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A mechanistic model of erythroblast growth inhibition providing a framework for optimisation of cell therapy manufacturing

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\textbf{A B S T R A C T}

Manufacture of Red Blood Cell based products in vitro requires highly efficient erythroblast culture for economic viability. It has previously been shown that efficiency of erythroblast culture in scalable bioreactors is not primarily limited by mass transfer, availability of medium components, or commonly recognised inhibitory metabolites or cytokines. We have developed a dynamic mechanistic model that describes an autocrine feedback loop in which a cell-derived factor accumulates in culture medium resulting in reversible erythroblast growth inhibition. Cells exhibited two phases of growth: a relatively uninhibited followed by an inhibited phase. Cell cycle analysis during inhibition identified slight accumulation of cells in G0 phase, distinct from the G1 accumulation anticipated in growth factor or nutrient deprivation. Substantial donor to donor growth rate variability (mean 0.047 h\textsuperscript{-1}, standard deviation 0.008 h\textsuperscript{-1}) required the growth rate parameter to be reftitted for different donors. The model could then be used to predict growth behaviour with full medium exchange, but showed some reduced predictive ability after partial medium exchange. The model could predict the growth inflexion point over a range of phenotypic maturities from early to late maturity erythroblasts; however the secondary phase of growth differed substantially with less inhibition observed in more mature cells. The model provided a framework to optimise culture economics based on cost of production time and input consumables. It also provided a framework to evaluate the benefits of biological process engineering in medium design or cell modification vs. operational optimisation depending on the specific cost scenario of a process developer.

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1. Introduction

A significant number of cell based therapeutic products (CBTs) are in development, and are projected to represent an increasing proportion of the total therapeutics market over the coming decade [1]. The manufacturing approach for CBTs builds on the extensive science and engineering base of the biologics industry and more than forty years of optimisation of cell line processing for protein production. However, CBTs pose certain unique challenges. In conventional biologics production the focus is on protein productivity and quality, allowing adaptation of other cell characteristics for manufacturing benefit. This opportunity is limited for CBTs due to the requirement for minimal alteration of many cell functions for safe and effective function in vivo [2]. Furthermore, CBT cell populations are often relatively heterogeneous and can produce paracrine signalling factors that alter cell function creating acute sensitivity to manufacturing operations such as medium supply regimens [3,4]. These factors combine to challenge the manufacturing goals of either maintaining fidelity with the source cell material or controlling phenotypic trajectory to a suitable endpoint.

CBTs require an appropriate level of bioprocess optimisation for quality, economics and risk for commercial success and regulatory approval. A validated mathematical model defining the manner in which bioprocess operation determines process outcomes could provide a powerful tool for such optimisation. Efficient empirical approaches, such as ‘Design of Experiments’, have been widely applied in bioprocess design to cost-effectively model control parameter effects on outcomes [5]. However, such techniques require substantial elaboration for complex non-linear relationships such as those that commonly occur over time series in biological systems. This decreases their efficiency for application to the dynamic processes underpinning cell culture [6]. Although high parameter approaches such as neural networks produce ‘black-box’ models that can achieve prediction and control goals, they are challenging to develop if data are scarce or expensive, a com-
Table 1

Erythroblasts were cultured in the ambr™ bioreactor under a series of different experimental process conditions, A1–D3, for development and validation of the model of growth and inhibition. The table details the starting density of each culture, the time at which any process operation was undertaken, and the nature (cell density adjustment or media exchange) and magnitude of the operation.

