A Methylcellulose Microculture Assay for the *In Vitro* Assessment of Drug Toxicity on Granulocyte/macrophage Progenitors (CFU-GM)

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Summary — In a recent prevalidation study, the use of a methylcellulose colony-forming unit-granulocyte/macrophage (CFU-GM) macroassay for two independent *in vitro* tests (human and murine cell based) was suggested for quantifying the potential haematotoxicity of xenobiotics. In this paper, we describe the transfer of the macroassay to a 96-well plate microassay, in which the linearity of the response was studied (both in terms of CFU-GM and optical density [OD] versus the number of cells cultured), and the inhibitory concentration (IC) values for doxorubicin, 5-fluorouracil and taxol were determined and compared with those obtained by using the original macroassay. Fresh murine bone marrow and human umbilical cord blood mononuclear cells were used as a source of myeloid progenitors. The cells were cultured in methylcellulose containing granulocyte/macrophage-colony-stimulating factor, and in the presence of increasing drug concentrations. The cloning capacity of the progenitors was measured both as the number of colonies counted manually (CFU-GM), and as OD evaluated with an automated plate reader in an MTT test. Our results show that, in the microassay, up to 20 colonies/well could be easily counted, and that this range (20 to zero) gave a regression line from which IC values were calculated, which were very close to those obtained by using the macroassay (where the range of colony numbers was from 100 to zero). The test did not give good results when the OD (instead of the colony count) was used as the endpoint, because, although a high coefficient of determination was obtained, the OD values ranged from 0.6 to zero and the IC values determined were not comparable to those obtained by manual counts. The use of the microassay dramatically reduces the quantity of methylcellulose needed, and permits hundreds of cultures to be processed in the same experiment, contributing to significant reductions in both the work involved and the cost. A further important benefit is a reduction of the amount of drug needed for testing, which is crucial for screening new molecules, when many different toxicological tests have to be carried out. The microassay is therefore a useful and reproducible tool for screening compounds (chemicals, drugs and xenobiotics) for potential haematotoxicity directly on human myeloid progenitors, and could contribute significantly to reducing the use of animals in toxicity testing.

Key words: *CFU-GM assay, drug, haematotoxicity, method, microcultures, myelotoxicity, toxicity.*

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Introduction

The original colony-forming unit-granulocyte/macrophage assay (CFU-GM) of Bradley & Metcalf (1) has been widely used for studying the physiology of haematopoiesis (2), for investigating the pathogenic mechanism of drug-induced blood disorders, and for the screening of chemicals for potential myelotoxic activity (3–8). The commercial availability of new media formulations based on methylcellulose, and the availability of specific recombinant cytokines, have allowed improvements which make this technique very versatile and applicable to the study of progenitors other than CFU-GM, such as erythroid (CFU-E) and megakaryocyte (CFU-MK; 9).

These basal techniques have been applied by many authors to the study of the effects of drugs and xenobiotics, by using various protocols that have been developed and proposed for *in vitro* haematotoxicity testing, as reviewed by Gribaldo *et al.* (10).

Recently, based on the recommendations of Balls *et al.* (11), a prevalidation study was used to set up a Standard Operating Procedure (SOP) for two independent *in vitro* tests (human and murine) for quantifying the potential haematotoxicity of xenobiotics from their direct adverse effect on clonal colony formation by the neutrophil progenitor CFU-GM (12).

The SOP was based on a CFU-GM assay that was modified to obtain the best response under experimental conditions in which only one factor, granulocyte/macrophage-colony-stimulating factor (GM-CSF), was used to stimulate the precursor

cells. The SOP was applied in a validation study on 20 drugs, which gave very good results and was also able to predict the maximum tolerated dose (MTD) of antitumoural molecules *in vivo* (13, 14). Because the method, as proposed, has limitations, in that it is laborious and expensive to perform, we have transferred the expertise acquired in the validation study to a micromethod. This permitted reductions in both the workload and the costs involved, and at the same time decreased the amount of each test compound needed and increased the number of compounds that could be tested within an experiment.

This paper describes the results of a study to assess a 96-well plate microassay based on methylcellulose. In the first phase, the linearity of the responses (colony number as a function of the number of cells cultured) was studied, and the toxicities of three drugs (doxorubicin, 5-fluorouracil [5-FU] and taxol) were evaluated. The inhibitory concentration (IC) values were compared with those obtained by using the original methylcellulose macroculture. The drugs were tested on both human and mouse haematopoietic progenitors.

