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Simply red: A novel spectrophotometric erythroid proliferation assay as a tool for erythropoiesis and erythrotoxicity studies

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ABSTRACT

Most mammalian cell proliferation assays rely on manual or automated cell counting or the assessment of metabolic activity in colorimetric assays, with the former being either labor and time intensive or expensive and the latter being multistep procedures requiring the addition of several reagents. The proliferation of erythroid cells from hematopoietic stem cells and their differentiation into mature red blood cells is characterized by the accumulation of large amounts of hemoglobin. Hemoglobin concentrations are easily quantifiable using spectrophotometric methods due to the specific absorbance peak of the molecule's heme moiety between 400 and 420 nm. Erythroid proliferation can therefore be readily assessed using spectrophotometric measurement in this range. We have used this feature of erythroid cells to develop a simple erythroid proliferation assay that is minimally labor/time- and reagent-intensive and could easily be automated for use in high-throughput screening. Such an assay can be a valuable tool for investigations into hematological disorders where erythropoiesis is dysregulated, i.e., either inhibited or enhanced, into the development of anemia as a side-effect of primary diseases such as parasitic infections and into cyto-(particularly erythro-) toxicity of chemical agents or drugs.

1. Introduction

Erythropoiesis is one of the body's most productive cell proliferation processes, yielding an average of 2×10^{11} new erythrocytes from hematopoietic stem cells of the bone marrow every day to replace those lost to senescence and destruction [25]. A reduced erythropoietic output or the production of malfunctioning erythrocytes leads to anemia which can have severe and even fatal consequences when tissues are insufficiently supplied with oxygen [17]. Homeostasis of erythrocyte production is primarily regulated by the hormone erythropoietin (EPO), whose production is upregulated upon tissue oxygen depletion [9,30]. However, numerous factors – both exogenous (such as toxins) and endogenous (such as inflammatory cytokines) – can inhibit proliferation and/or differentiation of erythroid cells [27]. In addition, the requirement for large amounts of iron for hemoglobinization makes the process highly dependent on the availability of sufficient concentrations of transferrin-bound iron [16]. In diseases of chronic inflammation such as rheumatoid arthritis, erythropoiesis is impaired both by the direct action of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ and by upregulation of the liver hormone hepcidin, the primary regulator of iron uptake and storage, leading to a reduction in the amount of bio-available iron in circulation [10]. Many chemotherapeutic agents have also been found to induce anemia either through an inhibition of the production of EPO or a direct cytotoxic/inhibitory effect on erythroid cells [6,31].

A number of infections with parasitic agents such as *Plasmodium*, *Schistosoma*, *Leishmania* or hookworms result in anemia [22,24]. In the case of intestinal infections, this anemia is believed to be caused primarily by intestinal hemorrhage, reduced iron absorption or decreased bioavailability of iron [29]. Inflammatory responses to the infections including the secretion of proinflammatory cytokines and/or the resultant upregulation of hepcidin additionally appear to inhibit erythropoiesis, as in the anemia of chronic disease [4,27]. In the case of malaria and leishmaniasis, evidence exists that parasitic products may also directly impede erythroid proliferation and/or differentiation [13,33].

On the other hand, erythropoiesis can become dysregulated in certain myeloproliferative disorders leading to uncontrolled proliferation of erythroid cells. In the erythroleukemia polycythemia vera for example a mutation in the Janus tyrosine kinase

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JAK2 renders erythroid proliferation independent of erythropoietin and causes excessive red cell production [20,23].

In vitro methods for the generation of erythroid cells from hematopoietic stem cells derived from various sources have been established and shown to yield both high proliferation of erythroid cells and produce functional, mature, enucleated reticulocytes or erythrocytes, thus faithfully recapitulating the in vivo process [3,11,12]. In general, the differentiation process of erythroid progenitor cells and their maturation is characterized by the acquisition of specific erythroid features including particular surface markers, an exit from the cell cycle and the accumulation of large amounts of hemoglobin that is responsible for the cells' ability to bind oxygen [35,39]. A tetramer of 4 globin chains with a central heme molecule, hemoglobin shows a spectrophotometric absorbance peak between 400 and 420 nm, which has been exploited for the quantification of hemoglobin in solution by Harboe and others [5,14,15]. As this characteristic can be used for hemoglobin quantification not only in solution but also when cell-bound, we have developed a spectrophotometric assay for assessing erythroid proliferation based on absorbance at 405 nm.

2. Materials and methods

All chemicals were obtained from Sigma–Aldrich (Arklow, Ireland) unless stated otherwise.

