000: **"*In Vitro* tests for haematotoxicity: prevalidation and validation of Colony Forming Unit Granulocyte/Macrophage (CFU-GM) assays for predicting acute neutropenia" (1997 – 2000)**. The Management Team of this ECVAM study has concluded that this validation stage, where an additional panel of 20 drugs were tested,confirmed the positive results obtained in the prevalidation phase (Anon., 2000; Pessina *et al. et al*., 2000, 2001 and 2003).

Furthermore, in 2006, the **ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the CFU-GM assay for predicting acute neutropenia in humans as a substitute to using a second species, such as the dog, for this purpose**. It should be noted that the test relies on the availability of mouse MTD data and is, therefore, not a full replacement method, but is intended to reduce the overall numbers of animals needed in toxicity testing. Performance standards for the assay should be developed to enable reasonable flexibility in the protocol used (24th meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 2006) (Pessina et al. 2001, 2003).

Last update: April 2006

PROCEDURE DETAILS, July 1998*

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INVITTOX n° 101

Note: This protocol presents the standard operating procedure used in the ECVAM validation study: "In Vitro tests for haematotoxicity: prevalidation and validation of Colony Forming Unit Granulocyte/Macrophage (CFU-GM) assays for predicting acute neutropenia" concluded in 2000.

* The accuracy of the SOP has been confirmed by the responsible laboratory in October 2000.

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1. Materials

1.1. EQUIPMENTS

- Microbiological Safety Cabinet
- centrifuge
- vortex
- incubator, 37°C, 5% CO_2, 95% relative humidity
- certified Eppendorf pipet 5-40µl, 40-200µl, 200-1000µl
- hemocytometer (e.g. Bürker, Neubauer)
- inverted microscope (20-25x magnification)
- warm waterbath at 37° C
- inverted microscope (20-25x magnification)
- freezer –80°C
- freezer –20°C
- refrigerator +4°C
- freezing container "Mr.Frosty" Nalgene, Cat. N° 5100

1.2. REAGENTS AND MATERIALS

- 70% ethyl alcohol
- gauze tissue
- surgical material: little pair of scissors, surgery tweezers

- needle 23 or 25 Ga, 18 Ga, 19 Ga

- antibiotics: penicillin 100 U/ml – streptomycin 100 µg/ml (Gibco, Ref. 15140)

- Iscove's Modified Dulbecco's medium 1X (IMDM)
(Gibco, Ref. 21980)

- Fetal Bovine Serum (FBS) (Gibco, Ref. 10084)

- Dimethyl Sulfoxide (DMSO) (SIGMA Ref. D-5879 or D 2650)

- PBS Dulbecco's Phosphate Buffer Saline without Calcium and Magnesium (GIBCO Ref. 14190)

- Ficoll-Paque Research Grade Ref. 17-0840-02 (Pharmacia Biotech.)(Store at 4°C and protect from direct light)

- Anticoagulant suggested CPD (Citrate-Phosphate-Dextrose solution) SIGMA Ref. C7165 (use at ratio 1.4:10)

- 0.22 µm disposable filter

- Human Serum Albumin (HSA) for clinical applications (Sigma, Ref. A 1653 Fraction V or Fluka Ref. 05418 Fraction V 99%)

- Dextran T 40: Rheomacrodex 10 %
(Dextran Mw 40,000)(Pharmacia Biotech.Ref. 17-0270-1)

- Dnase I: Rnase free, 10U/ml (Boeringher, Ref. 776785)

- Trypan Blue 0.4% (Sigma, Ref. T8154)

- Turk Solution (HBSS containing 2% acetic acid + 0.01% Methylen Blue, Sigma, Ref. M9140)

- methylcellulose culture media (MCM)(Stem Cell Technologies, specifically prepared)

- HBSS (Hanks balanced salt solution)

- cell strainer: 100 µm cell strainer (Falcon, Ref. 2360)

- cryotubes : 2,2 ml, Nalgène, Ref. A 12 984 75 or 1,2 ml,
Falcon Ref.4806

- petridish Æ 150 mm, Æ 60 mm

- petridish Æ 35 mm (Nunc, Ref. 153066)

- gridded petridish Æ 60 mm

- disposable syringe 5 ml, 10 ml, 1ml B-D insulin syringe

- 15 ml round bottom tubes (Falcon, Ref. 2057)

- tubes for density gradient : 15 ml conical tubes
(Falcon, Ref. 2097)

- 50 ml tube (Falcon, Ref. 2070)

- melting ice

- sterile disposable pipettes

1.3. INCUBATOR HUMIDIFICATION TEST

1. Prepare three Petri dishes of Æ 60 mm (Area = 28.27 cm^2) and fill each dish with 10 ml of distilled water (Vs = Starting Volume).