Materials and Methods

Drugs and reagents

Doxorubicin (Fluka, Buchs, Switzerland) and 5-FU (Oncology Research and Regulatory Consulting Associates, Grosse Pointe Woods, MI, USA) were dissolved in saline solution. Taxol (Oncology Research and Regulatory Consulting Associates) was dissolved in saline–cromophor–alcohol solution (Sigma, St Louis, MO, USA). The concentration of the standard drug preparations used was 10mg/ml. The final working concentrations used in the assay were obtained by serial dilution in Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, UK).

The methylcellulose culture medium (MCM; MethoCult H4001) was 2-mercaptoethanol-free and contained 10ng/ml GM-CSF. It was provided by StemCell Technologies (Vancouver, BC, Canada), and prepared as described previously (12). Aliquots of 1ml and 4ml MCM were stored at –20°C until required for use.

Human haematopoietic progenitors (hu-CBCs)

The source of the human haematopoietic progenitors was umbilical cord blood. The mononuclear cells were isolated by using the following technique.

One volume of cord blood was diluted with one volume of phosphate-buffered saline (PBS). 5ml of Ficoll-Paque (Amersham Biosciences, Piscataway, NJ, USA) was placed in a 15ml centrifuge tube, and 10ml of the diluted cord blood was carefully layered on top. After centrifugation at 400*g* for 30 minutes at 18–20°C, without braking, the upper layer was removed by using a pipette, leaving the mononuclear cell layer undisturbed at the interface.

The mononuclear cell layer was then carefully removed and placed in a 50ml centrifuge tube. It was diluted with three volumes of PBS and, after further centrifugation at 400*g* for 10 minutes at 18–20°C, the supernatant was removed and the wash procedure was repeated.

The cell pellet, containing isolated mononuclear cord blood cells (hu-CBCs), was resuspended at 10^7 cells/ml in IMDM, supplemented with 40% fetal calf serum (FCS; Euroclone, Paignton, UK) and 20% dimethylsulphoxide (Sigma), and cryopreserved in liquid nitrogen until use.

Thawing of hu-CBCs

A vial of frozen cells was rapidly thawed in a 37°C water bath, and 2ml of cell suspension was transferred to a 15ml conical tube. Medium (IMDM supplemented with 10% FCS and 10U/ml DNAse I [Euroclone]) was added dropwise to the cells over 3 minutes, to a total volume of 7ml. After each addition of several drops of medium, the suspension was gently swirled.

The cell suspension was centrifuged at 200*g* for 15 minutes at room temperature, and most of the supernatant (approximately 5ml) was carefully removed by using a pipette. The cell pellet was gently resuspended in the remaining 2ml of supernatant, and 5ml of the above medium was added slowly, then the centrifugation and resuspension procedure was repeated.

Finally, the cell suspension was diluted 1:2 with IMDM containing 50% FCS, and adjusted to give a standard cell density of 3×10^6 viable nucleated cells/ml (viability must be > 80%) in IMDM supplemented with 30% FCS.

Murine haematopoietic progenitors (mu-BMCs)

Murine myeloid precursors were obtained from fresh bone marrow from male C57BL/6J mice of about 18–20g (Charles River, Calco, Italy), as described previously (12). Briefly, mice were killed by cervical dislocation and bone marrow cells were flushed from the femurs and tibias with Hanks' balanced salt solution, by using a 27-gauge needle. The erythrocytes were lysed with 0.85% ammo-

nium chloride at 4°C, and the cells were washed twice in PBS (200*g* for 10 minutes) and resuspended in McCoy's 5A culture medium (Biochrom AG Seromed, Berlin, Germany), supplemented with 20% FCS, 100U/ml penicillin, 100μg/ml streptomycin and 2.5% L-glutamine, to give 3×10^6 viable mononucleated bone marrow cells (mu-BMCs)/ml.

CFU-GM macroassay

The assessment of the proliferation of the granulocyte/macrophage progenitors (CFU-GM) was based on *in vitro* agar clonogenic assays, as suggested by Bradley & Metcalf (1), according to the SOP described previously (12).