2.1. Cell isolation

Mononuclear cells (MNC) were isolated from peripheral blood buffy coats obtained from the Irish Blood Transfusion Services (Dublin, Ireland) using density gradient centrifugation with histopaque-1077. CD34+ cells were isolated from mononuclear cells via immuno-magnetic separation using anti-CD34 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bisley, UK).

2.2. Cell culture

Cultures were initiated from frozen or freshly isolated mononuclear cells or CD34+ cells. For depletion of leukocytes, mononuclear cells were pre-cultured for 2 days in IMDM with stabilized glutamine (Biochrom, Berlin, Germany) containing 5% (vol/vol) heparinized human plasma and 3 U/ml EPO (Janssen-Cilag, Dublin, Ireland). Cells were then washed and cultured in erythroid proliferation medium [12] consisting of IMDM with 330 μg/ml iron-saturated human transferrin and 10 μg/ml recombinant human insulin, supplemented with 5% heparinized human plasma, 100 ng/ml stem cell factor (SCF) (Cambridge Biosciences, Cambridge, UK), 5 ng/ml interleukin-3 (IL-3) (R&D Systems, Minneapolis, MN, USA), 3 U/ml EPO and 10^{-6} M hydrocortisone for 1–2 days. CD34+ cells were plated directly into the appropriate conditions. Manual cell counting was performed using the trypan blue exclusion method with trypan blue diluted 1/6 in phosphate buffered saline (PBS) and added to cells at 1:1 or 1:4 ratios.

2.3. Plasmodium falciparum *conditioned medium*

P. falciparum-conditioned medium was obtained from blood-stage cultures of *P. falciparum* 3D7 cultures grown in RPMI medium supplemented with 5% (wt/vol) Albumax® in a candle jar according to published methods [8]. *P. falciparum* cultures were grown to 10–15% parasitemia, washed two times with wash medium consisting of RPMI supplemented with 0.18 g/l sodium bicarbonate and one time with IMDM and then resuspended in IMDM. For higher protein content, cultures were concentrated 6–8 fold by resuspension in lower volumes of IMDM after washing and cultured for 3–4 h to obtain conditioned medium. Conditioned medium was obtained by centrifuging the culture supernatant for 10 min at 2000 rpm ($823 \times g$) followed by filtration through a 0.2 μm filter and used at up to 80% (vol/vol) in erythroid medium.

2.4. Absorbance-based assay

Cells (CD34+ cells or pre-cultured MNCs) were subsequently seeded in erythroid proliferation medium containing 5% heparinized human plasma, 100 ng/ml SCF, 5 ng/ml IL-3 and 3 U/ml EPO as standard conditions. These conditions served as a positive control for erythroid proliferation. As a negative control, cells were seeded in pure IMDM medium lacking growth factors and plasma. Erythropoiesis inhibiting agents were added at different concentrations or growth factor concentrations were varied to assess the effect on erythroid proliferation. Cells were seeded in 96-well flat-bottom plates at $1–5 \times 10^5$ cells/ml and cultured in a humidified incubator at 37 °C and 5% CO_2 for up to 21 days. All conditions were set up in triplicate and for each condition a well containing the appropriate medium blank without cells was included.

Absorbance measurements of plates with lid were taken at 405 nm using a Synergy H1 (Biotek, Potton, UK) plate reader preheated to 37 °C and following 2 min of linear shaking at 567 cycles per minute (cpm).

2.5. Analysis

Results from manual cell counting were determined as the mean and standard deviation of the cell concentrations of triplicate wells.

Results from spectrophotometric measurements were determined as the mean absorbance of triplicate wells and their standard deviation. The mean values were corrected by the absorbance of the medium blank and presented as percentage of the positive control. Statistical analysis was performed using Prism software (La Jolla, CA, USA).

3. Results

3.1. Method principle

The Harboe method has been established for determining hemoglobin concentrations in solution using spectrophotometric measurement at 415 nm and has been validated for assessing hemolysis in red cell samples [14,15]. We adapted this method for estimating erythroid cell concentrations in unlysed culture samples and determining erythroid proliferation in a non-invasive manner. Hemoglobin shows maximum light absorption between 400 and 420 nm and we found absorbance at 405 nm and 415 nm to correlate linearly ($R^2 = 0.9999$) allowing the use of 405 nm absorbance filters commonly available on standard plate readers (Fig. 1a). We established that the lysis of erythrocytes is not necessary for reliable hemoglobin quantification and that cell suspensions could be directly subjected to spectrophotometric measurement (Fig. 1b, $R^2 = 0.9905$). Initial assay set-up was performed using samples of native erythrocytes isolated from donor blood suspended in PBS and absorbance measurements at 405 nm were found to correlate linearly ($R^2 = 0.998$) with manual cell counts (Fig. 1c). Using the function obtained from the linear fit of such an erythrocyte standard curve using GraphPad software, cultures could be expressed as 'erythrocyte equivalents' based on

