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Stem Cell Predictive Hemotoxicology

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1. Introduction

Stem cells, those elusive entities that have the capacity for producing, maintaining and reconstituting the integrity of a biological system, also demonstrate the potential to predict partial or life-threatening damage in response to drugs, environmental compounds and other agents. It is ironic however, that in the animal or human, prior to the manifestation of such potential biological damage most, if not all of the stem cells might have been eradicated. To predict possible damage, surrogate *in vitro* stem cell assays have been developed that utilize specific properties and characteristics that divulge, through a measured response, how the system will react to different agents.

In vitro assays that detect toxicity to stem cells of a biological system allow potentially life-threatening damage to be predicted prior to human clinical trials taking place and environmental agents from causing harm. Discussions about stem cells usually focuses not on primary cells, but rather on embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, their ability to produce virtually any type of functional cell and their use in cellular therapy and regenerative medicine. The ES and iPS types of stem cells are, in fact, the least understood of all stem cell types. For many companies investigating or considering using these stem cells routinely as surrogate *in vitro* models for toxicity testing many questions remain, including (1) what is the relevance of these cells, (2) how do they compare with primary stem cell populations, and (3) can they be validated? In many cases, it is not the stem cells themselves, but rather the cells derived from ES and iPS cells that are of interest. Several companies already produce ES- or iPS-derived cardiomyocytes, hepatocytes, neural cells and many other cell types not only for toxicity testing, but also for basic research, cellular therapy and regenerative medicine.

How can stem cells be used to predict toxicity? The answer to this question lies in the characteristics and properties of stem cells and how they respond to different situations.

To understand this better, stem cell systems can be divided into "definitive" and "non-definitive" systems as illustrated in Fig. 1. Definitive stem cell systems are responsible for maintaining a specific biological system. They can be divided into continuously proliferating systems such as the blood-forming or lympho-hematopoietic system, the gastrointestinal system, hair and skin, reproductive organs and cells of the eye cornea. Although not necessarily a continuously proliferating system, the mesenchymal stem cell (MSC), also called the multipotent mesenchymal stromal cell [1] system can been included, because in culture, the MSCs proliferate and can be passaged over a long period of time. Definitive stem cells systems can also demonstrate partial proliferation. These include, but are not limited to, the liver, lung, kidney, heart, pancreas, and the neural/neuronal system. From a toxicological viewpoint, these are not usually considered stem cell systems. Yet, the different types of lineage cells present in these organs and the ability to maintain a specific cell mass has all the intricacies of a stem cell system, especially during development, even though the cell turnover in the adult may be very low. Non-definitive stem cells systems are represented by the ES and iPS cell systems, which can, theoretically, give rise to any of the definitive stem cell systems. Indeed, it is a prerequisite that the production of functionally, mature cells from ES or iPS cells first pass through a definitive stem cell compartment.

2. Stem cell characteristics and properties used for toxicity testing

Stem cells of primary, definitive systems always represent a very small proportion of the tissue or organ cellularity. This proportion is between 0.1 and 0.01% or less. The basic definition of a stem cell is that it possesses the capacity for self-renewal. In fact, stem cell systems are usually termed self-renewal cell systems, meaning that one stem cell can produce two daughter cells that are exact replicas of the parent. However, self-renewal is a difficult property to measure. The capacity for either serial *in vivo* repopulation or *in vitro* serial re-plating is considered a property of stem cells that implicates not only the presence of stem cells, but also their self-renewal capability. The fact that serial *in vitro* re-plating or *in vivo* repopulation cannot be performed ad infinitum is not only an indication for a stem cell hierarchy [2-7], but also for an alternative hypothesis to stem cell self-renewal. This hypothesis states that tissues and organs are endowed with a specific number of stem cells. Once used up, the system ceases to function [8]. Regardless of the hypothesis, this important property can be utilized in a toxicological setting by employing secondary re-plating technology. This allows not only the presence of residual stem cells to be detected that have not been affected by a compound, but any change in sensitivity to a compound that might be important during repeated dose administration.

Stem cells have two other important properties that can be applied to toxicity testing. The first is that they are undifferentiated. The second is that stem cells proliferate. Stem cells can be "determined" into one or more lineages of mature functional cells. When a stem cell becomes determined, it ceases to be a stem cell and becomes a progenitor cell that proliferates and differentiates. The fact that stem cells can be induced to differentiate means that what-

ever happens at the stem cell level will ultimately affect all downstream events. These characteristics enable stem cells to be the most important predictors of potential toxicity.
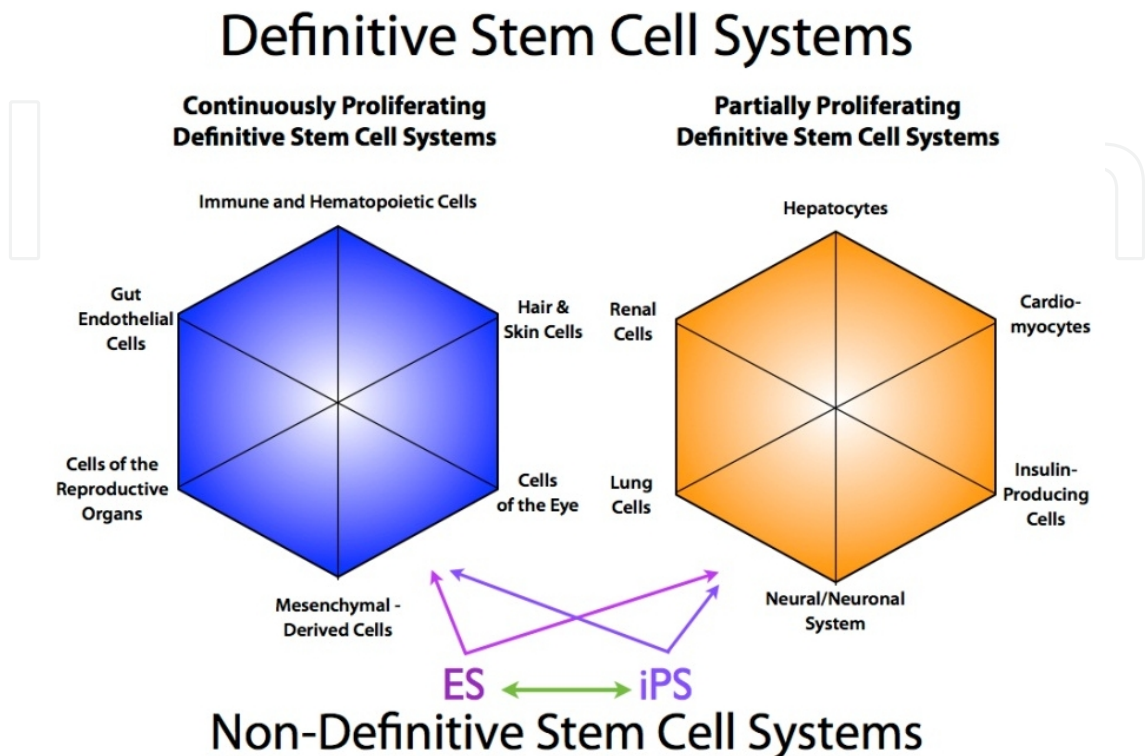

Figure 1. Definitive and Non-Definitive Stem Cell Systems. Definitive stem cell systems can be further divided into continuously and partially proliferating systems. Non-definitive stem cell systems such as embryonic stem cells and induced pluripotent stem cells can produce definitive stem cell systems, which in turn, give rise to mature functional cells.

All definitive stem cell systems have a common organization shown in Fig. 2. There is a continuum of stem cells within the stem cell compartment that exhibit different degrees of primitiveness or "stemness", which in turn, implies changing proliferating potential or potency as a stem cell moves through the compartment to the point of determination. These characteristic properties actually provide the information that allows stem cells to be predictors of potential toxicity. Once a stem cell becomes a progenitor cell, proliferation continues and actually increases for a certain time so that the compartment can be amplified, until it ceases completely and the differentiation and maturation processes takes over. These changes have important implications for the types of assays that can be used *in vitro* to detect potential toxicity.

From Fig. 2, it is clear that proliferation occurs prior to differentiation. Although there is considerable overlap between proliferation and differentiation, they are two separate processes that cannot be measured using the same assay readout. Since stem cells only proliferate, it follows that a proliferation assay is required to detect the presence and response of stem cells to a compound or agent. Using a differentiation assay to detect the effect of a compound or agent that targets one or more steps in the proliferation process can influence the interpretation

and conclusion of the results. This can have far-reaching consequences on the decision to move forward with the development of a new drug candidate.
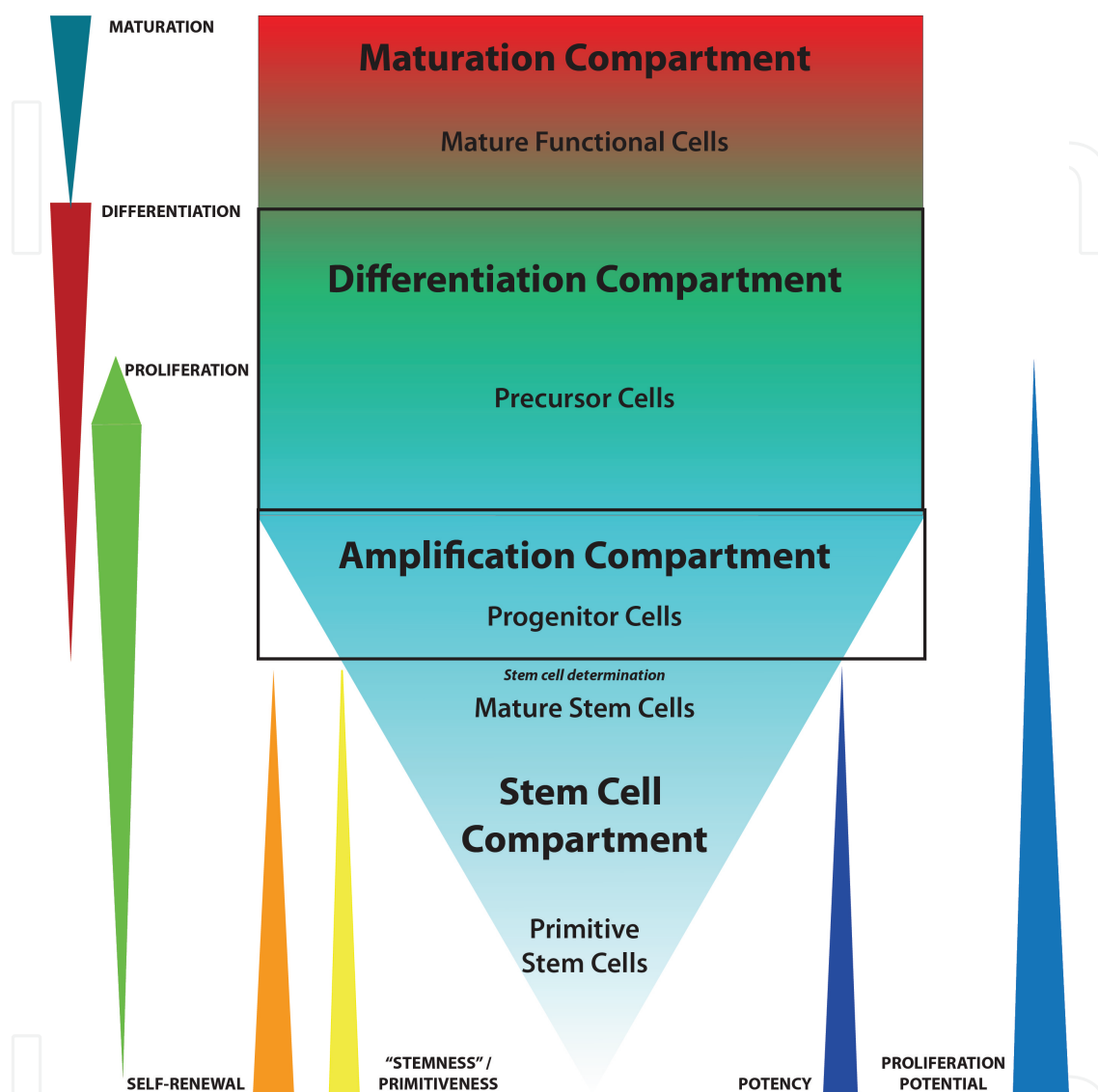