2. Put the dishes in the incubator in the centre of the middle plateau.
After 72h measure the volume of water in each dish (Vf = Final Volume)

3. Calculate the Evaporation Rate (ER) of each one as follow:

$$ER\left(\mu l \times h^{-1} \times cm^{-2}\right) = \frac{V_s - V_f}{h \times a} = \frac{V_{72}}{2{,}035}$$

4. Calculate the mean ± S.D. of the triplicate.

E.R. Values (ranges):

1.1 – 1.7	Very Good
1.7 – 2.1	Good
2.1 – 2.7	Acceptable
2.7 – 3.0	Poor
> 3.0	Unacceptable

2. Standard Operating Procedure

2.1 METHYLCELLULOSE CULTURE MEDIA (MCM)

2.1.1 Type A: formulation from Stem Cell Technologies, Vancouver, Canada

1 % Methylcellulose in IMDM

30% Fetal Bovine Serum

1% Bovine Serum Albumine

2 mM L- glutamine

10 ng/ml GM-CSF*

* Murine or human recombinant cytokines

2.1.2 Preparation of methylcellulose stocks

Thaw bottles of methylcellulose culture media (MCM) overnight at +4°C. Homogenize the MCM by inverting the bottle several times. With a 10 ml syringe and 18 Ga needle, dispense all of the MCM in the bottle into 4 ml aliquots in 15 ml round bottom tubes.

The precision in dispensing MCM represents a critical point in order to obtain the correct final concentration of methylcellulose and toxicant at later steps! If the medium runs along the tube wall, centrifuge the tube 140 X g, 30 sec to move it to the bottom. Dispense exactly 4 ml of MCM into a tared tube.

Aliquots must be stored at –20°C. Individual tubes can then be thawed and used as needed.

2.2 SOURCE OF MURINE HEMATOPOIETIC PROGENITORS

For murine model bone marrow cells are used, collected from male BDF/1 (C57Bl/6 x DBA-2) mice, 8-12 weeks old. Three mice per experiment are used and all the procedures are performed in rigorous sterile conditions by using sterile reagents and materials and operating in a Microbiological Safety Cabinet. Keep the cells at + 4 °C (melting ice).

2.2.1 Isolation of murine bone marrow cells (mu-BMC)

1. Sacrifice the animals by cervical dislocation (without anaesthesia). After the animals have been killed, proceed as soon as possible. Wash the mice thoroughly with 70 % ethyl alcohol and leave the animal for 1-2 minutes entwined into a gauze alcohol imbued.
2. Remove the skin of the legs and isolate the intact femura by cutting the muscle ligaments

using a little pair of scissors and tweezers in order to clean well the bones.

3. Put the six femura in a 150 mm petri dish containing 10 ml Iscove's Modified Dulbecco's medium (IMDM) supplemented with antibiotics (Penicillin 100 U/ml - Streptomycin 100 µg/ml) and maintain them at + 4°C on ice.
4. Clean the knee from the articular cartilage and cut both ends from the femura just below the head.
5. Hold the femur shaft with surgery tweezers, insert a 23 - 25 Ga needle mounted on a 5 ml syringe into the "knee end" and flush marrow out of the end with IMDM without antibiotics. Use 2 x 1.5 ml flushing per femur. Collect the bone marrow cells from all femura in a single 15 ml round bottom or 50 ml tube. Calculate 3 ml for each femur.
6. Disperse the BMCs with the syringe by repeated flushing (5 times) and transfer the cell suspension with the syringe into a 50 ml sterile tube by filtering them through a 100 µm disposable cell strainer.
7. Wash the cells at 400 x g, 10 minutes, discard the supernatant and resuspend the pellet in Iscove's medium (calculate 1 ml for each femur) supplemented with 30% FBS without antibiotics.
8. Dilute 50 ml of cells plus 450 ml of HBSS containing 0.04% Trypan Blue and evaluate the percentage of cell viability immediately after making the suspension in a haemocytometer (e.g.: Bürker, Neubauer, etc.). Thereafter count the cells in the same way by using 10 ml of cells plus 90 ml of Turk Solution.
9. Adjust the suspension at 1.5 x 10^6 viable nucleated cells/ml (viability must be > than 95%), then dilute the cell suspension according to the cell densities to use as specified in the experimental design (see table 2.1).

Table 2.1: Example of cell dilution procedure for murine bone marrow cells.
Starting cell suspension: 1.5 x 10^6/ ml

			Dilution for preparing 1 ml of cell suspension	
Cell dilution	**Tube**	**n° cells/dish (*)**	**Starting cells suspension (1.5 x 10^6 /ml)**	**IMDM+FBS 30%**
A	CTRL 1	2,500	0.025 ml	0.975 ml
B	CTRL 3, D0® D8	40,000	0.391 ml	0.609 ml

Note: The suspension must be constituted of single cells. If cell aggregates are present, they must be dispersed by gentle pipetting before counting!