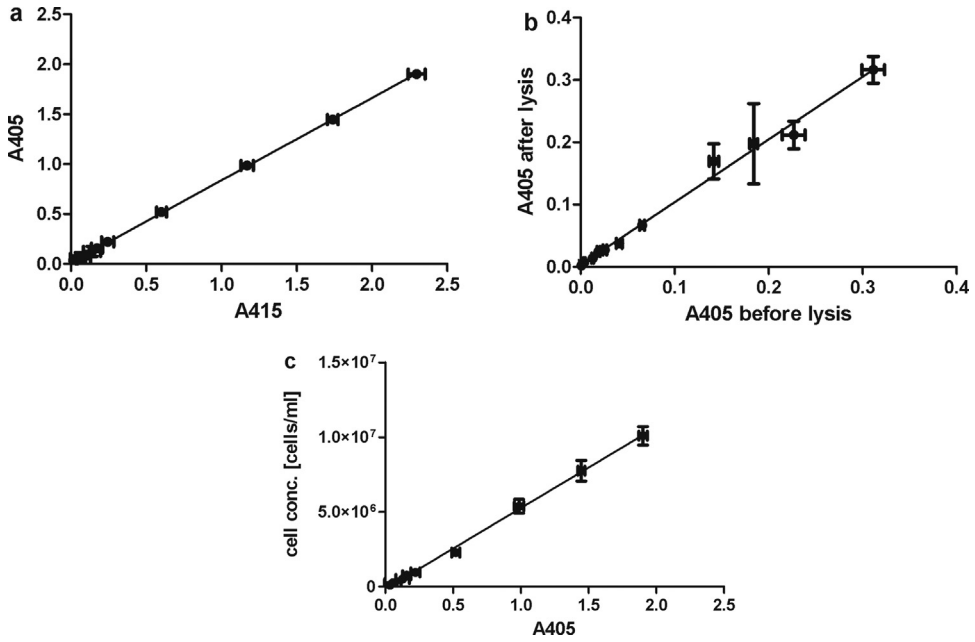

Fig. 1. (a–c) Hemoglobin measurement at 405 nm can be used to determine the cell concentrations of unlysed samples of erythrocytes: (a) hemoglobin absorbance of erythrocyte lysates at 415 and 405 nm correlated linearly with each other. (b) The absorbances at 405 nm of unlysed erythrocyte samples showed linear correlation with those of erythrocytes lysed osmotically in distilled water. (c) The concentrations of erythrocyte samples as determined by manual counting using trypan blue correlated linearly with absorbance measurements at 405 nm. Dilutions of native erythrocytes in PBS were used between 1×10^5 and 1×10^7 cells/ml; results are shown as mean and standard deviation of triplicate determinations.

their absorbance (erythrocyte equivalents/ml = (5,413,000 $\pm$ 91,210) $\times A_{405}$ − (154,700 $\pm$ 80,730)).

Absorbance measurements were obtained from in vitro erythroid cultures at various time points of culture using a plate reader pre-heated to 37 °C, and plates were agitated to disperse cells evenly in the microwells before measurement. The absorbance values were corrected using the absorbance of the medium of each condition and normalized to a positive control culture on the same plate to determine the hemoglobinization as percentage of the positive control that in turn correlated with the cell expansion (Fig. 2).

3.2. Stability

Hemoglobinization begins at the proerythroblast stage and two thirds of a cell's total hemoglobin are produced by the erythroblast while the remaining third is synthesized at the reticulocyte stage [35]. In culture, cells contained detectable amounts of hemoglobin from day 8 after seeding into erythroid medium, showed strong increase in hemoglobinization over the next 7 days and reached a plateau thereafter. Absorbance measurements based on hemoglobin remained stable over extended periods of time showing only slight decreases in