Figure 2. The Common Organization of Definitive Stem Cell Systems

Toxicity represents between 30-40% of the drug attrition rate [9,10]. It is therefore not surprising that biopharmaceutical companies are eager to employ assays that allow early prediction of toxicity prior to starting human clinical trials. Once the drug discovery phase has been concluded, the drug development phase begins (Fig. 3) by screening thousands of compounds in a battery of tests to determine absorption, distribution, metabolism and excretion (ADME) as well as preliminary toxicity (ADME/Tox). Many of the ADME/Tox assays as well as those in the lead optimization phase use transformed cell lines as cell targets, such as the NCI60 cell line panel [11,12]. Once these tests have whittled down the number of possible drug candidates, pre-clinical animal models are used. Neither cell lines (even if they are

of human origin) nor animal testing provide good extrapolation to the human situation. It is not uncommon for unexpected results or toxicity to rear its head during animal studies because of the lack of predictive information obtained during previous screening and testing [13]. Many published articles have dealt with this problem, one of the most notable being the monograph on Toxicity Testing in the 21st Century [14]. Despite the goals of the drug development pipeline and the considerable effort being undertaken by regulatory agencies [15-20] to determine the effect of environmental agents on human cells, interpretation and conclusions often fall short due to lack of understanding of the mechanism of action of the molecule, incorrect assay readout and/or incorrect target cell, to name but a few reasons. If the goal is to determine the effect on human cells, then mouse, rat, dog or even non-human primate cells will not provide the required information; human cells must be used. It goes without saying that drug development or testing xenobiotic agents cannot be performed on human subjects. It is for this reason why surrogate *in vitro* assays using primary human cells obtained from donors under the auspices of regulatory controlled internal review boards (IRBs) provide the best alternative. However, even under these circumstances, detailed knowledge of the biological system under study is necessary in order to interpret and make conclusions in the most objective manner.

Of all the biological systems of the body, the one most studied is also one of the systems that is given the least priority with respect to toxicity. The blood-forming or hematopoietic system and the gastrointestinal system are two continuously proliferating systems that are expected to be dramatically affected by anti-proliferating agents such as anti-cancer drugs. As a result, the only relevant questions are (a) how severe would toxicity be, and (b) would use of the drug provide a favorable therapeutic index?
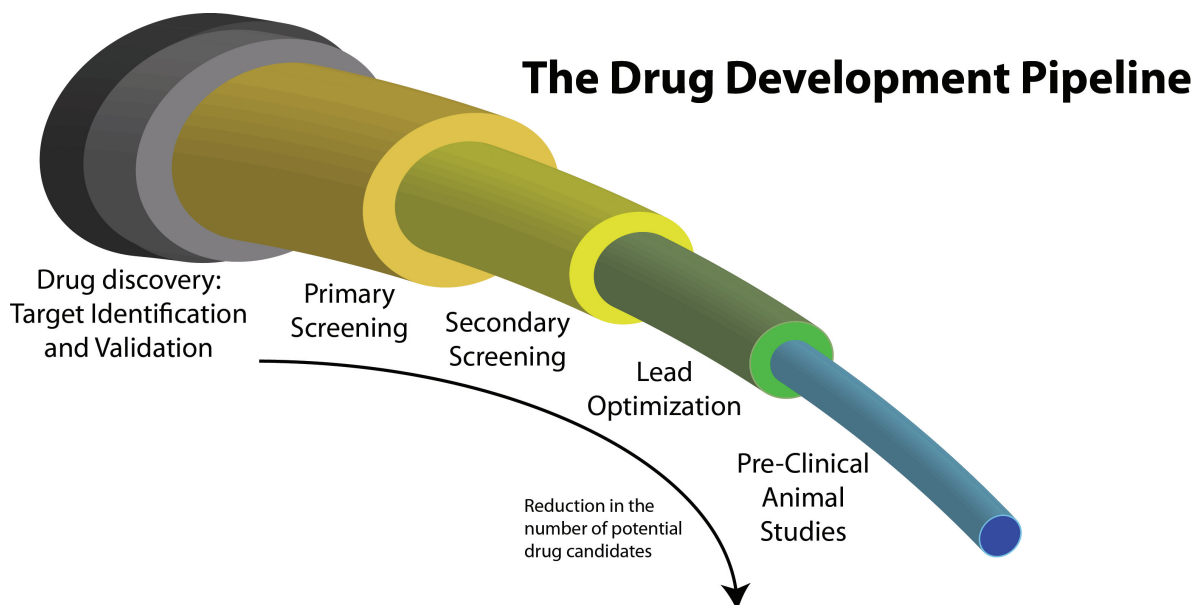

The Drug Development Pipeline

Drug discovery: Target Identification and Validation

Primary Screening

Secondary Screening

Lead Optimization

Pre-Clinical Animal Studies

Reduction in the number of potential drug candidates

Figure 3. The Major Stages of the Drug Development Pipeline

Hemotoxicity testing is traditionally performed during the last stage of drug development, namely pre-clinical animal testing. Circulating blood parameters are measured and at necropsy, bone marrow, spleen and even liver hematopathology are performed. Primary stem and progenitor cells cannot be morphologically identified. Morphological identification of cells is only possible once the cells start to differentiate and mature. Consequently, traditional hemotoxicity testing provides little, if any, predictive value since most of the toxic effects have occurred on more primitive cells.

Much of our knowledge about the characterization, properties and responses of hematopoietic stem cells and the system as a whole has been provided through the use of drugs and other agents (e.g. radiation) using both *in vivo* and later, *in vitro* assays. The information obtained has allowed the organization and hierarchy within the different compartments of the hematopoietic and lymphopoietic systems to be elucidated. By utilizing the knowledge that has accrued over more than six decades, analysis of the lympho-hematopoietic stem and progenitor cells provide the highest degree of predictive toxicity of any biological system.

3. The Colony-Forming Cell (CFC) assay and ECVAM studies

In 1966, Bradley and Metcalf in Melbourne, Australia [21] and Pluznik and Sachs in Rehovot, Israel, [22] independently published what is now known as the colony-forming unit (CFU) or colony-forming cell (CFC) assay. In its original form, mouse bone marrow target cells were suspended in agar containing a conditioned medium that we now know contained granulocyte-macrophage colony stimulating factor or GM-CSF as well as other soluble factors. In the semi-solid medium, the cells underwent proliferation and later differentiation to produce colonies of cells that were identified either as neutrophils, macrophages or a combination of the two cell types. The number of colonies counted under an inverted microscope was proportional to both the number of cells plated and the dose of the conditioned medium added. In the same year, Cole and Paul [23] in Glasgow, Scotland reported the first *in vitro* suspension culture of murine erythropoietic cells from the yolk sac and fetal liver. Culture of erythropoietic cells under clonal conditions did not occur until 1971, when the Axelrad group [24] in Toronto, Canada, demonstrated that erythroid colonies could be produced using a plasma clot technique. In 1974, Iscove and colleagues [25] in Basel, Switzerland introduced the methylcellulose CFC assay that is still used today. Since that time, colony assays have been developed to detect multiple cell populations of every blood cell lineage, including several different stem cell populations. In addition, conditioned media has been replaced with recombinant growth factors and cytokines.

In Section 2, emphasis was placed on the importance between proliferation and differentiation. The cell populations detected using the CFC assay must all be proliferating populations, otherwise the production of colonies would not occur. However, to identify the type of colony, the *in vitro* culture must be allowed to proceed long enough so that the cells produced can themselves be identified as being derived from a morphologically unidentifiable stem, progenitor or precursor cell, all of which are capable of proliferation, but to different extents.

As mentioned above, although proliferation is required to produce colonies, the number of cells produced as a quantitative measure of proliferation cannot be ascertained. Although proliferation is assumed, the CFC assay actually detects differentiation ability or potential. This has important consequences for toxicity testing.

Over several years, the European Center for the Validation of Alternative Methods (ECVAM) undertook a series of studies in which a number of drugs and chemicals were tested using the CFC assay. These studies are noteworthy because they represented the first attempt to validate a prediction model for assessing the maximum tolerated dose (MTD, equivalent to the IC90 value) for drugs that induce neutropenia [26,27] using the CFC assay. The studies were performed in different laboratories and were later extended to compounds that caused thrombocytopenia [28]. Potential neutropenia was detected by the effect on the granulocyte-macrophage colony-forming cell or GM-CFC (also called CFC or CFC-GM), while thrombo-cytopenia was detected by the effect on the megakaryocyte colony-forming cell or Mk-CFC (also called CFC-Mk). A decrease or inhibition in the number of colonies counted derived from GM-CFC or Mk-CFC predicted a reduction in neutrophils or platelets in the circulation. The authors demonstrated that the model could correctly predict the MTD of 20 out of 23 drugs tested (87% predictive rate).

There are two points worth emphasizing. First, not all compounds will produce an estimated IC90 value and may not even produce an IC50 value, when tested using the CFC assay. Does that mean that these compounds will not produce neutropenia or thrombocytopenia? It is interesting to note that the same CFC assay that is used to predict toxicity causing neutropenia or thrombocytopenia, is also used in an opposite manner to predict time to neutrophil or platelet engraftment after bone marrow, mobilized peripheral blood or umbilical cord blood stem cell transplantation for cellular therapy [39-31]. In either case, the GM-CFC or MK-CFC populations provide no information on the response of the more sensitive and more important stem cells. After all, it is the hematopoietic stem cells that give rise to both of these populations. This leads to the second point, namely that many compounds target one or more steps in the proliferation process, either at a molecular and/or cellular level. Although both GM-CFC and Mk-CFC populations are proliferating progenitor cell populations, they are not always the primary targets. When a compound affects more than one lineage, the primary effect is not on those lineages individually, but on the common cell that gives rise to those lineages, namely the stem cells [32]. From a practical viewpoint, however, the CFC assay posses daunting problems. The ECVAM studies summarized previously were exceptional in that the authors took the trouble to try and verify and standardize the readout of the assay that is inherently subjective and lacks the necessary external standards and controls by which the assay could be properly validated. In studies performed by the National Marrow Donor Program (NMDP), the results showed very high variability in CFU colony counting for cord blood [33]. This high variability, primarily due to the inaccuracy of dispensing methylcellulose and colony counting, together with the lack of high throughput capability does not provide the biopharmaceutical industry, environmental agencies or other areas of toxicology, risk or efficacy assessment with a routine and trustworthy assay platform. To negate all of these problems, the HALO Predic-tive Hemotoxicity Platform was developed.