Briefly, after overnight thawing of the MCM aliquots, the plating mixture was prepared by the addition of 0.3ml fresh mu-BMCs (6×10^5 cells/ml) or hu-CBCs (1.1×10^6 cells/ml) to each 4ml aliquot. The aliquots were vortexed vigorously for 5–8 seconds, and then 0.1ml of one of 44 drug dilutions at the appropriate concentration was added to each drug tube test (dose range: 0.1–0.00078μM for doxorubicin and taxol, and 20–0.156μM for 5-FU). 0.1ml of undiluted drug solvent was added to the solvent tube test, and 0.1ml IMDM was added to the control tube test. After being vortexed vigorously three times for 8 seconds, the tubes were allowed to stand for 5 minutes in melting ice to release air bubbles. 1ml of the cell–medium mixture (4×10^4 mu-BMCs or 7.5×10^4 hu-CBCs) was distributed into each of three Petri dishes, by using a 1ml BD insulin syringe (BD, Bedford, MA, USA) with a 19-gauge needle. After being incubated for 7 days (murine) or 14 days (human) at 37°C and 5% CO_2, under saturated humidity, the number of colonies (> 50 cells) was counted by using an inverted microscope.

CFU-GM microassay

Linearity test

The linearity of the response in terms of number of cells seeded and number of colonies counted in the microassay was determined as follows. 1ml aliquots of MCM were thawed overnight. Serial 1:2 dilutions of haematopoietic cells in IMDM plus 30% FCS were prepared to give supensions of 2.35×10^6 cells/ml to 1.47×10^5 cells/ml. 75μl of each cell dilution plus 25μl of IMDM were added to each tube of 1ml MCM, and vortexed twice for 8 seconds. The tubes were then allowed to stand to release air bubbles, and the suspensions were distributed into each of three wells (0.1ml/well) of a 96-well microtitre plate.

Drug assay

To test the effect of the drugs on CFU-GM, the method described above was used with the following modification. One cell suspension was prepared at a density of 1.17×10^6 cells/ml (8×10^3 cells seeded in each well). To each tube of 1ml MCM were added 75μl of cell suspension, 20μl of IMDM and 5μl of one of 200 drug dilutions that had been prepared previously by 1:2 serial dilution of the test compounds (dose range: 0.1–0.00078μM for doxorubicin and taxol, and 20–0.156μM for 5-FU).

The plates were incubated for 7 days (murine) or 14 days (human) at 37°C and 5% CO_2, and then the numbers of clusters and colonies (> 20 cells) were counted by using an inverted microscope. The plates were then processed according to the MTT method described below.

It is important to stress that for both tests (macroassay and microassay), the maintenance of efficient humidification of the cell cultures is crucial. For the microassay, this was achieved by wrapping the plates in transparent cellophane.

MTT assay

The method of Mossman (15), as modified previously (16), was used, with additional changes. After the number of colonies had been counted by using an inverted microscope, 20μl of MTT was added to each well, and the microtitre plates were incubated at 37°C. After 24 hours, 100μl of lysing buffer (20% w/v sodium dodecylsulphate in 50% *N*,*N*-dimethylformamide; pH 4.7) was added to each well, and the MCM was vigorously mixed by using a pipette, in order to obtain a good homogeneity of the cell–MCM mixture. After a further 24 hours, the optical density (OD) was measured at 550nm, by using a plate analyser (LP200; Diagnostic Pasteur, Paris, France).

Analysis of data

The number of colonies (CFU-GM) in triplicate cultures from at least three separate experiments were counted. Clonogenicity was expressed as a percentage relative to the number of colonies in the control dishes (cells treated with drug solvent). Inhibitory concentrations (IC50 and IC90) were calculated from the regression lines by using the statistical program InStat (GraphPad, San Diego, CA, USA), and the data were expressed as means ± SD. Because of the nature of the resulting data, and according to Festing (17), the mean values were compared by using a Mann-Whitney U test (InStat program). The differences were considered to be significant when $p < 0.05$.