2.3 SOURCE OF HUMAN HAEMATOPOIETIC PROGENITORS

The sources of haemopoietic cells used in the described procedure are cryopreserved human cord blood mononuclear cells (huCBMNC). A minimum of two frozen aliquots of CBCs from a single donor is required to complete the screening Phase and the IC Determination Phase of testing.

2.3.1 Collection of human umbilical cord blood cells (huCBC)

1- Add 7 ml of CPD in 50 ml tube (ratio 1.4 CPD : 10 cord blood).

2- Place the tube at room temperature.

3- Collect 43ml of cord blood after normal delivery in sterile conditions if possible.

4- Keep the sample at room temperature.

5- The cord blood cells has to be cryopreserved or cultivated at least 24 after collection.

2.3.2 Isolation of human umbilical cord blood mononuclear cells

Important: maintain the Ficoll-Paque and the cord blood samples at room temperature prior to and during the density gradient.

1. Dilute 1 volume of cord blood with 1 volume of PBS (1:1 dilution). For each 10 ml of diluted cord blood one gradient tube will be required.
2. Invert the Ficoll bottle several times to ensure thorough mixing.
3. Add 1 volume (i.e. 5 ml) of Ficoll-Paque in a 15 ml-centrifuge tube and carefully layer 2 volumes (i.e.: 10 ml) of the diluted cord blood sample. Do not mix the Ficoll with the diluted cord blood sample.
4. Centrifuge at 400xg for 30 minutes at 18-20 °C, **without braking.**
5. Draw off the upper layer using a pipette, leaving the Mononuclear Cell layer undisturbed at the interface.
6. Transfer the Mononuclear Cell layer of the gradients to one clean 50 ml centrifuge tube, using a pipette.
7. Add 3 volumes of PBS^- to the volume of Mononuclear Cells. Suspend the cells by gently drawing, using a pipette.
8. Centrifuge at 400xg for 10 minutes at 18-20°C, and draw off the supernatant with pipette.
9. Repeat the cell washing procedure (steps 7 and 8).
10. Resuspend cells in 0.5ml of PBS^- and place on melting ice.
11. Dilute 50 µl of cells in 450 µl of HBSS containing 0,04 % Trypan Blue and evaluate the percentage of viability immediately after making the suspension in a haemocytometer. Thereafter count the cells in the same way by using 50 µl of cells plus 450 µl of Turk solution.

2.3.3 Cryopreservation of huCBC

1. Prepare the freezing solution: 20 % DMSO and 40 % FBS in IMDM.
2. Adjust the cellular suspension to a concentration of 4 x 10^6 - 4 x 10^7 cell/ml in IMDM.
3. Dilute the cellular suspension 1:1 with the freezing solution to obtain a final concentration 2x 10^6 -2 x 10^7 cells/ml.
4. Fill the cryotubes with 1 ml of the cell suspension immediately after step 3 and place them at – 80°C for 24 h in a "Mr. Frosty" container.
5. After 24 h, introduce the cryotubes in Liquid nitrogen.

2.3.4 Thawing of Hu-CBMNC:

Note: All the procedures must be performed in rigorous sterile conditions operating in a Microbiological Safety Cabinet and using sterile reagents and materials.

1. Prepare 0.22 mm filtered solution I: 2.5% Human Serum Albumin and 5% Dextran 40 in IMDM (25mg HSA+50mg Dextran T 40/ml IMDM). (Prepare 1 volume of solution I per each volume of thawed cells).
2. Prepare 0.22 mm filtered solution II: FBS 10%, 3U DNase/ml in IMDM. (Prepare 8 volumes per each volume of thawed cells).
3. Rapidly thaw the required number of cryotubes in a waterbath at 37ºC. Do not agitate the tubes.
4. Combine the contents of the cryotubes and dilute the thawed cell suspension 1:1 (Vol/Vol) with Solution I in 15 ml round bottom tube, mix gently by hand, and maintain at room temperature for 10 min.
5. Add 4 volumes of solution II per each volume of the suspension obtained in step 4, mix gently by hand, and maintain at room temperature for 10 minutes.
6. Filter with 100 mm Falcon cell strainer to remove clump on a tube 50 ml.
7. Centrifuge at 800xg for 10 min at 18-20ºC, draw off the supernatant with pipette and resuspend the cells in 0.5 ml IMDM + 30% FBS. From now on put the cells in melting ice (+4°C).
8. Dilute 50 ml of cells into 450 ml of HBSS containing 0.04% Trypan Blue (Sigma, Ref.T8154) and count the cells in a hemocytometer (e.g.: Bürker, Neubauer, etc.).
9. Adjust the suspension at 1.5 x 10^6 viable nucleated cells/ml (viability must be > than 80%),

then dilute the cell suspension to achieve the cell densities required by the experimental design (e.g.table 2.2).

