WHITE PAPER

The CFU Assay in Preclinical Toxicity Testing: An In Vitro Tool for Predicting In Vivo Cytopenia

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Abstract

Toxicity is a major cause of attrition in therapeutic drug development and is a key factor in decision-making around the advancement of candidate drugs through the development pipeline. The expansion of preclinical testing to incorporate assays that better predict potential toxicities earlier in the drug development process has obvious advantages for the selection of successful lead candidates. In vitro testing on primary cells can allow investigators to preview in vivo responses, thus facilitating design of better dosing strategies and optimization of animal and Phase I clinical studies. Primary hematopoietic stem and progenitor cells are routinely utilized in the colony-forming unit (CFU) assay to predict in vivo marrow toxicity and clinical cytopenia. This review summarizes the application of the CFU assay for predicting hematopoietic toxicity and summarizes some key studies that illustrate its utility in this setting.

Introduction

During the standard lead optimization, the drug development process shifts from the utilization of high throughput to lower throughput, more clinically predictive screening assays. Testing for undesirable effects, both in vitro and in vivo, at the earlier stages of drug development helps to identify compounds with unacceptable levels of toxicity. A major challenge in the advancement of candidate drugs from preclinical development into Phase I trials is converting in vivo toxicology results drawn from relevant animal models into meaningful data to predict the response in humans.[1,7]

The colony-forming unit (CFU) assay is a predictive test that uses stem and progenitor cells from humans and traditional model organisms such as mice, rats and dogs. This in vitro assay offers a tool to investigate potential hematological disturbances seen in animal models and to design dosing regimens for human studies. The use of primary human cells that exhibit in vivo functionalities in culture allows the collection of information that has a greater likelihood of being clinically relevant.[2] By utilizing progenitor cells of the blood-forming (hematopoietic) system, the CFU assay can be used to investigate the toxic effects of small molecule compounds and biologics on specific subpopulations of blood cells. This assay can be used to accurately predict drug doses that may induce anemia, neutropenia and/or thrombocytopenia in animal models and in humans.

The CFU Assay in Toxicology

The CFU assay was first developed in the 1960's to identify and quantitate primitive cells at different stages of the hematopoietic cell hierarchy.[3] Primary hematopoietic progenitor cells obtained from bone marrow, peripheral blood or cord blood are cultured in a semi-solid medium that restricts their movement, and which supports proliferation and differentiation in situ. Individual clonogenic cells form distinct colonies that can be counted and "scored" on the basis of the type (i.e. lineage) of the cells they contain. In this way, the CFU assay is both quantitative and qualitative in nature.

Drugs of interest are tested in this system by addition to the culture medium at a range of concentrations to determine effects on the in vitro proliferation and differentiation of hematopoietic progenitor cells. By generating a dose-response curve of the total number of colonies as a function of drug concentration (normalized to solvent controls), it is possible to determine in vitro inhibitory concentrations, expressed as IC_{50} or IC_{90} values. In addition, changes seen in colony morphology (e.g. size and cellular composition) can be used to predict lineage-specific effects of the drug under investigation. Finally, IC values can be correlated to in vivo measures of toxicity (e.g., maximum tolerated doses, C_{max} in serum, etc.), to provide critical information for establishing effective dosing strategies both for animal and clinical studies.[2,4]

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Using the CFU Assay to Predict Cytopenia

Cytopenia is defined as a reduced number of blood cells in the peripheral blood. Different types of cytopenias are distinguished by the type of mature blood cell affected (Table 1).

CYTOPENIA	DEFINITION
Neutropenia	Decrease in the number of neutrophilic granulocytes
Thrombocytopenia	Decrease in the number of megakaryocytes and platelets
Anemia	Decrease in the number of erythrocytes, reduced hematocrit or hemoglobin levels.

TABLE 1. Cytopenias that result from disturbances of the hematopoietic system.

Many drug candidates being developed for oncology, antiviral and other therapeutic indications specifically target rapidly dividing cells, including normal hematopoietic progenitor cells, and consequently induce cytopenia. By investigating the acute effects of agents on progenitor cells of the granulocyte-macrophage (CFU-GM), erythroid (BFU-E, CFU-E), and megakaryocyte (CFU-Mk) lineages in vitro, it is often possible to predict dose-limiting hematotoxicity that may lead to cytopenia in vivo.

