



## CFU-GM ASSAY AS A DIAGNOSTIC TEST FOR TOXIC ASSESSMENT OF CHEMICALS

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### ABSTRACT

Pharmaceutical drugs, hazardous chemical compounds and other products must be evaluated for their hematological toxic potential for their safety use. *In vitro* CFU-GM clonogenic assay is one of the best test systems to evaluate the adverse toxic effects of xenobiotics on the blood forming system. The test involves exposing the mice bone marrow cells to various test substances at different concentrations and estimating the inhibition of the proliferation of the CFU-GM colonies. This technique is best use for determining the human maximum tolerated dose (MTD). It describes the use of *in vitro* CFU-GM assay for characterizing and predicting hazards to humans.

**KEY WORDS:** CFU-GM assay; *in vitro*; hematological; xenobiotics; hazards; pharmaceuticals

### INTRODUCTION

All blood cells arise from the hematopoietic stem cells (HSCs) that can differentiate into other cell types and are self renewing. This hematopoiesis process includes the proliferation, differentiation and maturation of many cell types, both local and systemic growth factors in their particular microenvironment, along one of the two pathways giving rise to either a common myeloid progenitor cell or a common lymphoid progenitor cell. The type and amount of growth factor (colony stimulating factor-CSF) controls the differentiation of a particular progenitor cell. The therapeutic drugs have common hematotoxic side effects [1]. Many drugs (anti-neoplastics), environmental contaminants (pesticides), food additives, chemicals (xenobiotics) interfere with the hematopoietic process at different stages and lead to acute or chronic hematotoxicity, such as agranulocytosis and granulocytopenia. The evaluation of hematotoxicity of drugs is necessary for the safe development of new drugs. The revolutionary changes in research such as use of molecular biology and development of advanced techniques have discarded the notion that animal models are the only way in toxicity testing. In addition to some limitations of *in vivo* study, the cost effectiveness of toxicity testing of more than thousands of newly synthesized drugs per year necessitates alternatives of animal use. While using animals, the results can vary from species to species but using *in vitro* culture method, the data represents the whole animal. The new *in vitro* method is a great progress in alternative testing method and is more reliable as they can test toxic chemicals and drugs more accurately and rapidly yet being cost effective [2]. The alternatives to whole animal testing include end point assays, cell and tissue culture, toxicokinetic modelling and structure active

relationship and databases [3]. *In vitro* hematotoxicity can help in reducing the risk related to chemicals, pharmaceuticals, food additives and other products [4]. The hematopoietic progenitor cells generate clonal colonies *in vitro* in semisolid media in response to specific growth factors, hence called colony forming unit (CFU) [5]. The colony forming units of the erythroid lineage (CFU-E), the less mature erythroid burst forming unit (BFU-E), the granulocyte-macrophage or myeloid lineage (CFU-GM) and the megakaryocyte lineage (CFU-Mk) are the bone marrow progenitors for *in vitro* study of toxic effects of compounds and drugs. All the test data either *in vivo* or *in vitro* shows some verification but greater in *in vivo* method due to greater genetic differences [2]. The CFU-GM assay uses the IMD media and granulocyte/macrophage colony stimulating factor (GM-CSF) that help in the proliferation of the precursor cells of the granulocytes and/or macrophages. The test detects the direct adverse effect of the xenobiotics on the proliferative capacities of the progenitors and its level that causes neutropenia after onset of acute exposure [4,6,7]. This short communication reviews the hematological process of risk assessment by *in vitro* toxicology, encompassing ongoing and future developments.