Table 2.2: Example of cell dilution procedure for human mononuclear cord blood cells (hu-CBMNC). Starting cell suspension: 1.5 x 10^6 /ml.

			Dilution for preparing 1 ml of cell suspension	
Cell dilution	**Tube**	**n° cells/dish (*)**	**Starting cells suspension (1.5 x 10^6 /ml)**	**IMDM + FBS 30%**
A	CTRL 1	10,000	0.098 ml	0.902 ml
B	CTRL 3, D0® D8	75,000	0.733 ml	0.267 ml

(*) Cell density obtained if 0.3 ml of each dilution is added to the corresponding tube.

2.4 TECHNICAL PROCEDURE FOR GM-CFU TEST

The methylcellulose, the test article and the cells are mixed prior to plating the culture dishes.

1. Aliquots of test article, paired vehicle, and diluent should be stored as directed by the supplier.
2. On the night before the testing day, completely thaw aliquots of methylcellulose at + 4°C.
3. There are eleven tubes of cell culture mixture for each experiment: CTRL1, CTRL3 (LINEARITY CONTROL DISHES), D_0 (VEHICLE CONTROLS), and D_1- D_8 (DRUG CURVE). Each tube should contain 4.0 ml of methylcellulose culture medium.
4. On an incubator tray, label 35 mm Petri dishes according to the experimental design.
5. Prepare the drug and solvent stocks immediately prior to use according to the specific SOP.
6. Add 100 ml of IMDM to CTRL1 and CTRL3. Add 78 ml IMDM to each of D_0- D_8.
7. Prepare the toxicant dilutions in sterile 1.5 ml Eppendorf tube as described in **Table 2.3a-b** for Prescreening phase and **Table 2.4** for IC determination phase.
   Using 2-200 ml certified pipettes with tips, add 22 ml of vehicle or 22 ml of each toxicant dilution to the methylcellulose tubes in melting ice. Vortex each tube 2x5 seconds. Final volume in each tube should be 4.1 ml after adding the toxicant.
8. Immediately add 0.3 ml of mouse or human MNC cell suspension, A or B, to each correspondent tube, move the tube gently to mix, and then vortex vigorously three times for 8 seconds.
9. Let the tubes stand for 5 minutes on melting ice to release air bubbles. Label the D_8 and D_4 dilution tube with toxicant name, dose level, date, and test location. Store these two tubes at –80°C for future analysis and record the stored samples on the Registration Form.
10. Distribute 1 ml of the cell-medium mixture into each of three Petri dishes using a 1 ml B-D insulin syringe with 19 Ga needle. Gently rotate the plate to spread the mixture evenly by allowing the meniscus to attach to the dish wall.
11. Incubate the cultures at 37°C in air + 5% CO_2 under saturated humidity for 7 days (murine assay) or 14 days (human assay).

Note: Sufficient humidity during incubation is critical because the drying of cultures drastically reduces colony formation. Saturated humidity can be achieved incubating six cultures dishes with one 60 mm dish (without lid) containing water inside a 150 mm Petri dish covered with a lid.

Table 2.3a

Toxicant Dose Level (ECVAM Nomenclature)	Vehicle Stock (ml)	20 mg/ml Toxicant Stock or Prepared Dose Level (ml)	Total Volume of Working Solution (Toxicant+Vehicle) (ml)
D8	0	1.000	1.000
D7	800	200	1.000
D6	980	20	1.000
D5	990	10 of D7	1.000
D4	990	10 of D6	1.000
D3	990	10 of D5	1.000
D2	990	10 of D4	1.000
D1	990	10 of D3	1.000
D0	1.000	0	1.000

Table 2.3b

Dilution of Toxicant into 200X Working Stock Solution	Concentration Toxicant into 200X Working Stock Solution (mg/ml)	Volume of Working Solution to add to the Culture Dish (ml)	Final Fold-Dilution of Toxicant When added to the Culture Dish	Concentration of Toxicant When added to the Culture Dish (mg/ml)	Concentration of Vehicle added to the Culture Dish (Vol %)
1	20.000	22	200	100	0,5
5	4.000	22	1.000	20	0,5
50	400	22	10.000	2	0,5
500	40	22	100.000	0,2	0,5
5.000	4	22	1.000.000	0,02	0,5
50.000	0,4	22	10.000.000	0,002	0,5
500.000	0,04	22	100.000.000	0,0002	0,5
5.000.000	0,004	22	1.000.000.000	0,00002	0,5
- -	0	22	N/a	0	0,5