4. Predictive stem cell hemotoxicity testing

Whereas the CFU assay may be used to predict neutropenia, thrombocytopenia, anemia and the MTD indicated by the IC90 values [27-29], stem cells assays allow potential hemotoxicity to be taken to a different system-wide "global" level. The reason is provided in Fig. 2 and in more detail in Fig. 4, which shows the different lympho-hematopoietic cell populations that can be detected using a hemotoxicity screening and testing platform specifically developed for this purpose. This platform, called HALO, will be described in more detail in the next section. Figures 2 and 4 demonstrate that functionally mature cells from definitive continuously proliferating and partially proliferating cell systems, are derived from stem cells. As such, any perturbation or damage to the stem cell compartment will ultimately affect all downstream cell populations. In other words, examining the effect on stem cells allows the "global" effect on the system to be predicted. Since more is known about the organization, hierarchy and regulation of the lympho-hematopoietic system than probably any other biological system in the body, this knowledge can be used to predict and explain potentially deleterious effects to the system. Changes in the response to hematopoietic stem cells will affect all three primary hematopoietic lineages, namely the erythropoietic, myelomonocytic and magakaryopoietic lineages. Changes in the response to lympho-hematopoietic stem cells, i.e. those stem cells that can give rise to both the lymphopoietic and hematopoietic cells, will be expected to affect most, if not all cell lineages, including the T- and B-cell lineages and therefore the immune system as a whole.

Predictive stem cell hemotoxicity testing is not simply the estimation of IC values so that compounds can be ranked in order of toxicity to different cell populations or species. There are several other important applications in which stem cell hemotoxicity, and indeed stem cell toxicity in general, can be used. Examples of these applications will be discussed later in this chapter. First, however, it is necessary to describe the principles, characteristics and properties of the assay that make this possible.

5. Materials and methods

HALO is the acronym for Hematopoietic/Hemotoxicity Assays via Luminescence Output. This platform was originally designed and developed to provide the biopharmaceutical industry with a high throughput, validated assay to examine the effects of virtually any compound on different cell populations of the lympho-hematopoietic system from multiple species. Initially, the assay platform was developed for fresh, primary human cells, as a surrogate assay that could be used at virtually at stage in the drug development pipeline (Fig. 3) to extrapolate to the human situation, and as an alternative to pre-clinical animal studies. The platform has since been further developed to include non-human primate, horse, pig, sheep, dog, rat and mouse, not only for toxicity studies, but also for basic research and veterinary applications.
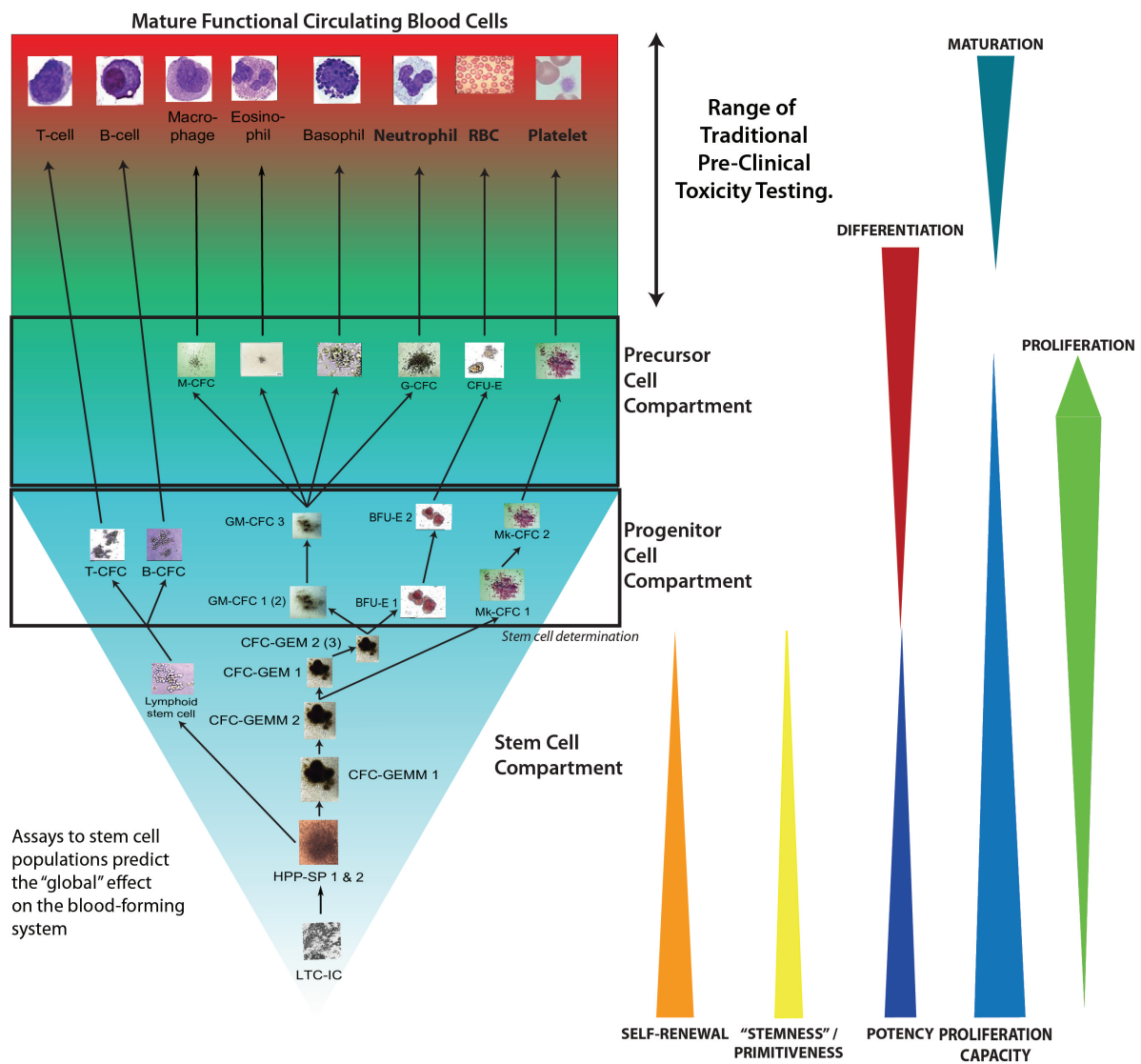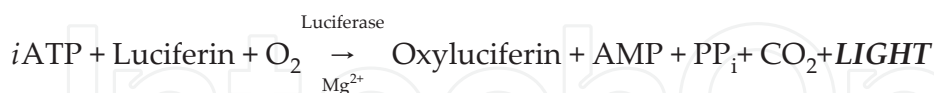
Figure 4. The Organization and Hierarchy of the Lympho-Hematopoietic System as a Model for a Definitive Continuously Proliferating Stem Cell System. The properties of stem cells play an integral part in predicting toxicity.

5.1. Concepts and principles of the HALO platform

When cells proliferate or are inhibited from proliferation by drugs or other agents, the concentration of intracellular adenosine triphosphate (iATP) changes proportionately. This biochemical marker is an indicator of cellular and mitochondrial integrity and therefore viability of the cells. Indeed, iATP is used as a metabolic viability assay (as opposed to a dye exclusion viability assay). Under normal conditions, stimulation of cell proliferation requires specific growth factors and/or cytokines either alone or in combination (cocktails). For continuously proliferating systems, growth factors or cytokines need to be present continuously, albeit, in very small concentrations, in order to maintain cell survival and production. Thus, to detect the effect of any agent on hematopoietic cells *in vitro*, the target cell population must be stimulated in order to detect changes in the cell

population response to the agent. The agent is usually added in a dose-dependent manner to the target cells, which are then incubated for a specific period of time. Thereafter, the cultures are removed from the incubator and the cells lysed to release the iATP. The latter then becomes a limiting substrate for a luciferin-luciferase reaction to produce bioluminescence in the form of light as shown in the equation below.

$$i\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[\text{Mg}^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PP}_i + CO_2 + \textbf{\textit{LIGHT}}$$

The light is measured in a plate luminometer. The amount of light produced correlates directly with any change in the iATP concentration and therefore with the state of proliferation or inhibition of the cells.

5.2. Cell sources

Cells from any hematopoietic or lymphopoietic organ can be used. For most of the studies described here, fresh or cryopreserved human bone marrow or peripheral blood cells were collected with prior authorization by an Internal Review Board (IRB). Human cells were obtained from Lonza (Walkerville, MD) or Allcells (Berkely, CA). A mononuclear cell (MNC) fraction was prepared using density gradient centrifugation. A nucleated cell count was performed using a Z2 particle counter (Beckman Coulter), while dye exclusion viability was performed using 7-aminoactinoycin D (7-AAD) and flow cytometry. Metabolic viability was performed using LIVEGlo (HemoGenix, Colorado Springs, CO).

5.3. Cell culture

For all toxicity studies, MNC were diluted so that the final cell concentration was either 7,500 or 10,000 cells/well. Either 96-well or 384-well, solid white-wall plates were used and all dispensing was performed using a liquid handler (Beckman Coulter, EPICS XL-MCL). After the cell suspension was prepared, it was added to a Master Mix containing reagents including growth factors and/or cytokines to stimulate the target cell population being studied. Five different hematopoietic stem cell populations have so far been developed for this assay, the most important being the Colony-Forming Cell – Granulocyte, Erythroid, Macrophage, Megakaryocyte or CFC-GEMM (referred to in Fig. 4 as CFC-GEMM 1). This particular stem cell population is stimulated with erythropoietin (EPO), granulocyte-macrophage and granulocyte colony stimulating factors (GM-CSF, G-CSF), Interleukins 3 and 6 (IL-3, IL-6), stem cell factor (SCF), Flt3-Ligand (Flt3-L) and thrombopoietin (TPO). Compared to a "classic" CFC assay, HALO does not incorporate methylcellulose and is therefore not a clonal assay. Instead HALO uses Suspension Expansion Culture (SEC) Technology, which has several advantages over methylcellulose assays. First, SEC assays allow more accurate dispensing using liquid handlers. This is in contrast to inaccurately dispensing methylcellulose with syringes and needles. Second, the use of liquid handlers allows for true high throughput capability with accurate dispensing even in 384-well plates. Third, as opposed to methylcellulose, where little or no cell interaction occurs, SEC technology allows cells to interact with each other. This has two

important consequences. Cell interaction reduces the time for the onset of cell proliferation by approximately 24 hours. This means that measurement of cell proliferation can be measured within 5 to 7 days. Indeed, for all of the studies described here, human cells were incubated for 5 days. Non-human primate cells are usually incubated for the same time, but all other animal cells only require 4 days of incubation. The second consequence of allowing cell interaction to occur is the two-fold increase in assay sensitivity. As with most cell cultures, cells are incubated at 37ºC in a fully humidified atmosphere containing CO_2. Incubating cells under low oxygen tension of 5% O_2, which is approx. equivalent to the venous oxygen tension, reduces oxygen toxicity due to free radical production and improves plating efficiency [34,35] for all lympho-hematopoietic cell populations as well as other cell types.

5.4. Controls and dosing

Four basic controls were always included for toxicity studies. A background control included cells, but no growth factors. A vehicle control was similar to the background control, but included the vehicle used to dissolve the compound. Growth of the target cell population without any compound or vehicle constituted the growth factor control. A similar control that included the vehicle was designated the growth factor + vehicle control. Drugs and other agents were investigated over 6 – 9 doses.