<table>
<thead>
<tr>
<th>Process Condition</th>
<th>Seeding Density (cells/mL)</th>
<th>Medium and/or Cell Density Adjustment Time (hrs)</th>
<th>Proportion of medium exchanged with fresh</th>
<th>Adjusted Cell Density (cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>$3.1 \times 10^6$</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A2</td>
<td>$4.2 \times 10^6$</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A3</td>
<td>$4.2 \times 10^6$</td>
<td>5</td>
<td>0.3</td>
<td>$4 \times 10^6$</td>
</tr>
<tr>
<td>B1</td>
<td>$2.5 \times 10^6$</td>
<td>16</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B2</td>
<td>$2.5 \times 10^6$</td>
<td>16</td>
<td>–</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>B3</td>
<td>$2.5 \times 10^6$</td>
<td>16</td>
<td>–</td>
<td>$0.65 \times 10^6$</td>
</tr>
<tr>
<td>C1</td>
<td>$2.5 \times 10^6$</td>
<td>18.5</td>
<td>1</td>
<td>$3.1 \times 10^6$</td>
</tr>
<tr>
<td>C2</td>
<td>$3.1 \times 10^6$</td>
<td>18.5</td>
<td>1</td>
<td>$0.5 \times 10^6$</td>
</tr>
<tr>
<td>D1</td>
<td>$2.8 \times 10^6$</td>
<td>18.5</td>
<td>0.4</td>
<td>$3.5 \times 10^6$</td>
</tr>
<tr>
<td>D2</td>
<td>$2.5 \times 10^6$</td>
<td>18.5</td>
<td>0.3</td>
<td>$0.5 \times 10^6$</td>
</tr>
<tr>
<td>D3</td>
<td>$2.4 \times 10^6$</td>
<td>18.5</td>
<td>–</td>
<td>$0.5 \times 10^6$</td>
</tr>
</tbody>
</table>

mon scenario during CBT product and process development [7,8]. Further, regulatory initiatives such as Quality by Design emphasise a degree of mechanistic understanding of process which such approaches alone rarely provide [9]. An alternative approach is to employ mechanistic models that describe known or hypothesised phenomena. This approach enables the description of non-linear dynamics in a relatively parsimonious form through incorporation of prior system knowledge (such as cell transitions or influences).

Systems biology researchers have developed highly parameterised dynamic mechanistic models, including considerations of parameter stochasticity, that can potentially describe complex biological systems such as intracellular signalling events [10]. Alternative approaches to describe non-intuitive outcomes based on mechanistic behaviour include agent based modelling, potentially in combination with more conventional approaches such as flux-balance models [11]. However, a CBT process developer has specific requirements of a modelling approach. It needs to be cost effective to develop and amenable to validation. Accessibility to the bioprocess team will ensure model development benefits from wide input. These drive towards the simplest model that will describe the system complexity sufficiently to deliver the predictive power required for optimisation and control goals. The complexity of goals, whether prediction of simple cell yield or complex definition of sub-populations, will dictate the model complexity required.

Manufactured red blood cells are a therapy of interest for transfusion medicine or as engineered delivery agents for other therapeutics [12,13]. We have previously shown that erythroblasts, the lineage committed and rapidly proliferating cell population underpinning red blood cell production, proliferate in vitro followed by relatively rapid growth inhibition [14]. We further identified that mass transfer, common metabolic limitations, or previously reported paracrine signals were not responsible for this inhibition creating a potential economic barrier to in vitro production of red blood cells. Here, we propose that a series of hypotheses regarding the mechanism of inhibition could be tested via the development of incrementally more complex deterministic mechanistic models based on the dominant phenomena of cell culture (such as growth and inhibition). We further aim to show that a sufficiently predictive model could be achieved in a relatively parsimonious form. Finally, we aim to show that this approach supports understanding of current manufacturing limits as well as hypotheses refinement for mechanism(s) of inhibition.

2. Materials and methods

2.1. CD34+ cell culture

Fresh umbilical cord-derived mononuclear cells (MNC) were supplied by the Anthony Nolan Cell Therapy Centre (http://www.anthonynolan.org/clinicians-and-researchers/cord-blood-services) with informed consent and NREC ethical approval. CD34+ cells were isolated via positive selection using CD34 antibody-labelled microbeads as per the manufacturer’s instructions (Miltenyi Biotec). Mixed donor CD34+ cells (>90% purity) were cryopreserved prior to cell culture. On thaw, CD34+ cells seeded at a density of $1–2 \times 10^5$/mL were cultured in IMDM (Merck Millipore) supplemented with 3% (v/v) AB Serum (Sigma), 2 mg/mL Human Serum Albumin (Irvine Scientific), 10 μg/mL Insulin (Sigma), 3 μL/mL heparin (Sigma), 500 μg/mL iron saturated Transferrin (R&D Systems), 10 ng/mL SCF (R&D Systems), 1 ng/mL IL-3 (R&D Systems) and 3 μL/mL EPO (Peprotech). Cells were cultured in tissue culture flasks at 37°C, 5% CO₂ prior to bioreactor culture; they were fed by addition of medium (including previously listed cytokines) on day 3 of culture; thereafter they were fed daily to maintain density between 2 and $3 \times 10^5$/mL. On day 5, 7, 8 or 9 cells were seeded according to Table 1 in the ambr™ bioreactor system (Sartorius Stedim); non-sparged vessels were pre-conditioned as described previously [15], and temperature, impeller speed, pH and DO were set to 37°C, 450 RPM, pH 7.5 and 52% respectively.