2.4.1 Passing from screening phase to IC determination phase

From the CFU-GM results in the Screening Phase, identify the lowest dose level that completely inhibits CFU-GM and name it MTC. Identify the highest dose level that did *not* inhibit CFU-GM and name it HNTC. Calculate the log dose differential c between HNTC and MTC:

c = log {final toxicant dilution @ HNTC} – log {final toxicant dilution @ MTC}

To determine the concentrations to use in the Testing Phase (IC determination), assign the MTC to D8 and the HNTC to D2.
Divide c into six parts of log-size f (= c: 6) and assign these parts to the ECVAM dose level D3-D8, where D8 will equal the MTC. Make the following dilutions for CFU-GM testing.
Note that required volumes of toxicant stock may be smaller than can be pipetted.
These concentrations will require dilution of the drug stock before making a working stock (for example pipes 100 ml of a 1:100 dilution of toxicant stock, rather than 1 ml of undiluted toxicant stock). Remember to adjust the volume of added vehicle so that the final volume of toxicant plus vehicle is 1 ml.

Table 2.4: Calculation Table

Toxicant Dose Level (ECVAM Nomenclature)	Vehicle Stock (ml)	20 mg/ml Toxicant Stock or Prepared Dose Level (ml)	Total Volume of Working Solution to add to Cell Cultures (ml)	Volume of Working Solution to add to Cell Cultures (ml)	Concentration of Vehicle added to the Culture Dish (Vol %)
D8 (MTC)	V (= amount from Table 2.3)	T (= amount from Table 2.3)	1.000 (= V + T)	22	0,5
D7	$V + T(1-10^{-f})$	10^{-f} T	1.000	22	0,5
D6	$V + T(1-10^{-2f})$	10^{-2f} T	1.000	22	0,5
D5	$V + T(1-10^{-3f})$	10^{-3f} T	1.000	22	0,5
D4	$V + T(1-10^{-4f})$	10^{-4f} T	1.000	22	0,5
D3	$V + T(1-10^{-5f})$	10^{-5f} T	1.000	22	0,5
D2	$V + T(1-10^{-6f})$ (= amount from Table 2.3)	10^{-6f} T (= amount from Table 2.3)	1.000	22	0,5
D1	$V + T(1-10^{-7f})$	10^{-7f} T	1.000	22	0,5
D0	1.000	0	1.000	22	0,5

Table 2.5 Calculation table

Toxicant Dose Level (ECVAM Nomenclature)	Vehicle Stock (ml)	20 mg/ml Toxicant Stock or Prepared Dose Level (ml)	Total Volume of Working Solution to add to Cell Cultures (ml)	Volume of Working Solution to add to Cell Cultures (ml)	Concentration of Vehicle added to the Culture Dish (Vol %)
D8			1.000	22	0,5
D7			1.000	22	0,5
D6			1.000	22	0,5
D5			1.000	22	0,5
D4			1.000	22	0,5
D3			1.000	22	0,5
D2			1.000	22	0,5
D1			1.000	22	0,5
D0			1.000	22	0,5

From CFU-GM results in the Screening Phase….

Dx completely inhibited CFU-GM, so it is the MTC

Dy did *not* inhibit CFU-GM but D3 did, so D2 is the HNTC

Log dose differential c between HNTC and MTC is

c = log{final toxicant dilution@HNTC} – log{final toxicant dilution@MTC}
= log{y}-{x}

Example 1

From CFU-GM results in the Screening Phase ….

D7 completely inhibited CFU-GM, so it is the MTC

D2 did *not* inhibit CFU-GM but D3 did, so D2 is the HNTC

Log dose differential c between HNTC and MTC is

c = log{final toxicant dilution @ HNTC} – log{final toxicant dilution @ MTC}
= log{100.000.000} - {1.000}
= 8 – 3 = 5

So concentration interval divided into six equal parts of
log-size f (= c: 6) = 5/6

Toxicant Dose Level (ECVAM Nomenclature)	Vehicle Stock (ml)	20 mg/ml Toxicant Stock or Prepared Dose Level (ml)	Total Volume of Working Solution (Toxicant +Vehicle) (ml)	Volume of Working Solution to add to Cell Cultures (ml)	Concentration of Vehicle added to the Culture Dish (Vol %)
D8 (=MTC, D7 from Screening Phase)	800	200	1.000	22	0,5
D7	971	29	1.000	22	0,5
D6	995,7	4.3	1.000	22	0,5
D5	937 (instead of 999,37)	63 of 1:100 diluted stock (instead of 0,63)	1.000	22	0,5
D4	990,7 (instead of 999,907)	9.3 of 1:100 diluted stock (instead of 0,093)	1.000	22	0,5
D3	860 (instead of 999,986)	140 of 1:10.000 diluted stock (instead of 0,014)	1.000	22	0,5
D2	980 (instead of 999,998)	20 of 1:10.000 diluted stock (instead of 0,0020)	1.000	22	0,5
D1	970 (instead of 999,99999)	30 of 1:100.000 of diluted stock (instead of 0,0003)	1.000	22	0,5
D0	1.000	0	1.000	22	0,5

Example 2:

From CFU-GM results in the Screening Phase....