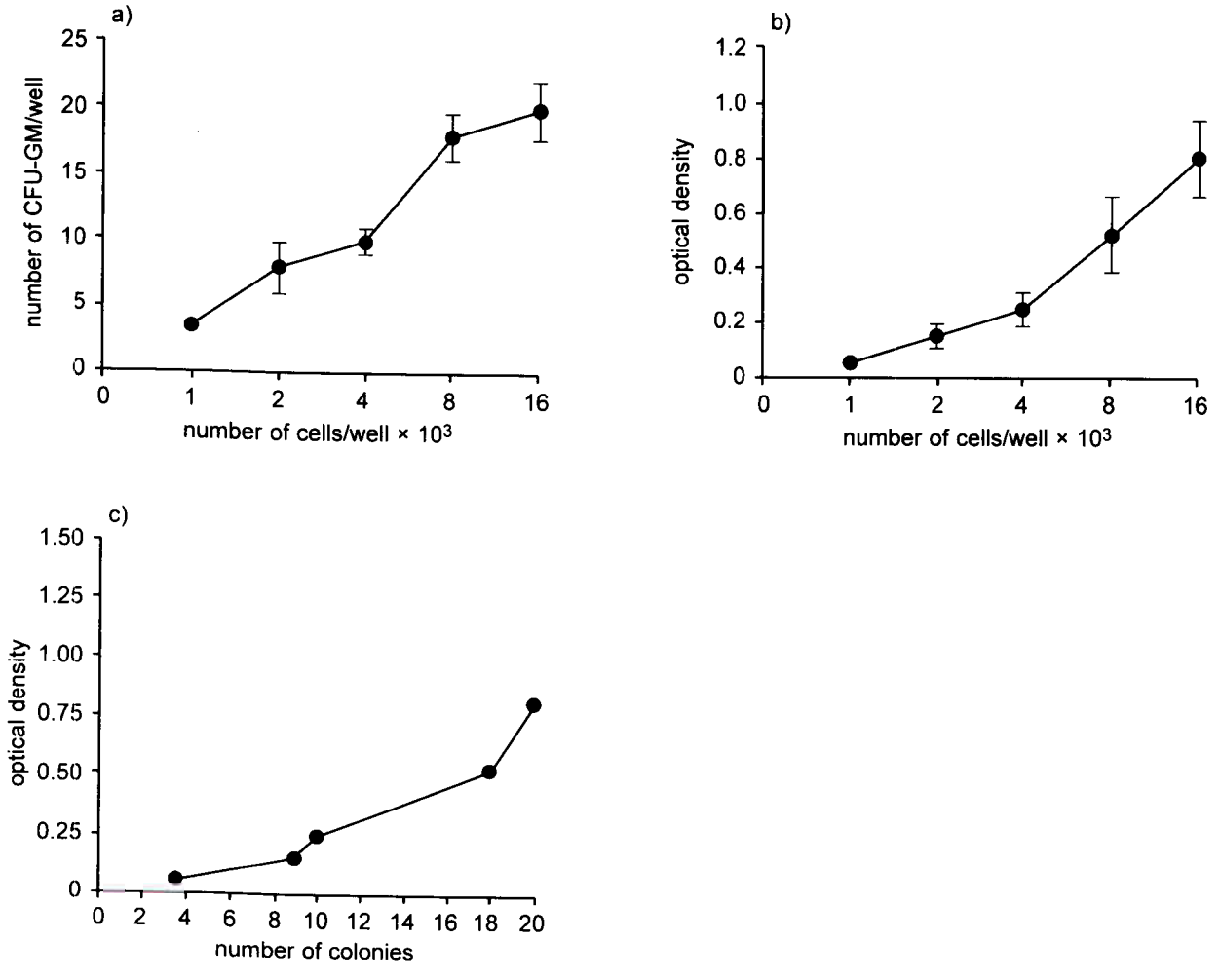
Results

Linearity of the response

The correlation between the number of mu-BMCs seeded and the number of colonies counted at 5 days of culture is shown in Figure 1a. A linear relationship was evident, with a maximum of 19.8 ± 3.4 CFU-GM/well with 1.6×10^4 cells seeded. As shown in Figure 1a, the numbers of colonies for 8×10^3 and 1.6×10^4 cells seeded appeared to be almost the same. This is probably because it is impossible to count more than 20–25 colonies per well, after they have become confluent. The linear regression showed a high coefficient of determination ($R^2 = 0.88$) and a significant slope ($p < 0.05$), without a significant deviation from linearity ($p = 0.8$).