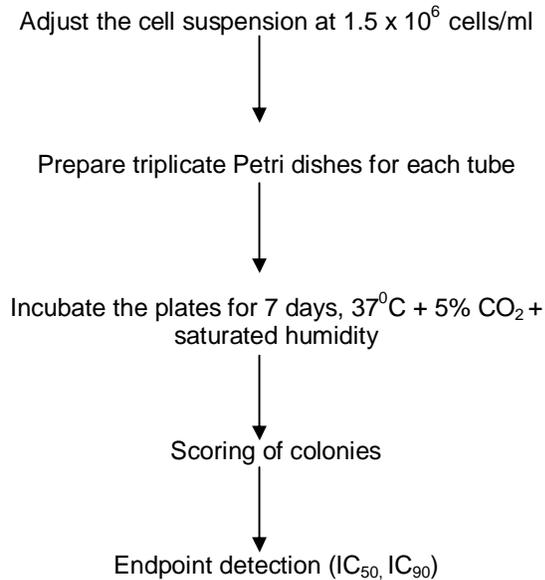
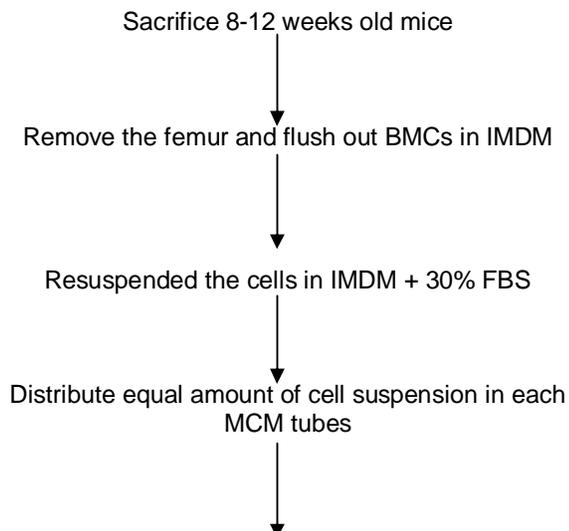
### PROCEDURE

CFU-GM assay was firstly performed by Pessina *et al.* in 2001[8]. The murine bone marrow cells (BMCs) are collected from the femur of 8-12 weeks old male Swiss mice and suspended in Iscoves Modified Dulbecco's Medium. All the operations should be performed under highly sterile conditions. Instruments should be autoclaved or oven sterilized before use. The suspension is filtered through 100µm cell strainer. The cell suspension is centrifuged at 400xg for 10 minutes and resuspended the pellet in

IMDM supplemented with 30% FBS. Counted the cells in hemocytometer to evaluate the cell viability percentage. The cell sample is diluted so as to adjust it at  $1.5 \times 10^6$  viable cells/ml of media. 4ml of methylcellulose culture media (MCM) is dispense in each tube and thawed overnight at  $+4^{\circ}\text{C}$  before use. 0.3ml of cell sample is added immediately to each of MCM tubes with different drug concentrations. Dispense 1ml of above into 35x10mm petridishes and prepared the triplicates for each concentration. In each experiment, a vehicle control (added IMDM only) and a negative control (added solvent only) are included. The Petri dishes are incubated at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  under saturated humidity. After the incubation period of seven days, the CFU-GM colonies are scored and counted under inverted microscope at 20x magnification. The highest drug level plate is considered first to determine the minimum aggregate to considered as colony. The aggregates containing 50 or more cells are defined as CFU-GM colonies. Four different morphological classes of CFU-GM colonies can be observed – (i) compact, (ii) diffuse and spread, (iii) muticentric and (iv) burst forming colonies. Compact colonies have a central dense nucleus and a peripheral halo. Diffuse and spread colonies are without apparent nucleus. Muticentric colonies have two or more dense nucleus with a common peripheral halo. Burst forming units are multifocal colonies ie. aggregate of several colonies or clusters, with or without a peripheral halo.  $\text{IC}_{50}$  value is calculated from the average value of the triplicates of each dilution according to Reed and Muench [9].  $\text{IC}_{90}$  is calculated using linear regression. The homogeneity of the results is verified by ANOVA – analysis of variance [4].

In our laboratory, standardization of CFU-GM assay using mice bone marrow cells with the model compound chloramphenicol is in progress.