D5 completely inhibited CFU-GM, so it is the MTC

D4 did *not* inhibit CFU-GM, did, so it is the HNTC

Log dose differential c between HNTC and MTC is

c = log {final toxicant dilution @ HNTC} – log{final toxicant dilution @ MTC} = log{1.000.000} - {100.000} = 6 – 5 = 1

So concentration interval divided into six equal parts of log-size f (= c: 6) = 1/6

Toxicant Dose Level (ECVAM Nomenclature)	Vehicle Stock (ml)	20 mg/ml Toxicant Stock or Prepared Dose Level (ml)	Total Volume of Working Solution (Toxicant + Vehicle) (ml)	Volume of Working Solution to add to Cell Cultures (ml)	Concentration of Vehicle added to the Culture Dish (Vol %)
D8 (=MTC, D5 from Screening Phase)	990	10 of 1:5 diluted stock (200 diluted with 800 –see Screening Phase)	1.000	22	0,5
D7	993,2	6,8 of 1:5 diluted stock	1.000	22	0,5
D6	995,4	4,6 of 1:5 diluted stock	1.000	22	0,5
D5	936 (instead of 996,8)	64 of 1:100 diluted stock (instead of 3,2 of 1:5 diluted stock)	1.000	22	0,5
D4	956 (instead of 997,8)	44 of 1:100 diluted stock (instead of 2,2 of 1:5 diluted stock)	1.000	22	0,5
D3	970 (instead of 998,5)	30 of 1:100 diluted stock (instead of 1,5 of 1:5 diluted stock)	1.000	22	0,5
D2 (HNTC)	980 (instead of 999)	20 of 1:100 diluted stock (instead of 1,0 of 1:5 diluted stock)	1.000	22	0,5
D1	986 (instead of 999,32)	14 of 1:100 diluted stock (instead of 0,68 of 1:5 diluted stock)	1.000	22	0,5
D0	1.000	0	1.000	22	0,5

2.5 SCORING THE COLONIES

Colonies are counted after 7 days (murine cells) or 14 days (human cells) of incubation as follows:

1. Place the culture dish inside a 60 mm gridded tissue culture dish.
2. CFU-GM colonies are scored by scanning the whole Petri dish by using an inverted microscope (about 20 - 25 X magnification). It is critical to use 20 - 25X magnification; DO NOT USE 40X magnification ! ! A D_8 plate should be scored first to detemine what is the minimal acceptable aggregate considered as a colony. In this highest drug level, the colonies

will be the smallest and most difficult to define because of toxicity. After scoring one D_8 replicate, randomly count one replicate from the other experimental groups. Then score a second D_8 replicate, and then randomly score the second replicate from the remaining groups. Repeat this sequence for the third replicates.
3. Aggregates containing 50 or more cells are defined as CFU-GM colonies.
4. Aggregates with 20-50 cells are defined as clusters.

Note: For correct discrimination between colonies and clusters, carefully evaluate the number of cells for each aggregate!

Note: It is important to look carefully at the edge of the plate. It is a place where a lot of colonies grow when a high cellular density is seeded.

2.5.1 Criteria for colony counting

Note: Applies to colony morphology at 20 - 25X magnification; DO NOT USE 40X

1. **Compact colonies**: with a central dense nucleus and a peripheral halo. These colonies are very easy to score.
2. **Diffuse and spread colonies**: Without apparent nucleus. Care must be taken with the magnification, since an excessive one (> 30X) can lead to lose this kind of colonies.
   With high densities of colonies in the plate (> 150 colonies/plate) it is really hard (sometimes impossible) to score these colonies.
   This is one of the reasons for suggesting not to score very high number of colonies (although correlations could be good, the scoring is really hard).
3. **Multicentric colonies**: are found frequently in Medium B but it is inusual to find them in Medium A. These are colonies with two or more dense nucleus nearby, with a common peripheral halo growing at the same depth in the plate.
   They should be considered as one colony.
4. **Burst-forming units** (BFUs): Multifocal colonies: are aggregates of several colonies or clusters, with or without a peripheral halo. These must be counted as one colony.

Table 2.6: Experimental Design (Module to perform 1 prescreening + 2IC Determination on 3 drugs).

Experiment	Drug	Doses (in triplicate)	Linearity control (·)	Total tubes	Total dishes
Prescreening	A	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
	B	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
	C	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
IC Determination 1	A	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
	B	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
	C	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
IC Determination 2	A	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
	B	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6
	C	D0;D1;D2;D3;D4;D5;D6;D7;D8	Ctrl 1; ctrl 3	11	27+6

Legend:

A-B-C: correspond to three codified drugs.