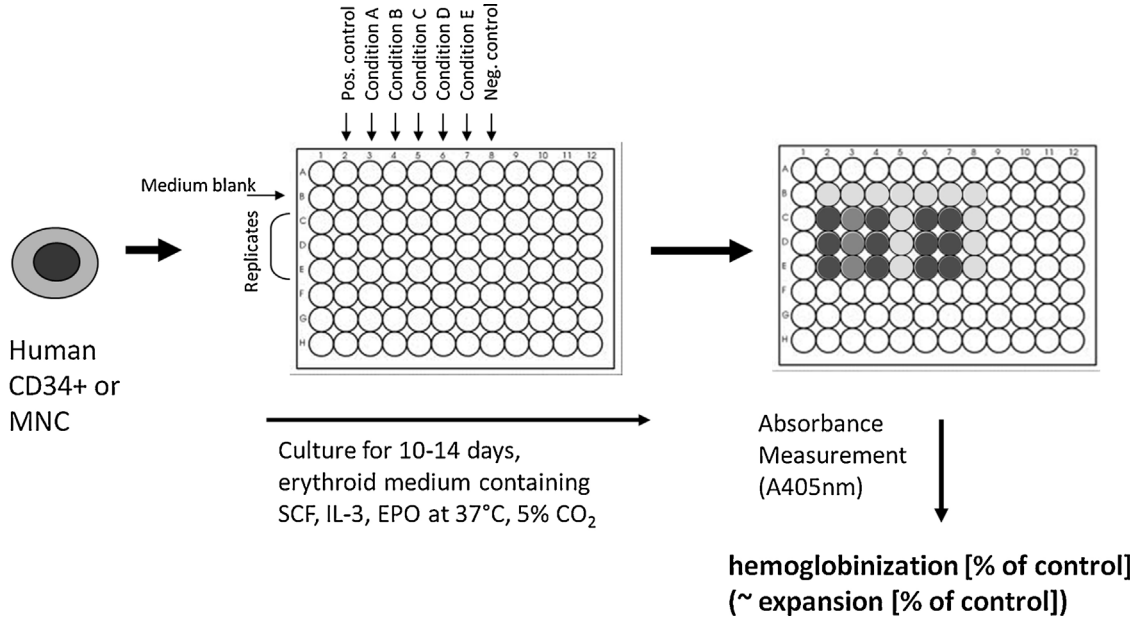

Fig. 2. Outline of set-up and measurement principle of spectrophotometric erythroid proliferation assay. MNC = mononuclear cell.

absorbance after further 10 days (Fig. 3), indicating that this molecule is not readily degraded even when it is released into the culture supernatant upon cell death and rupture.

3.3. Optimization of culture conditions

Cell concentrations and absorbance measurements for erythroid cultures correlated linearly and while standard deviations were larger than for native red blood cell samples, these varied comparably for both measurement principles due to higher biological variation between triplicate wells. The linear function obtained for a culture at day 15 of erythroid culture (Fig. 4b) indicated a lower absorbance for a given cell concentration than obtained for the erythrocyte standard curve as these erythroid cells had not yet fully hemoglobinized. While the ex vivo culture method does not yield fully mature erythrocytes but produces predominantly reticulocytes, a linear correlation between cell concentrations and absorbance could be demonstrated not only for

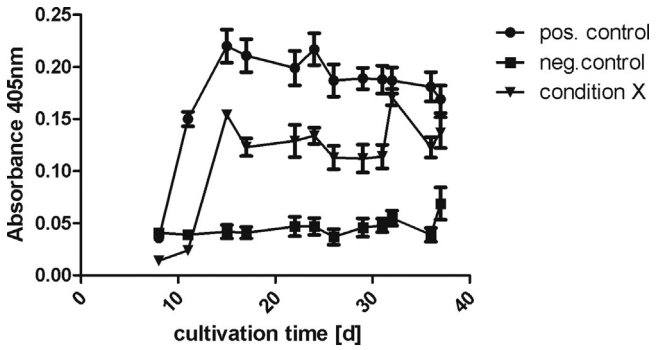

Fig. 3. Signal stability: hemoglobinization increased strongly between days 8 and 15 in erythroid culture and hemoglobin absorbance showed high stability for up to 20 days thereafter in cultures containing high growth (pos. control = optimal growth conditions), medium growth (condition X = suboptimal growth conditions) and very low growth (neg. control = cultivation without growth factors). Results are presented as mean and standard deviation of triplicate determinations.