5.5. Instrument calibration, assay standardization and sample processing

Prior to measuring any sample, the instrument was calibrated and the assay standardized using an external ATP standard and controls. The procedures have been described previously [32] and detailed procedures can also be obtained [36,37]. Calibration and standardization were also part of the assay validation process (see Section 5.6).

There are other advantages for calibrating and standardizing the assay. First results can be compared over time. Second, the output of a plate luminometer is in Relative Luminescence Units or RLU. The results are relative because different instruments demonstrate different ranges of RLU. These ranges may vary from 0 to 100 for one manufacturer or 0 to several million for another. This means that it would be very difficult to directly compare results within and between laboratories using RLU values. Performing an ATP standard curve allows all the results to be interpolated from RLU values into standardized ATP concentrations (µM).

5.6. Assay verification and validation

HALO was originally developed from the "classic" CFC assay because the latter was the only cell-based assay that could detect primitive hematopoietic cell populations. Since HALO is a proliferation assay, while the CFC detects differentiation of the same cells, and because proliferation occurs prior to differentiation, it follows that one assay can verify the other. Indeed, several publications have shown a direct correlation between the two assays [32,38,39].

Validation, on the other hand, is quite a different matter. Assay validation is defined as "establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes" [40]. When an assay is properly validated the accuracy (proportion of correct outcomes), sensitivity (proportion of correctly identified positive samples), selectivity (proportion of correctly identified negative samples), precision (intra and inter-laboratory variability) and robustness (the ability of the assay to withstand changes and transferability) all combine to give the user the assurance that the results obtained are correct. The ECVAM studies described in Section 2 above were, and still are, the closest the CFC assay has come to being validated. There have been many attempts to validate the CFC assay, but all have failed. Certainly the assay has shown, from a subjective viewpoint, some of the attributes. However, since there are no standards and controls by which the CFC assay can provide documented and quantitative evidence for each of the required parameters, the assay has never been properly validated. Like many assays that have been used for decades, the CFC assay has been "grandfathered" in and used despite the problematic trustworthiness and meaning of the results obtained [33,37].

HALO, from the outset, was designed to be validated. The assay was developed to incorporate the range values specified in the FDA Guidance on Bioanalytical Method Validation [40]. In summary, these values are as follows:

- Assay linearity: => 5 logs.

- Assay cell linearity: 1,000 - > 25,000 cells/well.

- Assay ATP sensitivity: ~ 0.001µM.

- Assay cell sensitivity: 20-25 cells/well, depending on cell purity).

- Accuracy: ~95%.

- Sensitivity & Selectivity by Receiver Operator Characteristics (ROC): Area Under Curve (AUC) 0.73 – 0.752 (lowest possible value: 0.5; highest possible value, 1).

- Precision: = < 15%. Lower limit of quantification (LLOQ): 20%.

- Robustness: ~95%.

- High throughput capability (Z-factor [57]): > 0.76.

- Log-log linear regression slope for ATP standard curve: 0.937 ± 15% (slope range: 0.796 – 1.07)

- Lowest ATP value indicating unsustainable cell proliferation: ~ 0.04µM.

- ATP value below which cells are not metabolically viable: ~0.01µM.

In addition, the assay has also been validated against the Registry of Cytotoxicity Prediction Model, which will be discussed in more detail in Section 5B.

5.7. Statistics

All of the results provided were produced using 8 replicate wells/point. Compound dose response curves were fitted to a 4- or 5-parameter logistic curve fit using SoftMax Pro software (Molecular Devices) from results exported directed from the plate luminometer and calculated automatically. To estimate IC values, raw data were converted to a percentage of the growth factor + vehicle control. Additional statistics, curve fitting or graphing was performed using Prism software (GraphPad) or OriginPro (OriginLab).

6. Results and discussion

6.1. Distinguishing the response of stem cells from progenitor cells

From a practical viewpoint, stem and progenitor cells are distinguished by at least two different characteristics. First, stem and progenitor cell populations are stimulated using different cocktails of growth factors and cytokines. In this way, specific cell populations can be targeted and studied, even though the cell suspension may contain other cell types. Combined with the culture conditions, this allows detection and measurement of specific cell populations. The other distinguishing characteristic is the difference in proliferation ability and potential between stem and progenitor cells. Even within the stem cell compartment, differences in proliferation potential will indicate the primitiveness or "stemness" of populations. This characteristic is shown in Fig. 5 for normal bone marrow cells. Since the stem cells are more primitive than the progenitor cells, it would be expected that their proliferation potential would be greater. Figure 5 shows that the two stem cell populations exhibit, not only greater ATP concentration values, but also greater linear regression cell dose response slopes than the hematopoietic or lymphopoietic progenitor cells. It is the slope of the cell dose response that measures proliferation potential. The greater the slope, the higher the proliferation potential, and the more primitive the cell population. Indeed, this is the basic principle for measuring potency of hematopoietic stem cell therapeutic products for transplantation [37]. In this way, it is possible to distinguish different stem cell populations, in this case the hematopoietic stem cell, CFC-GEMM 1, from the more primitive lympho-hematopoietic stem cell, HPP-SP (high proliferative potential – stem and progenitor cell). The HPP-SP stem cell will be discussed in more detail in Section 6.4. The three cell dose response clusters showing the differences in proliferation potential in Fig. 5 for stem cells, hematopoietic progenitor cells and lympho-poietic progenitor cells would be expected based on the organization of the blood-forming system shown in Fig. 4. Figure 6 demonstrates the expected proliferation ability of the seven different cell populations in response to mitomycin-C, with the stem cells showing the greatest ability to proliferate followed by the three hematopoietic lineages and lymphopoietic lineages.

The steepness of the linear regression slope of the cell dose response for a cell population provides a measure of the proliferation potential. Stem cells exhibit the greatest proliferation potential of all cells. Within the stem cell compartment, stem cells with different potentials for proliferation also indicate their primitiveness. Proliferation ability is measured at a single cell dose (see Fig. 6).
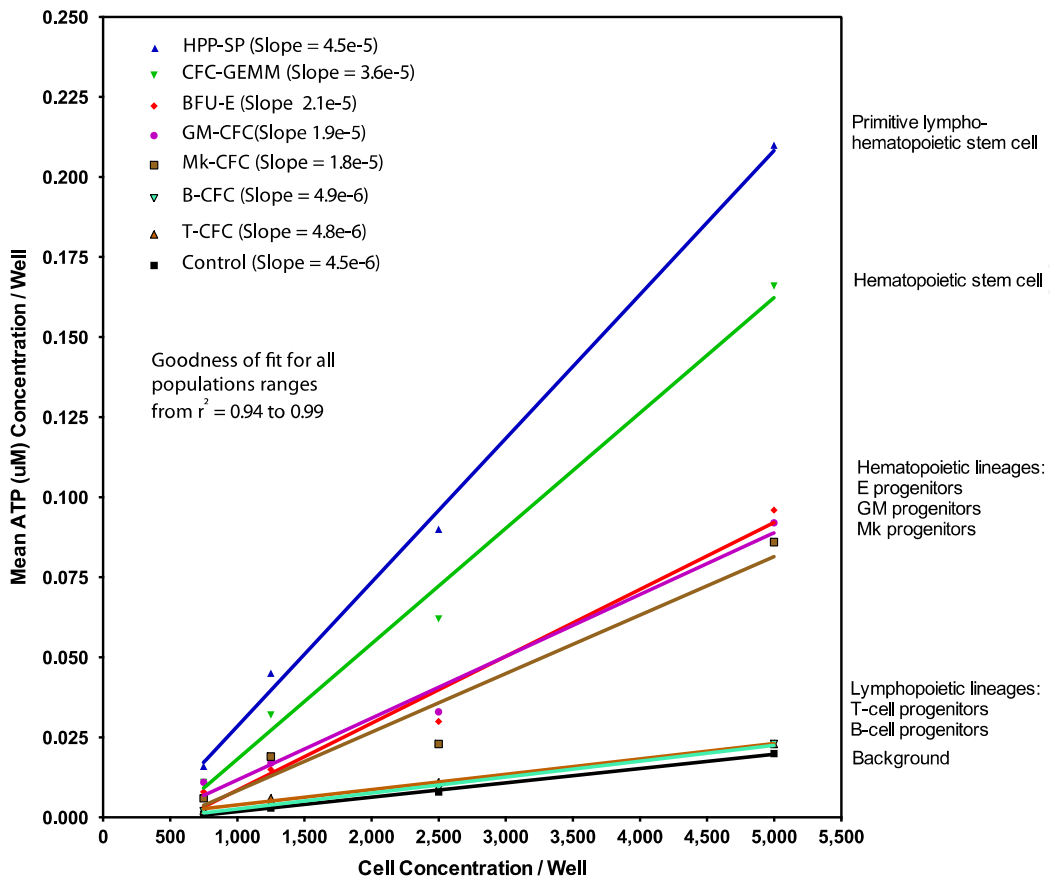
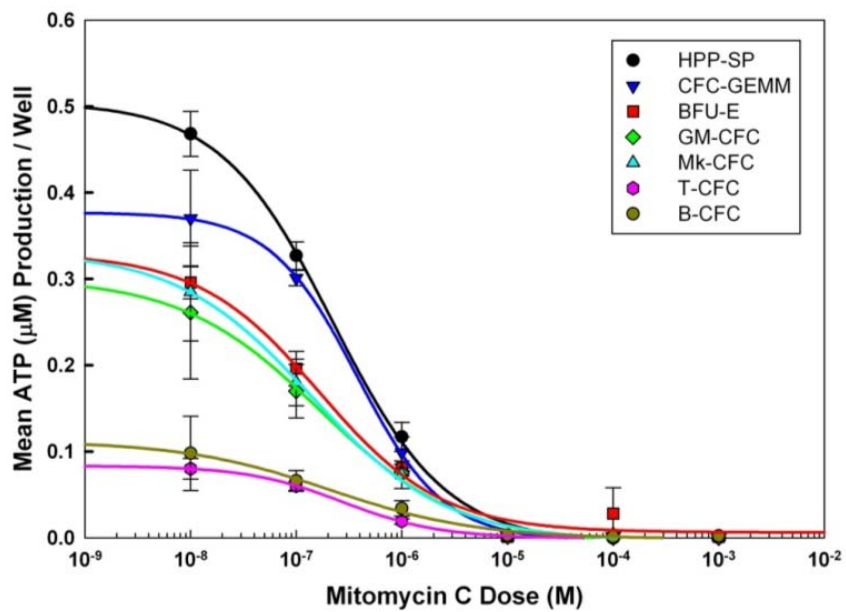**Figure 5.** Measuring Proliferation Potential of Cell Populations

Figure 6. Demonstration of Proliferation Ability between Cell Populations

6.2. Drug and compound screening for stem cell toxicity

In its most basic form, a single drug or compound is tested in a dose dependent manner on a target cell population. If the agent is cytotoxic to the cells, a negative sigmoidal dose response (Fig. 6) will result from which the estimated percent inhibitory concentrations (IC) can be calculated. Figure 7 shows the dose response curves from 13 drugs and compounds tested on hematopoietic stem cells (CFC-GEMM 1) derived from fresh, human bone marrow using the MNC fraction. Although different cell types are included in this fraction, stimulation of this particular stem cell population using a specific growth factor cocktail provides the relevant information. For each of the compounds tested a 4-parameter logistic curve fit was plotted from which the IC values could be calculated. Table 1 shows all of the compounds ranked in order of IC50 (μM) value from the most to the least toxic. The IC90 value (equivalent to the maximum tolerated dose, MTD) is also provided. Many of the compounds tested were also used in the ECVAM studies [26,27].