The correlation between the number of mu-BMCs seeded and the OD determined in the MTT test at 5 days of culture is shown in Figure 1b. A significant correlation ($R^2 = 0.72$) was observed, and the slope of the regression line was also significant ($p < 0.05$), with a maximum OD value of 0.81 ± 0.27 with 1.6×10^4 cells seeded.

Figure 1: Linearity of the response evaluated at 5 days of culture in the colony-forming unit-granulocyte/macrophage (CFU-GM) microassay for murine bone marrow cells (muBMCs)



a) Number of mu-BMCs seeded versus number of colonies counted (CFU-GM/well). $R^2 = 0.88$.

b) Number of mu-BMCs seeded versus optical density (OD) in MTT test. $R^2 = 0.72$.

c) Number of CFU-GM colonies scored versus OD in MTT test. $R^2 = 0.93$.

Each point represents the mean ± SD of three independent tests performed in triplicate.

R^2 = coefficient of determination.

The correlation between the number of mu-BMC colonies counted and the OD determined in the MTT test is shown in Figure 1c. A high coefficient of determination was obtained ($R^2 = 0.93$).

To confirm that clonogenicity does not change in relation to the number of cells cultured, the data were transformed and analysed as cloning efficiency (number of colonies/10^5 cells seeded) and as OD efficiency (OD/10^5 cells seeded). Neither parameter showed a significant regression (R^2 values ranged from 0.01 to 0.18; $p > 0.05$) with mean values of 285 ± 64 CFU/10^5 cells and 6.1 ± 0.4 OD/10^5 cells, respectively. This confirmed that cloning efficiency is a constant value that is not influenced by the number of cells seeded. Based on this result, and in order to have an adequate number of colonies to score (sufficient to provide a useful OD value, but also easy to count without colonies overlapping), a concentration of 8×10^3 cells/well was used for testing drug toxicity.

Determination of drug toxicity by CFU-GM

The IC values for the three drugs tested (doxorubicin, 5-FU and taxol), determined by using both the macroassay and the 96-well microtitre microassay, for mu-BMCs and hu-CBCs, are given in Tables 1 and 2, respectively. The results are expressed as the drug concentrations (μM) able to produce a 50% (IC50) and a 90% (IC90) inhibition of CFU-GM growth.

There was an acceptable degree of similarity between the results obtained for mu-BMCs for both the macroassay and microassay (Table 1). Because of the high SD values, the results were not significantly different ($p > 0.05$). For the mu-BMCs, the IC values were also calculated, based on the regression line for the OD reading taken as the endpoint for the CFU-GM growth. With the exception of doxorubicin, these IC values differed significantly ($p < 0.05$) from the values obtained in both the microassay and the macroassay, in which the colony count was used as the endpoint.

For the hu-CBCs (Table 2), essentially identical results were observed in the macroassay and in the microassay (in which the endpoint was the number of colonies counted). As confirmed by statistical analysis, the IC50 and IC90 values determined with the two tests were not significantly different ($p > 0.1$).

Discussion

As stated in the Introduction, the aim of our work was to determine the optimum conditions for a miniaturised CFU-GM assay, to be used for studying the potential haematotoxicity of chemicals, drugs or xenobiotics in general.

Table 1: IC values determined for murine bone marrow cells by macroassay and microassay in the clonogenicity and MTT test

	Clonogenicity						MTT test			
	Macroassay		Microassay		*p* value[a]		Microassay		*p* value[b]	
Drug	IC50 (μM)	IC90 (μM)	IC50 (μM)	IC90 (μM)	IC50	IC90	IC50 (μM)	IC90 (μM)	IC50	IC90
Doxorubicin	0.0096 ± 0.0021	0.021 ± 0.005	0.020 ± 0.003	0.039 ± 0.006	0.06	0.08	0.0095 ± 0.0023	0.019 ± 0.001	0.01	0.006
5-Fluorouracil	1.34 ± 0.21	2.31 ± 0.30	1.79 ± 0.29	3.19 ± 0.52	0.22	0.17	0.18 ± 0.06	0.41 ± 0.04	0.0007	0.0005
Taxol	0.0058 ± 0.0012	0.0082 ± 0.0014	0.005 ± 0.001	0.009 ± 0.001	0.5	0.61	0.057 ± 0.011	0.0324 ± 0.0025	0.0012	0.0001

[a] *Microassay versus macroassay in clonogenicity test.*

[b] *MTT test versus clonogenicity in microassay.*

Values are means ± SD of three independent tests performed in triplicate. Differences were considered significant when $p < 0.05$.