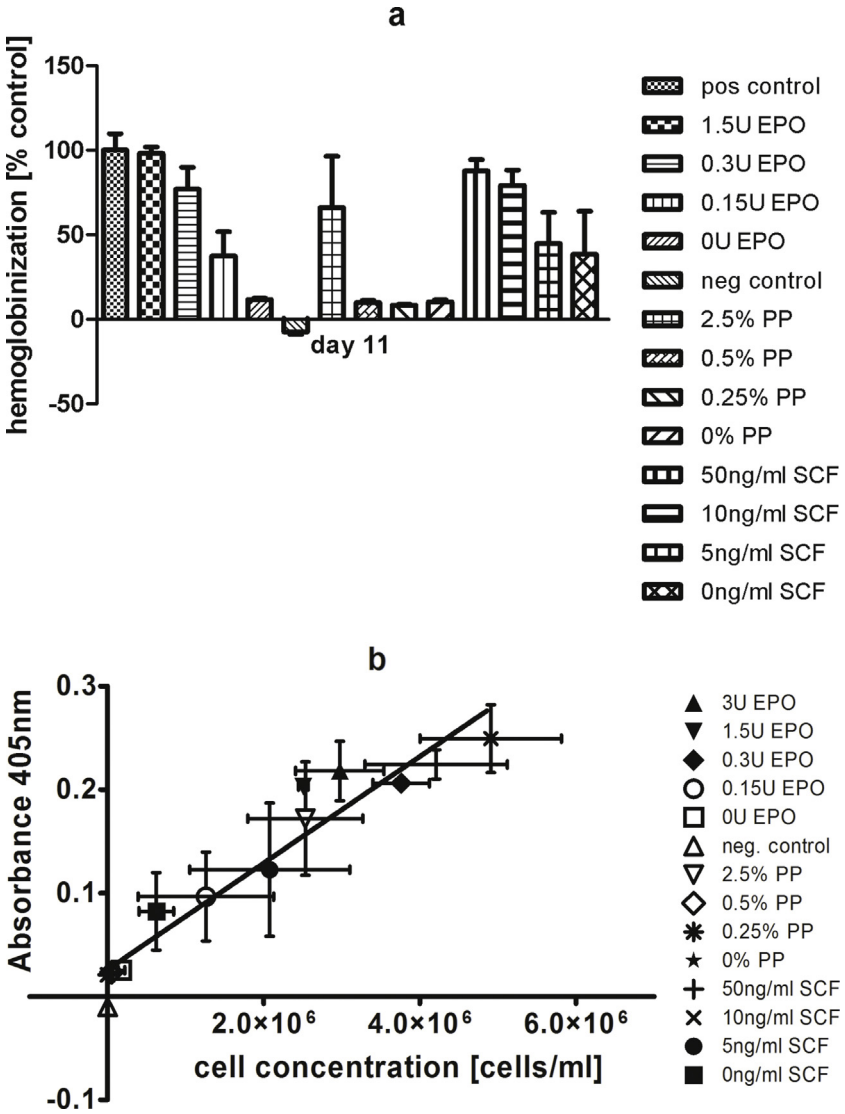

Fig. 4. (a and b) Optimization of cultivation conditions: (a) the effect of different growth factor (EPO, SCF) and plasma (PP) concentrations on erythroid proliferation could be determined using the spectrophotometric erythroid proliferation assay. The positive control was grown in standard conditions of 5% plasma, 100 ng/ml SCF, 5 ng/ml IL-3 and 3 U/ml EPO. Concentrations of plasma, SCF or EPO were varied individually while the other factors were maintained at standard concentrations. Results are the mean and standard deviation of triplicates expressed as percentage of positive control. (b) The assay could distinguish between conditions promoting or inhibiting erythroid proliferation equally well as manual cell counting, showing linear correlation ($R^2 = 0.9313$) between the cell concentration and the absorbance of respective samples. Results are shown as mean and standard deviation of triplicate determinations on day 15 of culture.

mature erythrocytes (Fig. 1c) but also for ex vivo generated erythroid cells which represent a mixed population of erythroid cells of different maturities. Comparison with the internal positive control (standard growth conditions) thus allowed for a determination of reduced or enhanced erythroid proliferation. Using this method, culture conditions which are less favorable to erythroid expansion, e.g., reduced concentrations of plasma or of the growth factors stem cell factor (SCF) or EPO in the erythroid medium, could be determined (Fig. 4a and b).

3.4. Erythroid inhibition by P. falciparum

Using this method, we were furthermore able to detect erythropoiesis inhibiting activity in medium conditioned by blood-stage cultures of the malarial parasite *P. falciparum*. The assay was able to distinguish between erythropoiesis-inhibiting and -promoting conditions to the same extent as manual cell counting using trypan blue exclusion (Fig. 5a) and was able to detect differential responses to different concentrations of the inhibitory medium (Fig. 5b).