Table 1 shows some compounds designated as NV or NE. The term NV indicates that an IC20 values was obtained, but no IC50 or IC90 value. The term NE means "no effect" in that no IC values could be estimated. As a result, methotrexate, which is an anti-cancer agent and expected to produce a more dramatic effect on stem cells, is actually ranked near the end of the list. Furthermore, compounds that do not allow an IC value to be calculated might actually produce some effect. The problem with ranking compounds based on their IC values is that it does not take into account the "form" of the dose response curve, which can actually provide more information than the IC value alone. Figure 7 shows a large number of different dose response curves. One of the most important parameters provided by the 4-parameter logistic curve fit is coefficient or parameter B, which describes the transition of the curve to the midpoint of the dose response. This is a measure of steepness or slope. In some cases the slope is shallow, while in other cases it is almost vertical. How can this and other parameters of the dose response curve be taken into account so that they are independent of the IC value? The answer lies in calculating the area under the curve (AUC) for the range of doses used. When the AUC is performed and plotted so that the compounds are ranked, a different and more plausible picture is obtained (Fig. 8).

In this case, the AUC values for both stem cells (CFC-GEMM 1) and granulocyte-macrophage colony-forming cells (GM-CFC) are shown. When the results for CFC-GEMM 1 are compared with those in Table 1, the results generally follow the IC50 values. However, the toxicity of methotrexate is significantly increased and cycloheximide is more toxic than paclitaxel. The results for the GM progenitor cells have been included to demonstrate that progenitor cells exhibit lower toxicities than stem cells. Unless there is evidence to demonstrate that a compound acts on a specific hematopoietic lineage, it is more prudent to analyze potential toxicity to the stem cell compartment first, rather than focusing on a particular lineage, since the latter will only provide limited information that could possibly result in a false interpretation and conclusion.
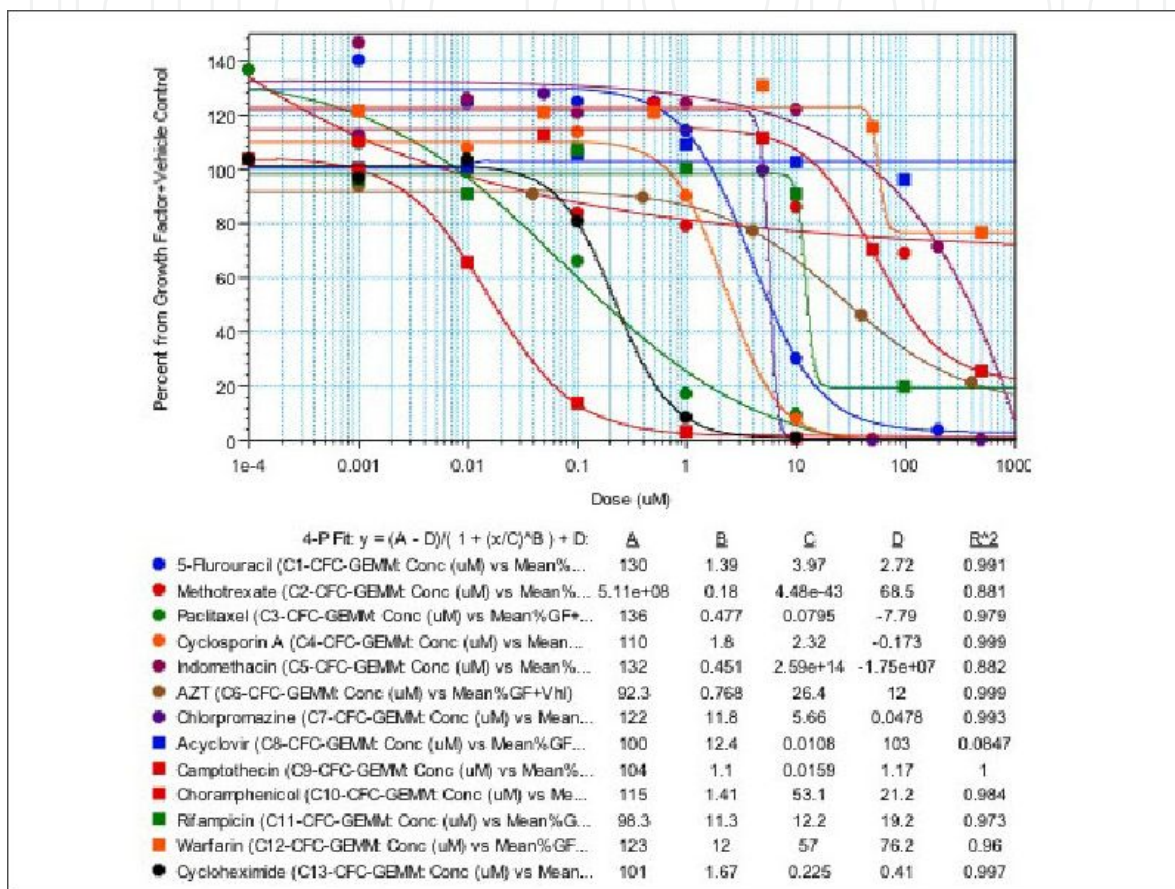
Figure 7. The Effect of 13 Compounds on Hematopoietic CFC-GEMM Stem Cells. Diagram showing the dose response plots produced automatically by SoftMax Pro software after the data was collected by the SpectraMax L plate luminometer. The parameters that define the 4-parameter logistic curve to which the dose responses of the compounds are fitted are as follows: Parameter A, asymptote (flat part of the curve) at low Y-values; Parameter D, asymptote at the highest Y-values; Parameter or coefficient B, the transition from the asymptotes to the center of the curve; Parameter or coefficient C, is the midpoint between parameters A and D, also called the IC50 or EC50. Data that cannot be properly fitted will result in ambiguous results.

Compound	Effect	Rank	IC50 (µM)	IC90 (µM)
Camptothecin	Anti-cancer	1	0.02	0.14
Paclitaxol	Anti-cancer	2	0.18	4.81
Cycloheximide	Pesticide	3	0.23	0.86
Cyclosporin A	Immunosuppressant	4	2.57	8.2
5-Fluorouracil	Anti-cancer	5	5.79	29.7
Chlorpromazine (Thorazine)	Anti-psychotic	6	5.88	7.03
Rifampicin	Anti-bacterial	7	12.8	NV
Zedovuidine(AZT)	Anti-viral	8	30.4	NV
Choramphenicol	Anti-bacterial	9	94.7	NV
Indomethacin	Anti-inflammatory	10	394.2	947.5
Methotrexate	Anti-cancer	11	NV	NV
Acyclovir	Anti-viral	12	NE	NE
Warfarin	Anti-coagulant	13	NE	NE

NV indicates No Value for these IC values. An IC20 value would have been estimated by the software program.

NE indicate No Effect. In this case, the dose response for the compound did not produce an IC values.

Table 1. Ranking of Stem Cell Toxicity According to IC50 Values
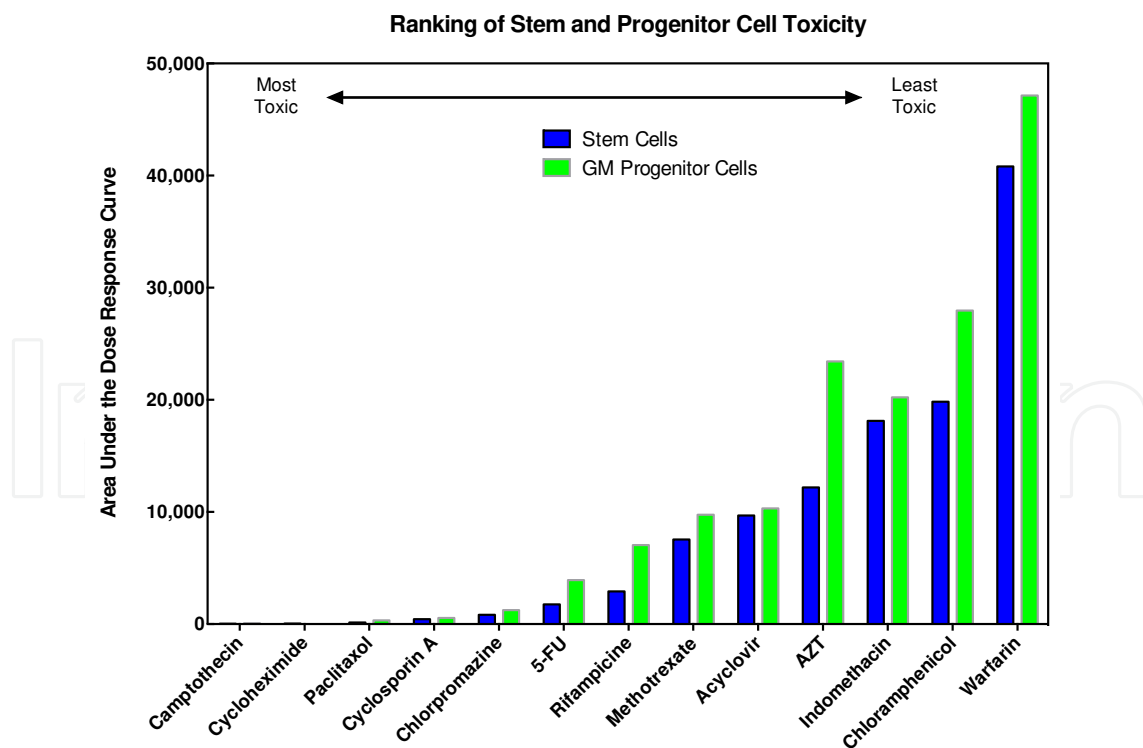

Figure 8. Ranking of Stem Cell and Granulocyte-Macrophage Progenitor (GM-CFC) Toxicity According to the Calculated Area Under the Curve (AUC) for the Dose Responses shown in Figure 7.

6.3. The registry of cytotoxicity prediction model [41]

The Registry of Cytotoxicity (RC) is a list of 347 compounds, for which the IC50 values using a neutral red uptake assay for human keratinocytes and mouse 3T3 cells and the oral LD50 values for rat or mouse, are known. When validating an *in vitro* assay against the RC, a sample of reference compounds is tested. The resulting IC50 values from the *in vitro* assay are then plotted against the LD50 values for the same compounds. A linear regression should be obtained exhibiting equation constants within a specific range. If this occurs, the *in vitro* assay is considered a validated cytotoxic test. The results validating HALO against the RC Prediction Model were first reported in 2005 [33]. One of the most interesting aspects of this prediction model is that once an assay has been validated, it can be used to convert *in vitro* IC values into clinically relevant doses that can be used as starting doses for pre-clinical animal models or human clinical trials. An example of this is shown in Table 2 where the results of converting IC20, IC50 and IC90 range values derived from the effects of 18 compounds on CFC-GEMM 1 bone marrow cells is shown. The predicted doses derived from the IC values are given in both milligrams/kilogram (mg/kg) and milligrams/meter^2 (mg/m^2). Doses used in the clinic to treat patients are also shown in mg/kg or mg/m^2 where available. With the exception of two drugs, namely acyclovir and warfarin, nearly all of the doses predicted by the *in vitro* CFC-GEMM 1 assay using ATP bioluminescence are in the same order of magnitude or very close to the doses used to treat patients. In some cases lower doses were predicted (e.g. 5-fluorouracil), while in other cases slightly higher doses were predicted (e.g. cyclosporine A, indomethacin, cisplatin and mitomycin-C). Thus these predicted starting values may be used in early toxicity and efficacy studies to "bracket" the lower and higher dose ranges.