The CFU Assay as an In Vitro Predictive Tool

In selecting an appropriate in vitro assay to assist in the prediction of in vivo drug toxicity, several assay attributes should be considered:[5]

1. Cells of interest should behave reproducibly and ideally be derived from a relevant tissue source to produce clinically relevant results.
2. The assay and culture conditions should be standardized and employ validated components to ensure high reproducibility .
3. The assay design should allow experimental results to be compared against benchmark compounds of known toxicity.
4. The predictive ability of the assay must be demonstrated through a reliable correlation with a specific clinical endpoint(s).

The CFU assay can be performed with primary hematopoietic cells from human tissues as well as from other species commonly used in toxicity testing. All of these different cell types exhibit well-defined and reproducible proliferation and differentiation potentials in vitro. The use of qualified media components, extensively screened and validated by STEMCELL Technologies, further ensures the reproducibility of results that can then be compared against benchmark compounds that have been selected on the basis of their recognized hematotoxicity. [2,4]

The CFU assay has been shown in several studies to be a reliable in vitro predictive tool for clinical cytopenia. Both the CFU-GM assay and CFU-Mk assay have been independently validated by the European Centre for the Validation of Alternative Methods (ECVAM) for the prediction of drug-induced neutropenia[2] and predicting the potential for thrombocytotoxicity.[4,8]

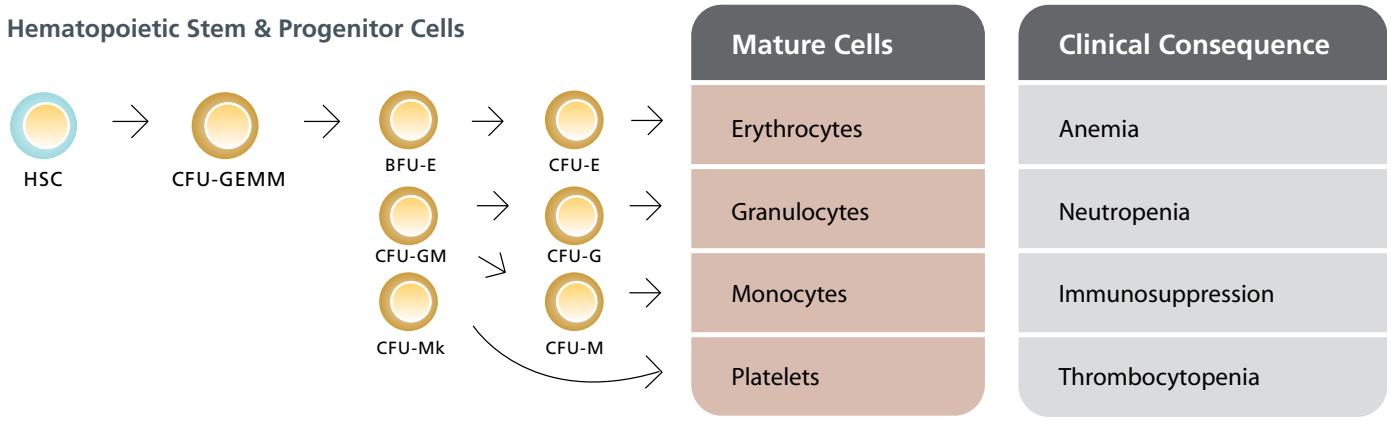

FIGURE 1. Progenitor populations that can be identified using the CFU assay and the clinical consequence of disturbing specific lineages of the hematopoietic compartment.

Effects on CFU-GM as an In Vitro Model of Clinical Neutropenia

In 2003, Pessina et al. (2) published a study validating the CFU-GM assay for predicting the maximum tolerated dose (MTD) in humans by adjusting a mouse-derived MTD for the differential sensitivity between these species. This study demonstrated a high predictivity (87%) of the murine CFU-GM assay.

DRUG	IC_{90} RATIOS (HUMAN/MURINE)	ACTUAL MURINE LD10 (mg/m^2/dose)	PREDICTED HUMAN MTD (mg/m^2/dose)	ACTUAL HUMAN MTD (mg/m^2/dose)	SUCCESSFUL PREDICTION?
5-Flurouracil	6.0	66.0	394.0	740.0	Yes
Etoposide	1.0	23.1	21.1	54.0	Yes
Taxol	1.2	69.6	82.8	40.0	Yes

TABLE 2. Prediction of maximum tolerated dose (MTD) in humans for drugs with determinable IC_{90} values.

"This validation study, performed with a panel of 20 drugs, confirmed that the SOP developed for assaying human and murine CFU-GM generates reproducible IC_{90} values that can be applied to our model for predicting acute systemic doses that will cause severe, reversible neutropenia in treated patients (marrow maximum tolerated dose)."