D0= Solvent (without drug) at higher concentration used in the drug dilution (3 dishes).

D1-D8= 1:10 serial dilution (3 dishes/dose) in the presence of 40.000 cells/dish (murine bone marrow cells) or 75000 cells/dish (human cord blood cells). The doses for prescreening phase and IC determination phase 1 and 2 were calculated according to the procedure reported in section 2.4).

(·): No drug, no solvent, only cells in culture medium.

Murine bone marrow cells: ctrl 1= 2,500 cells/dish, ctrl 3= 40,000 cells/dish.

Human cord blood cells: ctrl 1= 10,000 cells/dish, ctrl 3= 75,000 cells/dish.

Fig 1: Compact colony; cell density: 10000 cells/dish; murine medium A; colonies: 1.

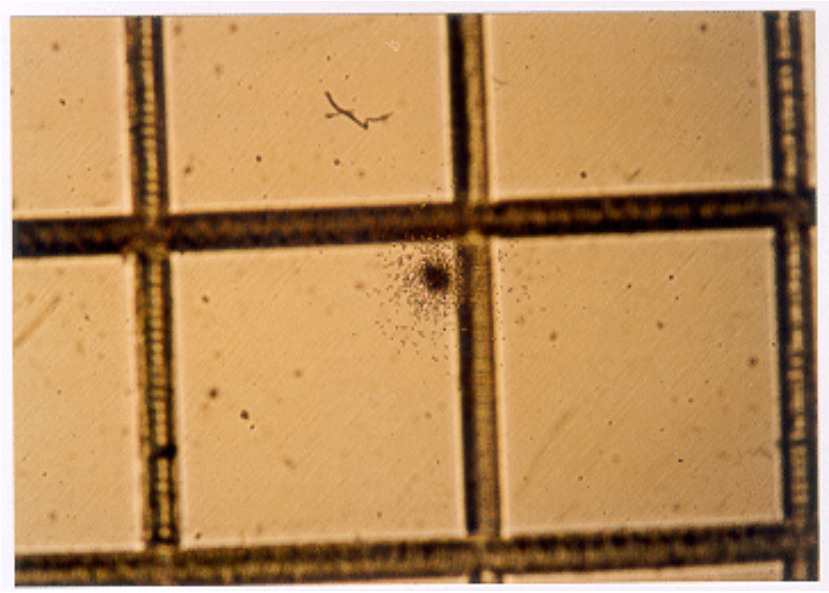


Fig 2: Diffuse colony; cell density: 10000 cells/dish; murine medium A; colonies: 1.

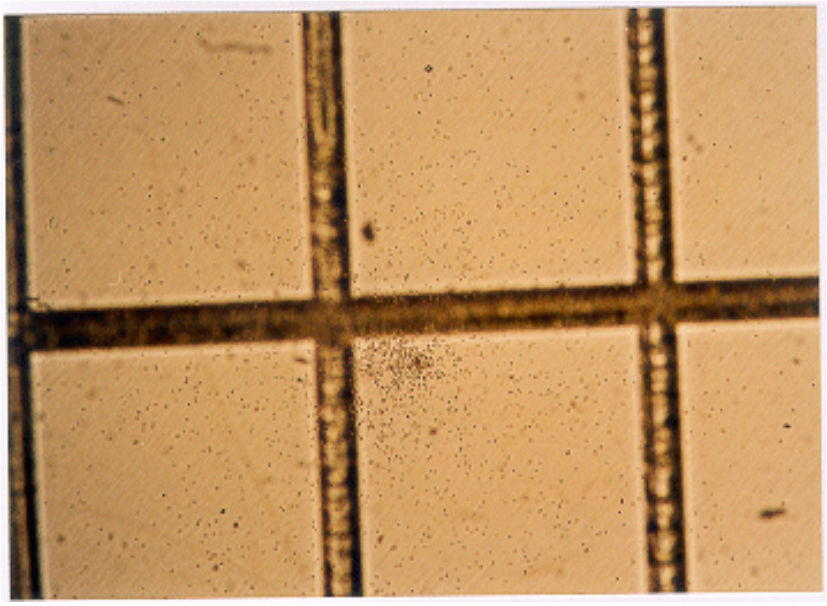


Fig 3: Multicentric colony; cell density: 80000 cells/dish; murine medium B; colonies: 1.