6.4. Residual stem cells after toxicity

Figure 7 shows that the response of stem cells to toxic agents can vary dramatically. In some cases, agents cause complete eradication of all stem and progenitor cells at high doses. In other cases, there is partial cytotoxicity at which, even at high doses, stem and progenitor cells are not eradicated. This is an indication that some stem cells survive or are possibly resistant to the drug or compound. If stem cells are not noticeably affected at high doses, there is a good chance that when the drug or compound is removed, the system will reconstitute itself. If no stem cells are available, this will not occur. However, there are other aspects to this phenomenon that are important.

Primitive stem cells are usually in a quiescent state; they are not proliferating and therefore not in cell cycle. This does not mean that they cannot be affected by an agent. Small molecules can enter a cell even if it is quiescent. When required to initiate the proliferation process and begin cell division, the process may be aborted because the agent inhibits the process. This is a potential dangerous situation for two reasons. First, the "backup plan" for reconstituting the system may not function. Second, if cells do begin to proliferate and divide, they may be more sensitive to the agent. The consequence of this is that repeated administration of the drug or compound will continually reduce the proportion of residual stem cells present.

Drug/Compound	Predicted Dosing Range from In Vitro Stem Cell Assay		Published Drug Doses Used to Treat Patients	
	Dose in mg/kg	Dose in mg/m²	Doses or Dose Range in mg/kg	Doses or Dose Range in mg/m²
Doxorubicin	2.6 – 6.9	97 – 255		25/50/60/75
Daunorubicin	0.5 – 2.6	19.6 – 97		30/45/60
5-Fluorouracil	2.0 – 7.0	79 – 259		400 – 2,600
Paclitaxel	2.0 – 17.5	72 – 647		75 – 250
Imatinib (Gleevec)	3.6 – 30.5	132 – 1,125		400/600
Methotrexate	5.8	215		10 – 8,000
Cyclosporin A	14.2 – 31.2	524 – 1,155	5 – 10	
Indomethacin	32 – 73	1,190 – 2,700	0.2 – 2	
Zedovudine (AZT)	4.3 – 12.2	161 – 452	1 – 7.4	
Chlorpromozine (Thorazine)	6.8 – 7.7	253 – 285	1 – 4.5	
Acyclovir	NV	NV	5 – 500	
Camptothecin	0.36 – 1.52	13.3 – 56		25/320/470
Choramphenicol	16 – 24	594 – 896	12.5/30 – 50	
Rifampicin	24 – 26	894 – 955	10	
Warfarin	NV	NV	0.1 – 5	
SJG-136	0.1 – 0.3	4 – 10		6 – 40
Cisplatin	6.3 – 9.8	233 – 363		30 – 100
Mitomycin-C	1.2 – 6.0	47 – 220		6/10 – 20

The IC values obtained from the validated *in vitro* assay are entered into the equation: $Y = 0.435 * Log (IC value) + 0.625$ [41]. The dose in mg/kg is then obtained by multiplying the value for Y with the molecular weight of the compound. The dose in mg/m² is obtained by multiplying the dose in mg/kg by a specific factor described in [42].

Table 2. Using the Registry of Cytotoxicity Prediction Model to Convert *In Vitro* IC Values into Clinically Relevant Starting Doses

To demonstrate this, we developed an *in vitro* secondary re-plating assay for primitive stem cells called high proliferation potential – stem and progenitor cells (HPP-SP). This stem cell population, within the stem cell compartment (Fig. 4), is approximately at the divergence of the lymphopoietic and hematopoietic systems. The majority of HPP-SP stem cells are quiescent. They can be induced or "primed" into proliferation with IL-3, IL-6, SCF and Fl3-L. This stem cell population is designated HPP-SP 1. Once the HPP-SP 1 cells begin proliferation, they can be expanded with a similar cocktail of growth factors and cytokines to that for CFC-GEMM 1, but with the addition of interleukins 2 and 7 (IL-2, IL-7). This fully stimulated primitive stem cell population is designated HPP-SP 2. In this two-stage assay, the HPP-SP 1, present in the MNC fraction of bone marrow are cultured in the presence of the drug or compound in a dose-dependent manner. Thereafter, the cells are removed from culture, washed and re-plated in a secondary culture system in which the HPP-SP 2 population is measured. By performing a secondary re-plating step, the assay is substantiating the presence of primitive stem cells

present in the first "priming" step of culture. The proliferation at both stages is determined using ATP bioluminescence technology. The results using busulphan and daunorubicin are shown in Figs. 9A and 9B, respectively. The effect of busulphan (Fig. 9A) on HPP-SP 1 demonstrates partial cytotoxicity to the stem cells and the presence of residual stem cells. However, when the treated cells are removed from primary culture and placed into secondary cultured to reveal their expansion potential, there are few residual cells that are available for expansion and the high doses used in the primary culture eradicated any remaining cells. There was also little change in the IC50 values. This indicates that busulphan continued to act on primitive stem cells leaving no residual stem cells (secondary culture results minus primary culture results) for possible repopulation. Daunorubicin (Fig. 9B) is highly toxic to stem cells with an IC50 value in the nanomolar range compared to the micromolar range for busulphan. At low doses of daunorubicin, residual stem cells would be available, but secondary culture demonstrates that both these and the residual cells have increased their sensitivity by approx. 3 fold, indicating that repeated drug administration would incur increased sensitivity of the stem cells to the drug.
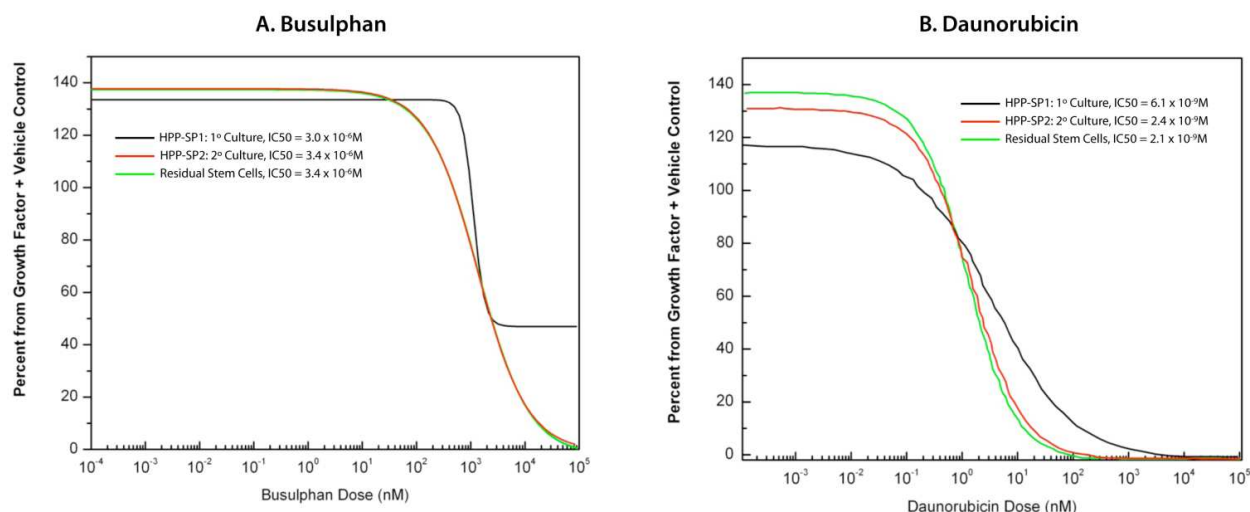

Figure 9. Assessing Residual Stem Cell Activity and Change in Stem Cell Sensitivity to Agents by Measuring the Response of Primitive Stem Cells in a Two-Step Secondary Re-Plating In Vitro Assay.

6.5. Stem cells and drug-drug interactions

Drug-drug interaction (DDI) can lead to dangerous consequences if not investigated properly. Traditionally, DDI are investigated using cultured hepatocytes since the liver is the organ primarily responsible for detoxification. The main enzymes investigated during DDI studies are those of the cytochrome P450 (CYP450) system present in the endoplasmic reticulum of the cells. CYP450 enzymes are present not only in hepatocytes, but in virtually all cells. There are a large number of CYP450 enzymes and assays are available for many of these. Depending on the drug or compound, one or more CYP450 enzymes can be induced or inhibited [43,44]. The response by different enzymes provides an indication as to whether an interaction between different drugs will occur. However, measurement of CYP450 activities does not indicate a

response at the cellular level. To investigate this, we developed an assay in which drugs could be titrated against each other to determine potential DDI on stem cells.
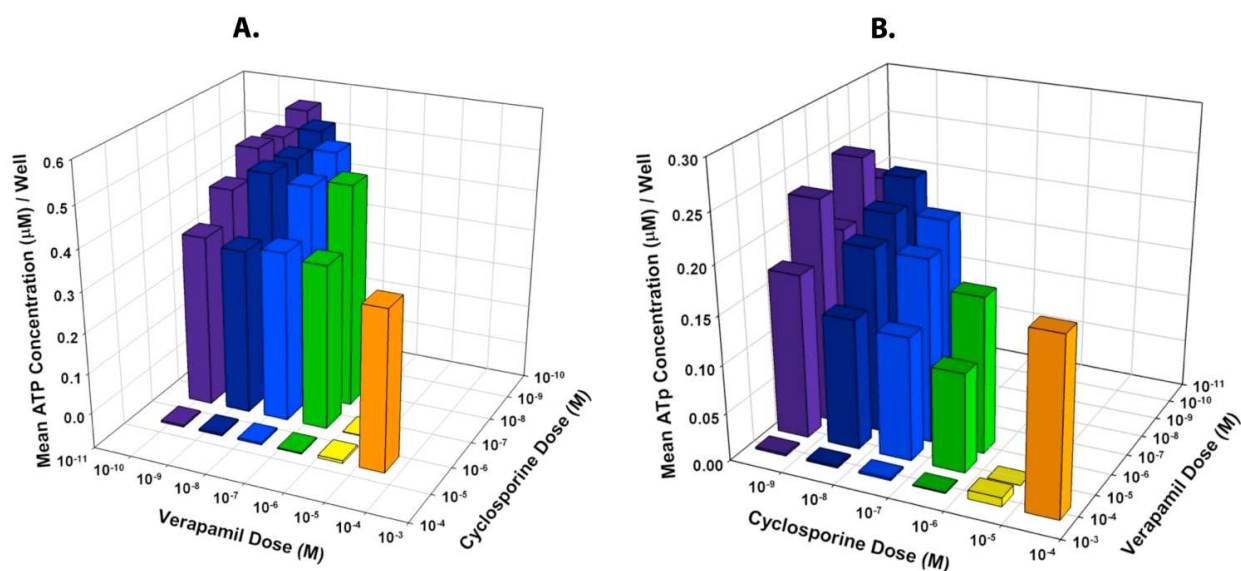

Figure 10. Examples of Drug-Drug Interactions at the Stem Cell Level

Figure 10A shows the response when verapamil is titrated against cyclosporin A, while Fig. 10B shows the effect when cyclosporin A is titrated against verapamil. Both drugs inhibit 3A4 CYP450 enzyme. Individually, both drugs are cytotoxic to CFC-GEMM 1 stem cells. However, when titrated against each other, cytotoxicity may be observed initially, but may be followed by an opposite effect at higher doses. The cells appear to overcome the inhibitory effects. In terms of DDI, this would indicate that one or other drug is present at concentrations that could cause serious harm to the patient. This unusual dose response behavior produces a U-shaped or inverted dose response curve that has been observed for many compounds, including dopamine [45] and endostatins [46]. Although often attributed to solubility, these effects appear to be pharmacologically and physiologically important, but in most cases, the mechanism is not understood. This is the first indication that DDI can occur at the stem cell level. Considering the importance of assessing toxicity to stem cells and the predictive value afforded by these cells, it is obvious that more has to be learnt before the consequences of these reactions on a stem cell system can be understood.