Effects on CFU-Mk as an In Vitro Model of Clinical Thrombocytopenia

In 2008, Pessina et al.[4] demonstrated that a relationship can be established between the maximal concentration of drug in plasma (C_{max}) and the IC_{10}/IC_{50}/IC_{90} values determined in vitro, illustrating that it is possible to predict the direct toxic effect of a compound. Comparing the C_{max} and the IC values generated in the CFU-Mk assay showed that it was possible to accurately predict the induction of thrombocytopenia due to bone marrow damage. When the C_{max} exceeds the IC_{90} and/or IC_{50} values, thrombocytopenia can occur due to direct toxicity of the compound on megakaryocytic progenitor cells.

DRUG	C_{MAX} (µg/mL)	IC VALUES (µg/mL)		
		IC_{90}	IC_{50}	IC_{10}
5-Fluororacil	50–300	8.6	2.7	0.6
Busulfan	0.8	4.0	1.3	0.3
Warfarin	11.2	620.6	198.0	43.7

TABLE 3. Comparison between the C_{max} and IC values for benchmark drugs.

DRUG	RISK OF THROMBO-CYTOPENIA	C_{max} VS IC VALUES
5-Fluorouracil	High (level 3)	$C_{max} > IC_{90}$
Busulfan	Low (level 1)	$IC_{50} > C_{max} > IC_{10}$
Warfarin	None (level 0)	$C_{max} < IC_{10}$

TABLE 4. Suggested prediction model based on comparison between the C_{max} and IC values.

The CFU Assay in Preclinical Toxicity Testing: An In Vitro Tool for Predicting In Vivo Cytopenia

The authors of the above studies have concluded that mathematical modeling of IC values derived from CFU assays can be:

1. Used to refine in vivo dosing regimens, thereby reducing the number of animals required for preclinical toxicology studies and informing the establishment of appropriate starting doses for phase I clinical trials.
2. Considered as a validated in vitro model for neutropenia and thrombocytopenia that can be utilized to address a number of preclinical drug development considerations.[2]

The CFU assay can provide accurate answers to questions that are crucial to the development of effective drug dosing strategies:

- Is the compound toxic to the bone marrow in patients (e.g., does it induce cytopenia)?
- If so, at which dose is toxicity seen and to what extent (e.g., what are the IC90, IC50 values)?
- Is the drug toxic only to specific lineages of blood cells (e.g. does it cause neutropenia, thrombocytopenia and/or anemia)?

Retrospective Investigation of Drug-Induced Cytopenia

CFU assays can also be utilized as a tool for dissecting the cause of unexpected hematological disturbances seen in animal models or clinical studies following treatment. If a deleterious effect on hematopoiesis is observed in vivo, the CFU assay can be employed to help identify whether the drug is exerting its effects directly on hematopoietic progenitor cells, or if the observed effects is occurring as a result of off-target or other ADME-related parameters.[6,9] For example, if a drug has no effect on colony numbers or their composition in the CFU assay, this suggests that its toxicity on the hematopoietic system are manifested indirectly, e.g. by inducing the release of inhibitory cytokines, inhibiting the release of stimulatory cytokines, or processing in vivo that produces metabolites with these or other effects.

Summary

The CFU assay has been validated as an in vitro tool to measure hematopoietic toxicity of candidate therapeutics at all stages of preclinical drug development. The use of primary hematopoietic stem and progenitor cells allows the CFU assay to predict in vivo responses with a high level of accuracy. This improves the ability to successfully balance drug efficacy against the likelihood of bone marrow suppression and subsequent cytopenic effects. The inhibitory concentrations of a drug measured in vitro can be correlated to in vivo measures of toxicity. This allows investigators to refine dosing and reduce the number of animals used for in vivo modeling, as well as to design more effective dosing regimens prior to human trials to ensure that fewer patients will be exposed to sub-therapeutic levels of the drug.

Several groups are currently investigating the effects of different classes of reference compounds with known in vivo profiles, both positive (stimulatory) and negative (inhibitory), on various developmental stages and lineages of hematopoietic cells. STEMCELL Technologies' Contract Assay Services performs customizable CFU assays to evaluate the hematotoxic effects of drug candidates in vitro. By identifying the potential for in vivo toxicity early, this assay helps to minimize the likelihood of late-stage drug failure and allows drug development resources to be allocated toward advancing those candidates with the best chances for success.

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