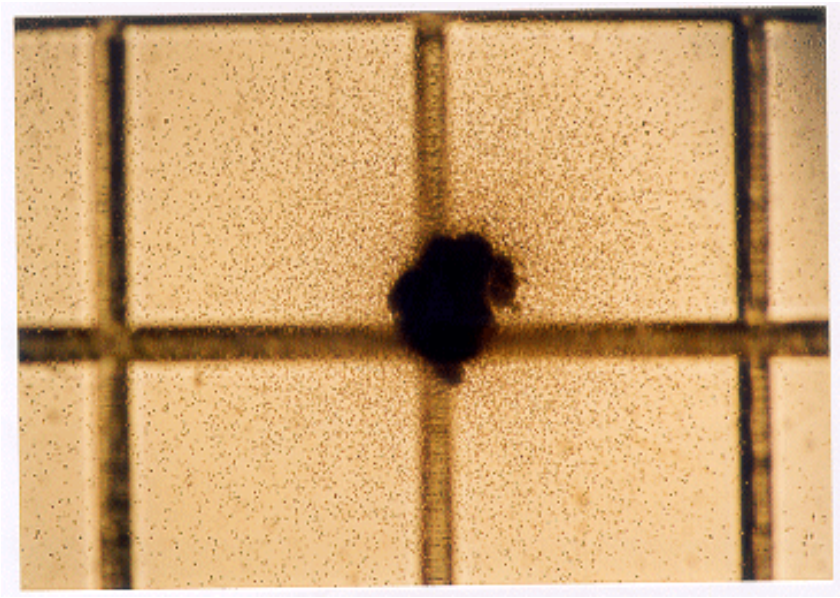


Fig 4: Burst-forming unit or multifocal colony; cell density: 80000 cells/dish; murine medium B; colonies: 1.

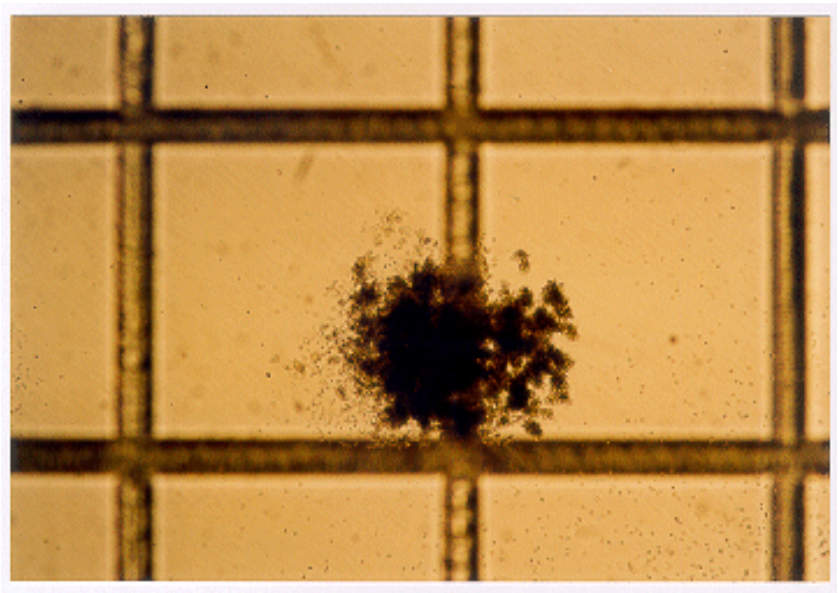
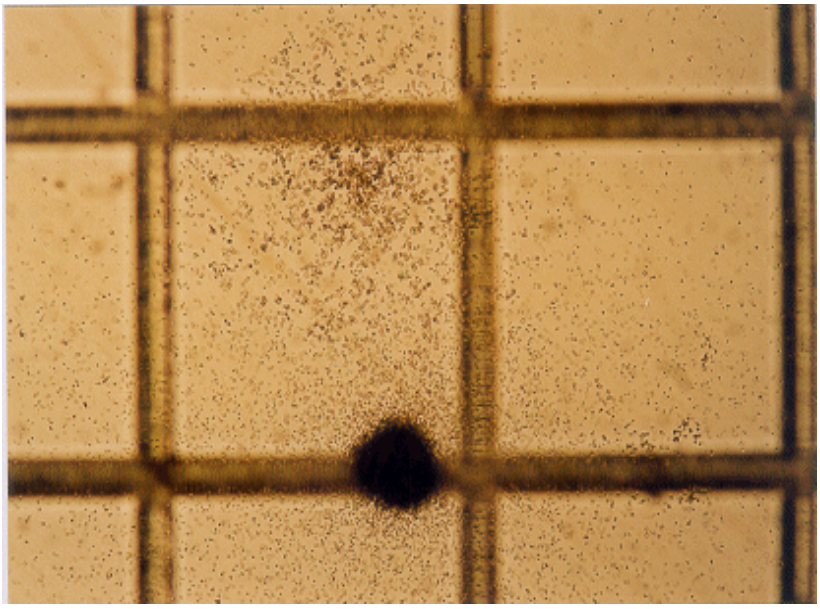


Fig 5: Compact colony (under) and Diffuse/spread colony (upper); cell density: 80000 cells/dish; murine medium B; colonies: 2.



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