3.5. Cytotoxic agents

Dimethylsulfoxide (DMSO) is a commonly used solvent for drugs that show limited solubility in aqueous solutions but it

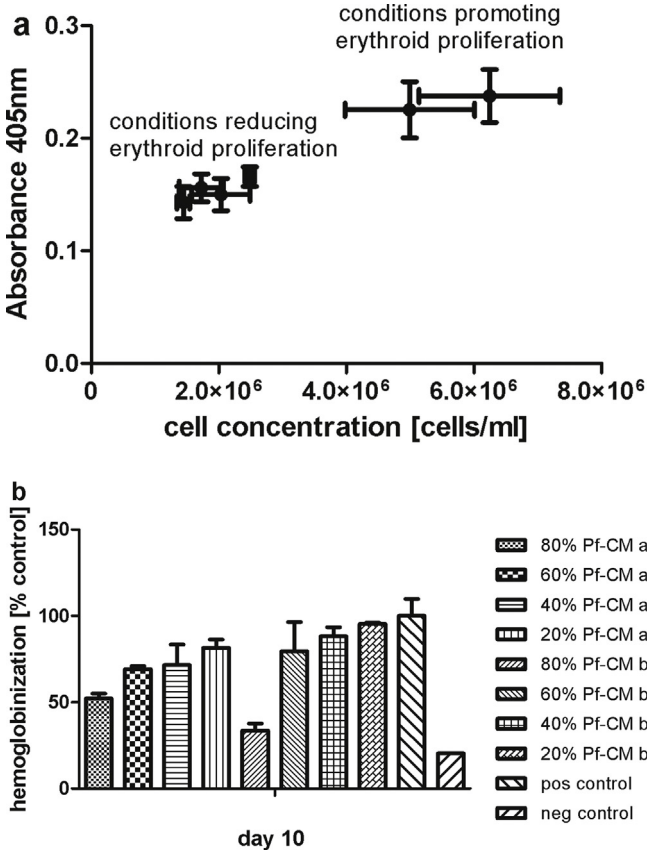

Fig. 5. (a and b) Erythroid inhibition by products of *P. falciparum*: (a) absorbance measurement and manual cell counting could distinguish between conditions promoting erythroid proliferation (containing optimal concentrations of growth factors [100 ng/ml SCF, 5 ng/ml IL-3, 3 U/ml EPO] and medium [5% plasma]) or reducing erythroid proliferation (medium conditioned with products of *P. falciparum*). Results are presented as mean and standard deviation of triplicate determinations. (b) Erythroid proliferation was affected by different concentrations of two different batches (a and b) of *P. falciparum* conditioned medium (Pf-CM). Results are the mean and standard deviation of triplicates expressed as percentage of positive control.

effects a range of biological functions and can cause toxic side effects in vivo [37]. DMSO is also used as the primary cryoprotective agent for hematopoietic stem cells for transplantation as it reduces cell damage due to crystal formation and protects cells from dehydration. It has, however, been shown to be toxic to these cells at elevated concentrations resulting in around 25% of viable cell loss at 5% (vol/vol) DMSO and up to 50% at 10% DMSO [1]. DMSO therefore presents a useful candidate molecule for evaluating the potential of this assay for the assessment of chemical cytotoxicity. High concentrations (20% and 10%) abrogated all cell growth and very low levels of hemoglobin formation were detected at 5% and 2% DMSO whereas concentrations below 1% showed no inhibition (Fig. 6a). The applicability of the assay for toxicological studies was further demonstrated by the use of the antibiotic chloramphenicol which has been found to cause bone marrow suppression and aplastic anemia in vivo [34]. Using our in vitro system, concentration-dependent inhibition of erythroid growth was observed, with a 1 mg/ml concentration of the drug almost abolishing erythropoiesis whereas 12.5 μg chloramphenicol/ml still caused about 50% growth reduction (Fig. 6b). The suitability of an assay for high-throughput screening (HTS) depends on its performance and sensitivity which can be assessed using a number of statistical parameters including the Z' factor commonly used to quantify the quality of an assay [19]. In a total of 10 different assays that were validated, our spectrophotometric erythroid proliferation assay performed well within the acceptable limits and showed an average Z' of 0.67 (Table 1).

4. Discussion

Erythropoiesis is one of the body's most proliferative cell production processes and dysregulation of this process can have life-threatening consequences. In the absorbance based erythroid proliferation assay presented here, we exploit the features of erythroid cultures – high cell expansion in vitro and accumulation of large amounts of spectrophotometrically quantifiable hemoglobin – to develop a novel research tool.