2.2. Culture analysis

2.2.1. Cell count and viability

Online cell counting and viability was measured using a Vi-Cell XR (Beckman Coulter) set to the following parameters: Minimum diameter (μm) = 6, Max diameter (μm) = 20, Cell brightness (%) = 85, Cell Sharpness = 100%, Viable cell spot brightness (%) = 65, Viable spot area (%) = 10, Minimum circularity = 0.8. A coefficient of variation of 4% was calculated for counts from parallel bioreactor technical replicates.

2.2.2. Assessment of cell morphology

1 $\times 10^5$ cells were sampled, re-suspended in 200 μL of medium, and centrifuged onto poly-lysine coated microscope slides (Sigma 3–16 PK centrifuge with a cytolgy rotor) at 60g for 5 min at room temperature. Slides were left to air dry overnight, stained using Leishman’s stain (VWR International) and mounted. Slides were examined by bright field microscopy using a Nikon Eclipse Ti (Nikon) at 40× magnification.

2.2.3. Flow cytometry of erythroid lineage markers

Cells were sampled (1 $\times 10^5$/tube) into flow buffer (PBS containing 1% BSA) and centrifuged at 300g for 5 min. Cell pellets were re-suspended and mixed with the appropriate volume of antibody in three separate panels to a final volume of 100 μL in flow buffer, incubated for 30 min at room temperature, washed once and analysed using a BD FACS Canto™ II flow cytometer (BD Biosciences) and gated against specific isotype and fluorescence-minus-one
controls. Panels were: (A) 1.5 μL CD34-PE (BD), 1.5 μL CD123-PECy7 (eBioscience), 0.5 μL CD235a-BV421 (BD), 0.5 μL CD36-APC (BD); (B) 1 μL CD71-PE (eBioscience), 1 μL CD117-PECy7 (eBioscience), 0.5 μL CD235a-BV421 (BD); (C) 1.25 μL of 1:10 dilution of CD225-PE (BD Biosciences), 1 μL CD49d-PEVio770 (BD Biosciences), 0.5 μL CD235a-BV421 (BD) 

2.2.4. Apoptosis staining

1 × 10⁶ cells were sampled at time points throughout the intensification process, re-suspended to a total volume of 500 μL Annexin buffer containing 5 μL Annexin V-FITC (Ab-Cam) and 5 μL Draq 7 (Biostatus), and incubated at room temperature in the dark for 10 min. Samples were analysed using a BD FACScanto™ II flow cytometer (BD Biosciences), and gated against unstained cells for viable, apoptotic and dead populations.

2.2.5. Cell cycle staining

1 × 10⁶ cells were sampled at time points throughout the intensification process, permeabilized with 1 mL of ice-cold ethanol. Samples were stored at -20°C for up to one week. Samples were washed twice in wash perm buffer (WBP; BD) and stained for 30 min at room temperature in the dark in 200 μL WBP containing 5 μL Alexa Fluor 488-conjugated anti-human Ki67 antibody (B56; BD), 3 μL Alexa Fluor 400-conjugated anti-Phospho (Ser 10)-Histone H3 antibody (Cell Signalling Technology) and 25 μg 7AAD (BD). An isotype control sample was prepared containing 5 μL Alexa Fluor 488-conjugated mouse IgG1 (Biosciences) and 25 μg 7-AAD. Samples were analysed using a BD FACScanto™ II flow cytometer (BD Biosciences). This method allows discrimination of cells in a quiescent stage (G0 phase), mitotic cells (M phase) from cells in the G1, S and G2 phases [16].

2.3. Modelling and model evaluation for manufacturing

Hypotheses for alternative mechanisms of growth inhibition were proposed and expressed mathematically (as described in the results) using a tailored Ordinary Differential Equation (ODE) modelling framework previously described [17]. Proliferating pro-erythroblasts in the ambr bioreactor were subject to varying media exchange and initial density culture regimes and hourly cell counts recorded for 36 h. Candidate models were fitted to minimise least squares deviation and residuals evaluated for systematic deviation from the experimental data. The underlying hypotheses were iterated in the light of deviations between predicted and actual growth trajectories. The best fitting model was tested against independent data sets to validate predictive capability.