6.6. Circadian rhythm and stem cells

One of the most interesting aspects of drug treatment is the field of chronotherapy; the administration of drugs in accordance with circadian rhythms. Although studied for decades, the role of circadian rhythms to reduce toxicity and improve drug efficacy has been largely ignored by the biopharmaceutical industry. The primary reason for this is because chronotherapeutic studies are difficult, time-consuming and expensive to perform. Nevertheless, many areas of chronotherapy, especially using anti-cancer drugs. have proved to be successful [47-49]. Many cellular functions are dependent upon circadian rhythms. It is not the purpose

of this section to describe or even summarize this field. The intention is to instead provide an example in which the circadian rhythm of cells, especially hematopoietic stem cells [50-52], can be used to predict the best time of day to administer an anti-cancer drug, which in this case, is 5-fluorouracil (5-FU) [38].

These studies were performed using normal peripheral blood mononuclear cells. Blood was obtained from the same donor every 4 hours over a 24 hours period. The MNCs were fractionated at each time point and cryopreserved into aliquots. Prior to cryopreservation, an aliquot of fresh cells was used to measure the proliferation ability of hematopoietic stem cells (CFC-GEMM 1), erythropoietic progenitor cells (burst-forming units – erythroid, BFU-E), GM-CFC and megakaryopoietic progenitor cells (megakaryopoietic colony-forming cells, Mk-CFC) at each time point using HALO. After collection of the cells, an aliquot from each time point was thawed and the circadian rhythms compared to fresh cells. A cosinor curve fitting analysis was performed to produce all the circadian rhythms shown in Fig. 11 [53]. The results for hematopoietic stem cells (Fig. 11A) and all progenitor cells (not shown) demonstrate that even after cryopreservation, the cell populations maintain their circadian rhythm. This was a prerequisite to use cryopreserved cells for the remainder of the study.
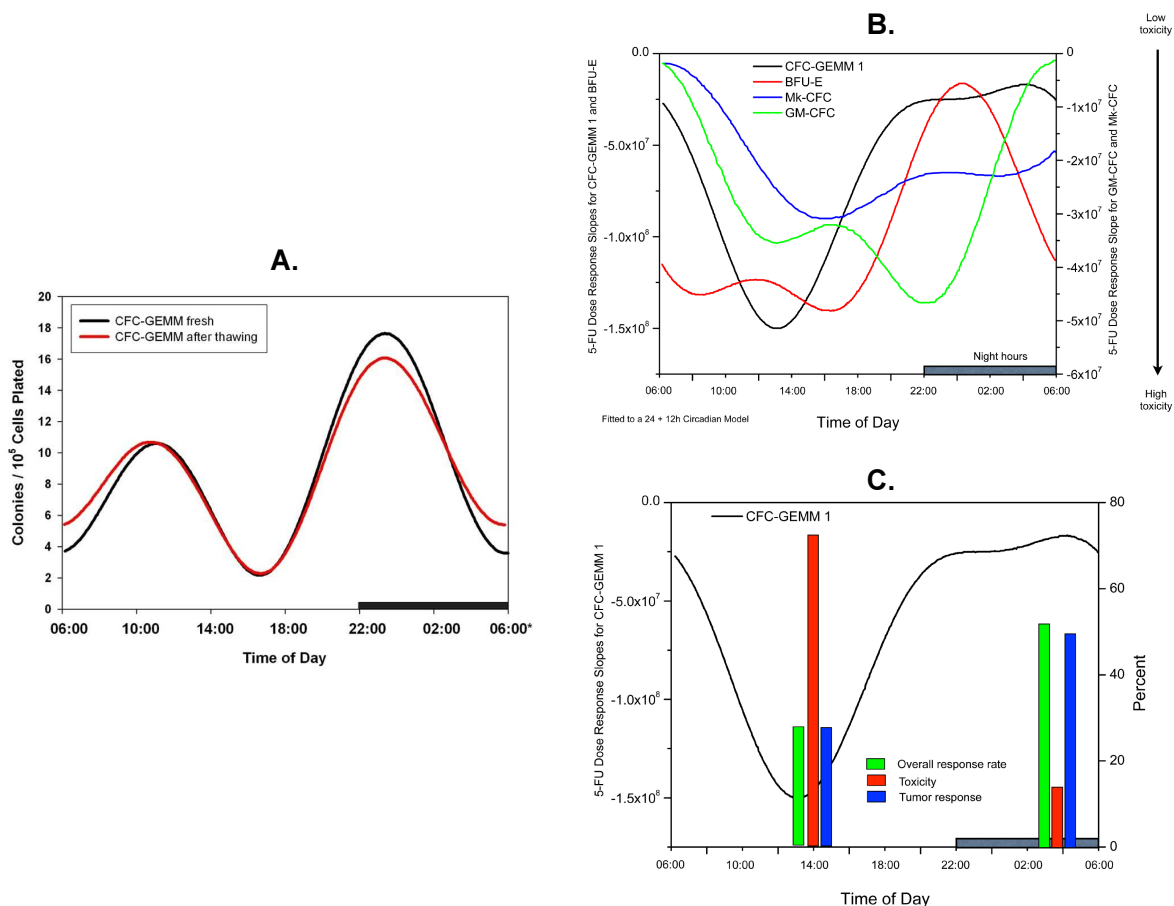

Figure 11. Using the Circadian Rhythm of Hematopoietic Stem Cells to Predict the Best Time of Day to Administer 5-Fluorouracil to Reduce Toxicity and Improve Efficacy of the Drug.

For each time point, cells were thawed and treated with 5-FU at six doses to measure the response of CFC-GEMM 1, BFU-E, GM-CFC and Mk-CFC. The slope of each negative sigmoidal dose response curve was then calculated from the 4-parameter logistic curve fit. The dose response slope values were then analyzed by cosinor analysis for each time point and for each cell population to obtain the circadian rhythms as a function of 5-FU treatment. The results are shown in Fig. 10B. Each of the hematopoietic cell populations exhibited its own circadian rhythm in response to 5-FU. When these circadian rhythms were correlated with either the continuous infusion of 5-FU that is normally used to treat patients and that of chronomodulated infusion of 5-FU as reported by Dogliotti and colleagues in 1998 [54], the results shown in Fig. 10C were obtained. For each of the administration types, the percent overall patient response rate, toxicity and tumor response are shown. These were overlaid onto the circadian rhythm for the CFC-GEMM 1 stem cell response to 5-FU and demonstrated that the lowest toxicity and highest overall and tumor response occurred when 5-FU was administered in a chronomodulated manner in the early morning hours rather than at any other time of the day. The nadir of the CFC-GEMM 1 circadian rhythm to 5-FU occurred at 14:00 hours in the afternoon. This was approximately the same time at which the highest toxicity to 5-FU was found. As expected, these results did not correlate nearly as well for the hematopoietic progenitor cells. In addition, the results clearly demonstrate that the potential for toxicity can be dramatically reduced if the circadian rhythm of the target cells is taken into account. From the brief description here, it follows that to ascertain the best time of day to administer a drug a considerable amount of work must be undertaken. The question is whether the patient response and well-being outweigh the time and cost to perform these types of studies.

7. Conclusions and future trends

To use *in vitro* stem cell assays to predict potential toxicity to the hematopoietic system, and any stem cell system for that matter, knowledge of the biology, physiology, regulation and response is required for an *in vitro* to *in vivo* concordance to be justified. This concordance plays an integral role in predicting toxicity since it allows for *in vitro* surrogate assays to be used in place of animals and therefore comply with the principle of the 3Rs (replacement, reduction and refinement) [55]. More importantly, to allows extrapolation to the human situation. Previous literature on stem cell and hematopoietic research demonstrates that *in vitro* assays show a high concordance with *in vivo* data. Using the HALO platform, Olaharski et al. demonstrated an *in vitro* to *in vivo* concordance of greater than 80% [56]. This high degree of concordance provides the basis to predict the response of the lympho-hematopoietic and other stem cell systems to potential toxic insults. This has been described previously [32], but it is worth reiterating some of these paradigms. First, virtually any compound can be toxic to stem cells. Second, toxicity to the most primitive, definitive stem cells will affect all cells of the system. Third, since stem cells only proliferate and proliferation occurs prior to differentiation, stem cell cytotoxicity will affect all downstream cell types. Fourth, if more than one cell lineage is affected by toxicity, the target is not the cells that constitute the lineages, but the stem cells producing the lineages. Finally, stem cells are more sensitive to toxicity than the progenitor

cells. When considering using stem cells to predict potential toxicity, at least two considerations need to be taken into account. The first is the primitiveness of the stem cell population being measured, while the second is variation between human donors. The former will depend, among other things, upon the ability and sensitivity of the assay to detect specific stem cell populations and the latter will be dependent upon the state and demographics of the donors that can, in turn, affect the stem cells. Both are difficult to control, but can provide a more realistic view.

Based on these paradigms, it is worth briefly considering how the non-definitive stem cells systems (Fig. 1), ES and iPS cells, fit into predictive stem cell toxicity testing. At the present time, these cells are used to produce functionally, mature lineage-specific cells such as hepatocytes, cardiomyocytes and neurons. These and other cell types can be produced in larger numbers and presumably at a lower cost than their primary counterparts. Embryonic stem cells are used as an *in vitro* developmental toxicity model to predict teratogenicity. The use of ES and/or iPS cells for definitive stem cell system toxicity testing is certainly on the horizon. It should be remembered however, that even to produce functionally, mature hepatocytes, cardiomycytes and other cells, the ES and iPS cells must pass through the definitive stem cell compartment specific for the cells being produced. In other words, the ES and iPS cells should produce an organization analogous to that shown in Fig. 2. If this transpires, then the face of toxicity testing, and stem cell toxicity testing in particular, as well as many other applications, could significantly change the face of biological and toxicological research in the future.

Acknowledgements

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References

[1] Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A; The International Society for Cellular Therapy:

Clarification of the Nomenclature for MSC: the International Society for Cellular Therapy Position Statement. Cytotherapy 2005; 7:393-395.

[2] Potten, CS. Cell cycles in cell hierarchies. Int J Radiat Biol Relat Stud Phys Chem Med. 1986; 49:257-278.

[3] Lemischka IR. The haematopoietic stem cell and its clonal progeny: mechanism regulating the hierarchy of primitive haematopoietic cells. Cancer Surv. 1992; 15:3-18.