#### FLOW CHART SHOWING METHODOLOGY OF CFU-GM ASSAY



#### SENSITIVITY OF THE ASSAY

Although many protocols were developed for *in vitro* toxicity [10,11,12,13], the European Centre for Validation of Alternative Methods (ECVAM) accepted the clonogenic CFU-GM assay for predicting acute neutropenia and developed a standard operating procedure (SOP) for murine and human, based on the previous original assay suggested by Bradely and Metcalf [14]. With the support of European Union, Pessina and his colleagues conducted the *in vitro* clonogenic assays particularly on CFU-GM assay to predict acute neutropenia [8] and CFU-Mk assay to predict thrombocytopenia [15]. Though CFU-Mk progenitor cells rapidly give rise to megakaryocytes [16], CFU-GM is getting more attention because of its technical simplicity and correlation to drug induced neutropenia [17,18]. However, in many cases the administration of drugs initially causes thrombocytopenia while regular use produces total aplasia [19]. This prediction model shows a relation between *in vitro* CFU-GM inhibition and *in vivo* absolute neutrophil count (ANC) nadir [4,8,20].

An *in vivo* and *in vitro* study of 9-methoxy pyrazoloacridine in murine, canine and human bone marrow cells predicted the MTD in animal models and humans [17]. The CFU-GM assay has been used by many researchers to evaluate *in vitro* hematotoxicity. Potassium oxonate (Oxo) has the potential to reduce 5-fluorouracil (5-FU) induced myelotoxicity in human CFU-GM but a greater degree in the mouse CFU-GM assay [21]. *In vivo* data using rat showed that human G-CSF increases the peripheral neutrophil count [22] while *in vitro* studies showed similar effect to murine G-CSF in CFU-GM *in vitro* assay, indicating that murine CFU-G induced by human G-CSF can be used for the evaluation of drug induced hematotoxicity [23]. The

MTD prediction model using *in vitro* CFU-GM assay is considered scientifically validated with a large number of anti-neoplastics, myelosuppressive xenobiotics [6,24]. It is reported that CFU-GM assay is the most suitable method and informative progenitor for *in vitro* hematotoxicity studies as compared to other myeloid progenitor CFU-G, CFU-GEMM and HPP-CFC with different alkylating drugs – mechlorethamine, busulfan, melphalan, carmustine and lomustine [25]. Many new alternatives to the CFU-GM clonogenic assay have been developed to assess the hematotoxic potential of new drugs. Malerba *et al.* developed an advanced system – high throughput *in vitro* method using 96 well plate for evaluating the toxic effects of drugs and xenobiotics on the proliferation of BFU-E and CFU-GM by studying three anticancerous drugs – cyclophosphamide (CTX), 5-FU and taxol (TAX) which is helpful and reliable tool in early stages of drug development [26]. An alternative to CFU-GM assay a new *in vitro* time saving experiment has been set up with culture in liquid medium and flow cytometric analysis of bone marrow cells which enable to speed up the procedure, reduce inter experimental variability and enhance result accuracy [27]. Though, the results of the experiment with AZT do not support the validity of this protocol, it can be a successful approach for full validation process. The CFU-GM assay is an important key indicator of toxicity and can be involved in multiple assay system [28]. A research team refined the CFU-Mk assay that can be used for *in vitro* toxicology studies with cord blood cells and bone marrow cells. They used collagen based clonogenic assay to examine the sensitivity of mononuclear human cord blood cells (CBC), mononuclear human bone marrow cells (BMC), cord blood enriched CD34+ CD38+ cells and bone marrow enriched CD34+ CD38+ cells, to test the toxicity of drugs that causes thrombocytopenia [29]. A recent study investigated the correlation between birth-weight and stem cell burden as a risk factor for carcinogenesis in adulthood by using CFU-GM culture to measure the proliferative capacity of CD34+ stem cells [30].