Research continues into the development of suitable drug treatments for erythroleukemias such as polycythemia vera (PV). These drugs currently include hydroxycarbamide (hydroxyurea), pipobroman or interferon, but these therapies can increase the risk of transformation to myelofibrosis or leukemia [21,26]. An erythroid proliferation assay based on PV hematopoietic stem cells could therefore significantly facilitate the screening for novel compounds that reduce erythroid proliferation to normal levels in this type of myeloproliferative disorder. As venesection in an attempt to lower hematocrit levels is one of the primary treatments for PV, mononuclear cells from PV patients would be readily available from these phlebotomies and a patient's own cells could even be used to test for responsiveness to specific drug treatments.

On the other hand, such screening may enable identification of erythropoiesis stimulating agents in conditions where the process is inhibited such as those of Diamond Blackfan anemia, a congenital hypoplastic anemia characterized by mutations in genes encoding ribosomal proteins leading to reduced production of erythrocytes [7].

Anemic conditions where erythroid inhibition may be a direct result of the action of inhibitory pathogen-derived factors as suspected in malaria [2] or *Leishmania* infections could also benefit from a screening tool for the identification of the causative factors and methods of their inactivation.

Finally, drug cytotoxicity studies – and erythrotoxicity of cancer chemotherapeutics in particular – may be significantly facilitated by a high-throughput assay, reducing the need for animal models and cutting both time and cost requirements. A number of

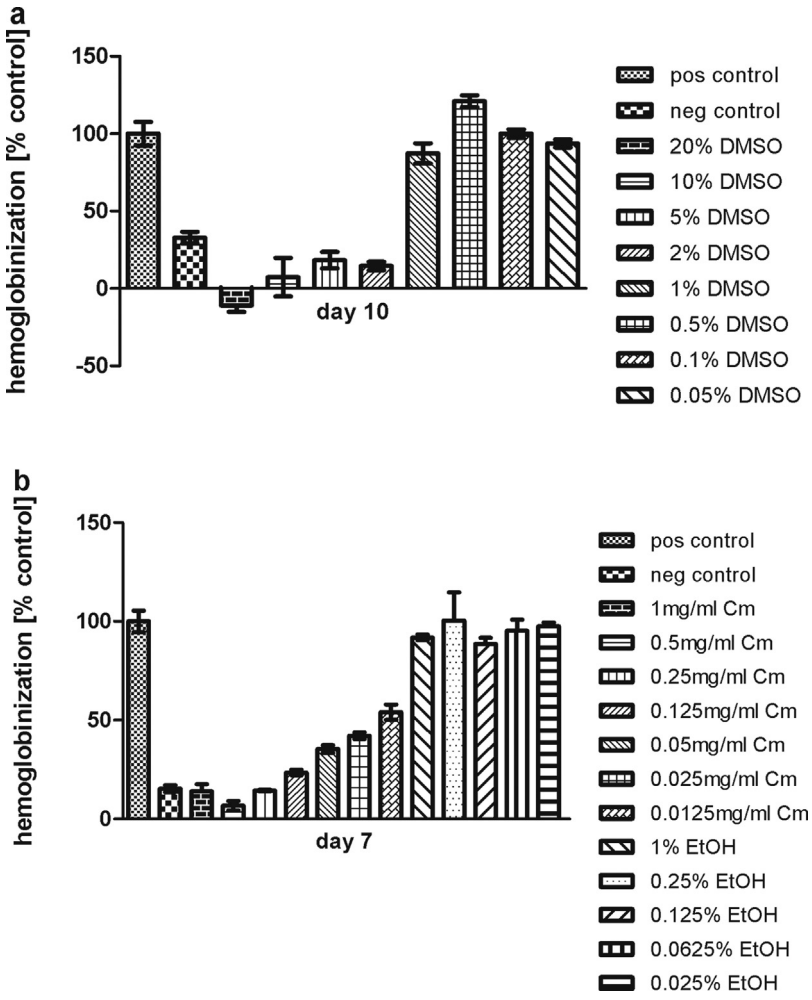

Fig. 6. (a and b) Determination of cytotoxicity using hemoglobin-based absorbance assay: (a) DMSO strongly inhibited erythropoiesis at concentrations higher than 2%. (b) Chloramphenicol (Cm) reduced erythroid proliferation in a concentration-dependent manner whereas this effect was not seen by the ethanol (EtOH) solvent control. Results are the mean and standard deviation of triplicates expressed as percentage of positive control.