[4] Yahata, T, Muguruma, Y, Yumino, S, Sheng, Y, Uno, T, Matsuzawa, H, Ito, M, Kato, S, Hotta, T, Ando, K. Quiescent human hematopoietic stem cells in the bone marrow niches organize the hierarchical structure of hematopoiesis. Stem Cells. 2008; 26: 3228-3236.

[5] Campbell, CJ, Lee, JB, Levadoux-Martin, M, Wynder, T, Xenocostas, A, Leber, B, Bhatia, M. The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity. Blood, 2010; 116:1433-1442.

[6] Levesque, JP, Winkler, IG. Hierarchy of immature hematopoietic cells related to blood flow and niche. Curr. Opin. Hematol. 2011; 18: 220-225.

[7] Staal FJ, Baum C, Cowan C, Dzierzak E, Hacein-Bey-Abina S, Karlsson S, Lapidot T, Lemischka I, Mendez-Ferrer S, Mikkers H, Moore K, Moreno E, Mummery CL, Robin C, Suda T, Van Pel M, Vanden Brink G, Zwaginga JJ, Fibbe WE. Stem cell self-renewal; lessons from bone marrow, gut and iPS towards clinical applications. Leukemia. 2011; 25:1095-1102.

[8] Van Zant G, de Haan G, Rich IN. Alternatives to stem cell renewal from a developmental viewpoint. Exp Hematol. 1997; 25:187-192.

[9] Kola I, Landis J. Can the pharmaceutical industry reduce attrition rats? Nature Reviews: Drug Discovery. 2004; 3:711-715.

[10] Mahajan R, Gupta K. Food and drug administration's critical path iniative and innovations in drug development paradigm: Challenges, progress, and controversies. J Pharm Bioallied Sci 2010; 2: 307-313.

[11] National Cancer Institute, Division of Cancer Treatment and Diagnosis, Developmental Therapeutics Program. http://dctd.cancer.gov/ProgramPages/dtp/tools_drug_discovery.htm.

[12] Yamori T. Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics. Cancer Chemother Pharmacol. 2003. 52 Suppl 1:S74-S79.

[13] Shanks N, Greek R, Greek L. Are animal models predictive for humans? Philos Ethics Humanit Med. 2009; 15: 2.

[14] Toxicity Testing in the 21st Century. A Vision and a Strategy. Committee on toxicity testing and assessment of environmental agents. National Research Council of the

National Academies. National Academic Press. ISBN 0-309-10993-0. 2007. http://www.nap.edu/catalog/11970.html.

[15] Senior JR. Monitoring for hepatotoxicity: what is the predictive value of liver "function" tests? Clin Pharmacol Ther 2009; 85: 331-334.

[16] Maziasz T, Kadmabi VJ, Silverman L, Fedyk E, Alden CL. Predictive toxicology approaches for small molecule oncology drugs. Toxicol Pathol 2012; 38: 148-164.

[17] Firestone M, Kavlock R, Zenick H, Kramer M. The U.S. environmental protection agency strategic plan for evaluating the toxicity of chemicals. J Toxicol Environ Health B Crit Rev. 2010; 13: 139-162.

[18] Benigni R, Bossa C. Mechanism of chemical carcinogenicity and mutagenicity: a review with implications for predictive toxicology. Chem Rev. 2011; 111: 2507-2536.

[19] Gleeson MP, Modi S, Bender A, Robinson RL, Kirchmair J, Promkatkaew M, Hannonqbua S, Glen RC. The challenges involved in modeling toxicity data in silico: a review. Curr Pharm Des. 2012; 18: 1266-1291.

[20] REACH Directive, http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm

[21] Bradley TR, Metcalf D. The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci. 1966; 44: 287-299.

[22] Pluznik DH, Sachs L. The induction of clones of normal mast cells by a substance from conditioned medium. Exp Cell Res. 1966; 43: 553-563.

[23] Cole RJ, Paul J. The effects of erythropoietin on haem synthesis in mouse yolk sac and cultured foetal liver cells. J Embryol Exp Morphol. 1966; 15: 245-260.

[24] Stephenson JR, Axelrad AA, McLeod DL, Shreeve MM. Induction of colonies of haemoglobin-synthesizing cells by erythropoietin in vitro. Pro Natl Acad Sci USA. 1971; 68:1542-1546.

[25] Iscove NN, Sieber F, Winterhalter. Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. J Cell Physiol. 1974; 83:309-320.

[26] Pessina A, Albella B, Bueren J, Brantom P, Casati S, Gribaldo L, Croera C, Gagliardi G, Foti P, Parchment R, Parent-Massin D, Sibiril Y, van Den Heuvel R. Prevalidation of a model for predicting acute neutropenia by colony forming unit granulocyte/macrophage (CFU-GM) assay. Toxicol In Vitro. 2001; 15: 729-740.

[27] Pessina A. Albella B, Bayo M, Bueren J, Brantom P, Casati S, Croera C, Gagliardi G, Foti P, Parchment R, Parent-Massin D, Schoeters G, Sibiril Y, Van Den Heuvel R, Gribaldo L. Application of the CFU-GM assay to predict acute drug-induced neutropenia: an international blind trial to validate a prediction model for the maximum tolerated dose (MTD) of myelosuppressive xenobiotics. Tox Sci. 2003; 75:355-367.

[28] Pessina A, Parent-Massin D, Albella B, Van Den Heuvel R, Casati S, Croera C, Malerba I, Sibiril Y, Gomez, S, de Smedt A, Gribaldo L. Application of human CFU-Mk assay to predict potential thrombocytotoxicity of drugs. Toxicol In Vitro. 2009; 23:194-200.

[29] Bacigalupo A, Piaggio G, Podesta M, Figari O, Benvenuto F, Sogno G, Tedone E, Raffp MR, Grassia L, Ferrero R, et al. Bone Marrow Transplant. 1995; 15:221-226.

[30] Jansen EM, Hanks, SG, Terry C, Akard LP, Thompson JM, Dugan MJ, Jansen J. Prediction of engraftment after autologous peripheral blood progenitor cell transplantation: CD34, colony-forming unit granulocyte-macrophage, or both? Transfusion. 2007; 47:817-823.

[31] Gluckman E. Milestones in umbilical cord blood transplantation. Blood. 2011; 25:255-259.

[32] Rich IN, Hall KM. Validation and development of a predictive paradigm for hemotoxicology using a multifunctional bioluminescence colony-forming proliferation assay. Toxicol Sci. 2005; 87: 427-441.

[33] Spellman S, Hurley CK, Brady C, Phillips-Johnson L, Chow R, Laughlin M, McMannis J, Reems JA, Regan D, Rubinstein P, Kurtzberg J. Guidelines for the development and validation of new potency assays for the evaluation of umbilical cord blood. Cytotherapy. 2011; 13: 848-855.

[34] Rich IN, Kubanek B. The effect of reduced oxygen tension on colony formation of erythropoietic cells in vitro. Br. J Haematol. 1982; 52: 579-588.

[35] Rich IN. The role of the macrophage in normal hemopoiesis. II. Effect of varying physiological oxygen tensions on the release of hemopoietic growth factors from bone marrow-derived macrophages in vitro. Exp Hematol. 1986; 14: 746-751.

[36] Hall KM, Rich IN. Bioluminescence assays for assessing potency of cellular therapeutic products. In: Cellular Therapy: Principles, Methods and Regulations. ISBN: 978-1-56395-296-8. Bethesda, MD. 2009. p 581-591.

[37] Hall KM, Harper H, Rich IN. Hematopoietic Stem Cell Potency for Cellular Therapeutic Transplantation. In: Advances in Hematopoietic Stem Cell Research, Rosana Pelayo (Ed.), ISBN: 978-953-307-930-1, InTech, 2012. http://www.intechopen.com/books/advances-in-hematopoietic-stem-cell-research/hematopoietic-stem-cell-potency-for-cellular-therapeutic-transplantation.

[38] Rich IN. In vitro hematotoxicity testing in drug development: a review of past, present and future applications. Curr Opin Drug Discov Devel. 2003; 6:100-109.

[39] Rich IN. High-throughput in vitro hemotoxicity testing and in vitro cross-platform comparative toxicity. Expert Opin Drug Metab Toxicol. 2007; 3: 295-307.

[40] FDA Guidance for Industry, Bioanalytical Method Validation, 2001. http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf.

[41] Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity. 2001. NIH Publication No.: 0.1-4500. http://iccvam.niehs.nih.gov/docs/acutetox_docs/guidance0801/iv_guide.pdf.

[42] FDA Guidance for Industry and Reviewers. Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers. 2002. http://www.fda.gov/downloads/Drugs/.../Guidances/UCM078932.pdf.

[43] Mann HJ. Drug-associated disease: cytochrome P450 interactions. Crit Care Clin 2006; 22:329-345.

[44] The Cytochrome P-450 Enzyme System. http://www.edhayes.com/startp450.html.

[45] Monte-Silva K, Fuo M-F, Thirugnanasambandam N, Liebetanz D, Paulus W, Nitsche MA. Dose-dependent inverted U-shaped effect of dopamine (D2-like) receptor activation on focal and nonfocal plasticity in humans. J Neurosci. 2009; 29: 6124-6131.

[46] Celik I, Sürücü O, Dietz C, Haymach JV, Force J, Höschele I, Becker CM, Folkman J, Kisker O. Therapeutic efficacy of endostatin exhibits a biphasic dose-response curve. Cancer Res 2005; 65: 11044-11050.

[47] Innominato PF, Levi FA, Bjarnason GA. Chronotherapy and the molecular clock: Clinical implications in oncology. Adv Drug Deliv Rev 2010; 61: 979-1001.

[48] Takeda N, Maemura K. Circadian clock and cardiovascular disease. J Cardiol. 2011; 57: 249-256.

[49] Cutolo M. Chronobiology and the treatment of rheumatoid arthritis. Curr Opin Rheumatol. 2012; 24: 312-318.

[50] Haus E, Lakatua DJ, Swoyer J, Sackett-Lundeen L. Chronobiology in hematology and immunology. Am J Anat. 1983; 168: 467-517.

[51] Laerum OD. Hematopoiesis occurs in rhythms. Exp Hematol. 1995; 23: 1145-1147.

[52] Mendez-Ferrer S, Chow A, Merad M, Frenette PS. Circadian rhythms influence hematopoietic stem cells. Curr Opin Hematol. 2009; 16: 235-242.

[53] Naitoh P, Englund CE, Ryman DH. Circadian rhythms determined by cosine curve fitting: Analysis of continuous work and sleep-loss data. Behav Res Metho Instrum Comp. 1985; 17: 630-641.

[54] Dogliotti L, Tampellini M, Levi F. Chronochemotherapy of colorectal cancer. Biological Clock: Mechanisms and Applications, Touitou Y (ed); 1998; 475-481.

[55] Liebsch M, Grune B, Seller A, Butzke D, Oelgeschlaeger M, Pirow R, Adler S, Riebeling C, Luch A. Alternatives to animal testing: current status and future perspectives. Arch Toxicol. 2011; 85: 841-858.

[56] Olaharski AJ, Uppal H, Cooper M. Platz S, Zabka TS, Kolaja KL. In vitro to in vivo concordance of a high throughput assay of bone marrow toxicity across a diverse set of drug candidates. Toxicol Let. 2009; 188: 98-103.

[57] Zang JH, Chung TDY and Oldenburg Kr: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 1999; 4: 67-73