IC50 = concentration giving 50% inhibition; IC90 = concentration giving 90% inhibition.

Table 2: IC values determined for human umbilical cord blood cells by macroassay and microassay in the clonogenicity test

Drug	Macroassay		Microassay		*p* value[a]	
	IC50 (μM)	IC90 (μM)	IC50 (μM)	IC90 (μM)	IC50	IC90
Doxorubicin	0.037 ± 0.004	0.063 ± 0.007	0.033 ± 0.009	0.060 ± 0.009	0.44	0.63
5-Fluorouracil	7.95 ± 3.92	15.26 ± 7.10	9.67 ± 1.09	17.68 ± 1.79	0.50	0.59
Taxol	0.0047 ± 0.0002	0.0087 ± 0.0011	0.005 ± 0.001	0.009 ± 0.001	0.29	0.7

[a]*Microassay versus macroassay.*

Values are means ± SD of three independent tests performed in triplicate.

IC50 = concentration giving 50% inhibition; IC90 = concentration giving 90% inhibition.

The data reported in this paper show that, with microcultures in 96-well plates, it is possible for up to 20 mu-BMC colonies/well to be counted, and that this range (20 to zero) results in a regression line with IC values that are very close to those obtained when using the macroassay (where the range of colony numbers was from 100 to zero). The same result was observed for hu-CBC (data not shown).

In a prevalidation study performed by using a traditional macrotest (12), it was demonstrated that it was possible to determine the toxic effect of a drug on myeloid precursors under experimental conditions in which the progenitors were stimulated with a unique factor (GM-CSF), instead of the traditional cocktail of cytokines which is able to recruit new stem cells to commit toward the myeloid lineage. This demonstrated that the toxic activity of a chemical could be correctly determined by stimulating progenitors with receptors for GM-CSF.

The cost of the microassay studied in our laboratory was about one-tenth of that of the validated macrotest (14). The number of cells required was reduced (from 7.5×10^4 cells/culture to 8×10^3 cells/culture), and the amount of MCM used was also reduced (from 1ml/culture to 0.1ml/culture). The microassay also permits the assessment of hundreds of cultures in the course of one experiment, with significantly less space being taken up in the incubator (one plate for 96 Petri dishes). In addition, the time taken to count the colonies is reduced by about 50%.

Another very important advantage of the microtest is the possibility of reducing the amount of test drug or xenobiotic required. This could be very important, and is sometimes crucial, in the testing of new molecules, because, during the first phase of screening, only a small amount of the chemical is often available, and many different tests have to be carried out. In addition, the microassay could be used to study other toxicants, such as microbial toxins or environment pollutants.

Unfortunately, satisfactory results were not obtained in the MTT test in which the OD (instead of the colony count) was used as the endpoint. Although the regression line was significant, with a high coefficient of determination (see Figure 1), the OD response ranged from 0.6 to zero and the IC values determined did not agree with those for the colony counts in the macroassay (see Table 1). The low range of OD values obtained was probably because the MTT test only provides indirect information on the number of living cells, by measuring the enzymatic activity of mitochondria. The cells present in GM colonies poorly express this activity and, in addition, MCM quenches the activity, due to its high basal OD at 550nm. Other strategies for reducing the high absorbance of MCM are currently being studied (for example, by using other dyes or substrates), in order to solve this limitation of the micromethod, and to make possible the automated reading of plates. In fact, the requirement for microscopic counting of the colonies is a limitation, because of the time required and also as it could be a potential source of variability.

Despite these limitations, we consider the micromethod presented in this paper to be a reproducible tool that could be very useful for screening large numbers of compounds. The method could

also be relevant to various fields of research, and also in regulatory and epidemiological monitoring, where information about the potential haematotoxicity of chemicals, drugs and xenobiotics is very important. Finally, the use of this test for screening for toxicity directly in a human cell model could contribute significantly to reducing the number of animals needed in toxicological studies.

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