Gianni and his colleagues for the first time refined CFU-GM assay by introducing liver microsomes and co-factors as inclusions of a metabolic system. Nevertheless, it is not significant further refinement of the experimental protocol is needed to obtain better data. The  $IC_{50}$ ,  $IC_{70}$  and  $IC_{90}$  value of CFU-GM progenitors can be better correlated with human  $LC_{50}$  values than the sensitivity of HL-60 cells, more accurately predicting the human acute systemic toxicity of the hematopoietic myeloid system [31]. The sensitivity of the CFU-GM progenitors exposed to drugs is quite similar by using either h-BMCs or h-CBCs in human model [32]. The CFU-GM assays using cryopreserved rat bone marrow cells (r-BMCs)

is a useful technique for testing *in vitro* hematotoxicity or relative toxicity of different compounds [33]. The h-CFU-Mk progenitors are more sensitive to PNU-159548 [(4-demethoxy-3'-deamino-3'-aziridinyl -4'-methylsulphonyl-daunorubicin), a dose limiting myelosuppressive agent] than m-CFU-GM and m-CFU-Mk [34]. Giovanni and his colleagues designed an automated scoring procedure, an algorithm that scored CFU-GM colonies by data fusion from a 3D colony model applied to image stack, acquired by a digital camera [35]. Aplidine, a cyclic depsipeptide is less toxic to human hematopoietic progenitors than tumor cell types. However, CFU-GM and BFU-E progenitors from cord blood cells are less sensitive than isolated from bone marrow of humans [36]. Gender dependent naphthalene toxicity in animal models was reported but *in vitro* it does not affect CFU-GM proliferation while its metabolites 1-naphthol, 2-naphthol and particularly 1,4-naphthoquinone strongly inhibit CFU-GM [37]. The relationship between the plasma concentration ( $C_{max}$ ) and *in vitro* inhibitory concentrations ( $IC_{10}$ ,  $IC_{50}$ , and  $IC_{90}$ ) can detect the drug induced thrombocytopenia [15]. An advanced technique – automated non-clonogenic fluorometric microculture cytotoxicity assays – FMCA-GM7 and FMCA-GM14 offers a simple and robust alternative method to CFU-GM assay in preclinical *in vitro* hematotoxicity [38].

#### LIMITATIONS

While these *in vitro* clonogenic assays have advantage in the development of new drugs, there are also certain limitations. In CFU-GM assay accurate number of colonies cannot be predicted either due to death of progenitor cells or suppressed proliferation. Firstly, a minimum of 50 cells is considered as a colony. So, the dishes containing the same number of colonies having different number of cells have same value. Secondly, a particular different morphology of colony is not counted. In addition, CFU-GM clonogenic assays requires 7 days and 14 days of incubation for murine and human cells, respectively, which is a weak point in pharmaceutical industries where fast *in vitro* test is required for a large number of new chemical compounds. Moreover, drugs that undergo metabolism require metabolites to be added in culture otherwise cannot be studied easily [25]. In few years new experimental proposals have been proposed [26,35,39] but further refinement of the procedure is needed that reduce variability and produces better data.

#### DISCUSSION

*In vivo* myelotoxicity of drugs is studied in animal models such as rat, dog or monkey. *In vitro* also there are some tools for testing myelotoxicity but bone marrow is the most convenient [21]. Rat is the

most important animal for *in vivo* study. Thus, *in vitro* CFU-GM assay using rat is useful for evaluation of hematotoxicity of newly synthesized compounds [25]. The cytotoxic drugs can be acute or subacute myelotoxic [40]. Acute toxic drugs are much more toxic to mature blood precursor cells [41,42]. Comparison of *in vivo* and *in vitro* toxicity data helps in the selection of animal model for toxicological studies [43]. Therefore, *in vitro* CFU-GM assay opens new criteria for developing validated hematotoxicology tests to study the effect of toxicants on human and murine hematopoietic progenitor cells which reduces the animal use [5].

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