cytotoxicity assays are commercially available and have been used for high-throughput screening. Most of these are colorimetric or fluorescent assays that rely on either the measurement of enzyme activities in viable cells or detect enzymes released into culture supernatants upon cell death using established cell lines [28,40]. Among the most common assays are those based on the measurement of activities of ATP, lactate dehydrogenase (LDH), aminopeptidase, or proteases, or determination of redox potential via resazurin or tetrazolium based compounds (MTT, MTS, XTT). While many of the aforementioned assays have established track records for cytotoxicity studies, their limitation lies in the requirement for the addition of reagents making them both more cost and labor-intensive and preventing continuous measurement of a culture. Recent advances in drug cytotoxicity testing include

Table 1
Assay performance: evaluation of standard parameters using the mean (μ) and standard deviation (σ) of the positive (max = standard erythroid growth conditions) and negative control (min = no growth factors) shows good assay performance and sensitivity for the spectrophotometric assay when used to analyze the effect of DMSO, chloramphenicol and in a total of 10 different assays. Results are calculated using the given equations and the average assay performance is shown as mean and standard deviation of the respective parameters for 10 assays performed between days 7 and 12 of erythroid cultures; value ranges are given in parentheses.

Parameter	DMSO day 10	Cm day 7	Average of $n = 10$ assays day 10 ± 1.5 (7–12)	Acceptable limits
Coefficient of variation (max) $\%CV = \sigma_{max}/\mu_{max} \times 100$	7.6%	5.4%	$6.0 \pm 2.6\%$ (2.5–9.9%)	<15%
Coefficient of variation (min) $\%CV = \sigma_{min}/\mu_{min} \times 100$	11.6%	11.3%	$10.5 \pm 5.4\%$ (0–17.2%)	<15%
Signal to noise ratio $S{:}N = (\mu_{max} - \mu_{min})/\sigma_{min}$	17.8	49.1	34.1 ± 16.5 (14.3–63.8)	
Signal to background ratio $S{:}B = \mu_{max}/\mu_{min}$	3.1	6.5	4.6 ± 1.1 (3.1–6.5)	>2-fold
Signal window $SW = (\mu_{max} - \mu_{min} - 3(\sigma_{min} + \sigma_{min}))/\sigma_{max}$	4.4	11.7	9.9 ± 4.6 (4–17.4)	>2-fold
Z' factor $Z' = (3\sigma_{max} + 3\sigma_{min})/\lvert\mu_{max} - \mu_{min}\rvert$	0.49	0.75	0.67 ± 0.11 (0.49–0.86)	>0.5

the development of microfluidic cell culture systems or 'lab-on-a-chip' devices [36,38]. These devices have the advantage of allowing non-invasive and continuous monitoring but are more complex and costly in terms of equipment.

Automation of the spectrophotometric assay described here should be easily achievable through an adaptation to common microplate-handling robotic set-ups. While an internal positive control to which results are normalized reduces the need for plate-to-plate standardization, this could nonetheless be facilitated, e.g., for cytotoxicity studies of candidate drugs, by establishing large MNC pools or using erythroleukemia cell lines.

A limiting factor to the use of a hemoglobin-based assay for cytotoxicity studies is however its inability to distinguish between live and dead cells, as it can only determine effects on the growth/hemoglobinization of erythroid cells but cannot detect the death of already fully hemoglobinized cells. Hemoglobinization continues past the stage where erythroid cells become cell cycle arrested and cease proliferating and large amounts of hemoglobin are still synthesized at the reticulocyte stage [35]. The assay is thus able to detect cytotoxic effects on erythroid cells during growth phase, as hemoglobinization would cease prematurely, but unable to differentiate between intact highly hemoglobinized reticulocytes and hemoglobin which may have been released into solution by lysed cells in late stages of culture.

The spectrophotometric assay has been successfully used for the detection of erythropoiesis inhibiting activity in medium from *P. falciparum* cultures and to determine preferential growth factor concentrations for erythroid expansion. It can further detect cytotoxic components and react in a concentration-responsive manner. Overall, this method provides the means for rapid assessment of erythroid proliferation – either enhanced or inhibited – compared to a standard control and can thus be highly beneficial in initial screening stages to select potential conditions or candidate molecules of interest. Design of experiment (DoE) has been growing in significance for process optimization and drug design applications [18,32]. Coupling DoE for erythroid systems with an automatable assay such as this one to obtain the experimental results on which to build the design and verify its prediction could allow for the acquisition of large amounts of data in short periods of time. For an assay to be implementable in high-throughput screening it has to meet a number of criteria: it should be simple, automatable, show sensitive and reproducible performance and last but not least be economically feasible. We believe this assay fulfills all of these criteria and presents a good candidate for HTS.

Few cells in the human body lend themselves to the establishment of a colorimetric proliferation assay as readily as erythroid cells which simply produce the red read-out dye themselves – the next step is developing the applications.

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