An ODE paradigm was used to model over time (t) the evolution of cell density (ρ) in terms of growth, and, as required, the dynamics of an inhibitory factor (I) in terms of accumulation. Media change events were modelled as step changes in ρ and I (I has an initial value of zero in fresh medium), with forward Euler used to obtain model variable evolution between these time-points. A 0.3 proportional change in medium describes removal of 30% of the medium volume and replacement of the same volume with fresh medium; this result in a reduction of 30% in the value of I immediately post exchange. Optimisation was performed via an exhaustive search of parameter space (i.e. a brute force screen of all combinations of parameter values) implemented in C#.

The values for optimally efficient full medium exchange described in Section 3.3 were calculated as follows: Model 3 (Section 3.1) was evaluated using the estimated parameter set for day 7 erythroblasts from the initial donor (r_g = 0.057 h⁻¹, kiện = 3.4, k_i = 2.6, r_d = 0.005). Cost was considered to be a composite of that related to facility time and that related to media that is:

\[ C(t, M) = C_t + C_m M \]  

(2.1)

where C is total cost, C_t is the per unit time-cost, C_m is the per unit media cost, t is time and M is the volume of media.

The efficiency of the system for a given time is defined as the cell yield per cost if the total medium is exchanged at that time i.e. the total new cells created in the system at that time point divided by the cost:

\[ E(t) = \frac{M}{(C_t + C_m M)} \]  

(2.2)

where \( \rho(t) \) is the cell density at time t.

Optimally efficient medium exchange time (and corresponding cell yield/$) was determined with systematic variation in the cost of medium volume and cost of time. This data was used to graph the impact of input costs on optimal possible efficiency (given optimal exchange time). The effect of a change in inhibitor threshold (k_i) was similarly evaluated.

3. Results

3.1. Constructing a mechanistic model of erythroblast growth and inhibition

CD34+ cells derived from cord blood were grown in culture under erythroid differentiation conditions for 7 days to generate populations of erythroblasts for growth models. Phenotype and morphology were assessed to identify the stage of erythroid differentiation. Surface marker analysis with flow cytometry indicated cells were low CD34, high CD71 & CD49d (alpha-4 integrin), with split population levels of CD36, CD117, CD233 (band 3), and CD235a (glycoporphin A). This is consistent with a heterogeneous population of pro-erythroblasts with some cells (rising band 3/CD235a) entering early basophilic stage [18] (Fig. 1). At this point cells were transferred to fresh culture medium and subject to different operating conditions: two different initial culture densities and a partial media exchange (Table 1, treatments A1-A3). Cells were counted hourly to generate high time resolution datasets to distinguish
Fig. 2. Erythroblasts were cultured under three different medium supply and cell density treatments (Table 1: A1, A2, A3) to test alternative mechanistic models of growth inhibition. Models were selected for high $R^2$ values and lack of structure in residual vs. time plots. i, ii) the cell growth data fits poorly to a model representing a simple cell density inhibition of growth (model 1). iii, iv) a model representing cell exhaustion of the media at a constant specific rate (model 2) is an improvement over model 1, but still shows significant structure in residual plots. v, vi) a model representing production of a growth inhibitor by the cells at a rate proportional to cell growth rate provides a good fit to the data (model 3).
between alternative models of growth and inhibition. As previously observed [14], cells placed in fresh medium exhibited two distinct phases of growth, a relatively uninhibited period of exponential growth followed by a prolonged period of slower growth. The simplest mechanistic hypothesis, cell density mediated inhibition of growth, was formalised as an exponential growth function with sigmoidal dose-response inhibition of growth rate by cells (Model 1).

Experimental data from all operations were fitted simultaneously (Fig. 2i).

Model 1

\[ \frac{d\rho}{dt} = r_\rho (1 + e^{k_c (\rho - \kappa)})^{-1} \] (3.1)

Best fit: Growth rate \((r_\rho) = 0.049\ h^{-1}\), Inhibition critical value \((\kappa) = 8\ \text{cells}\cdot\text{mL}^{-1}\), Inhibition sensitivity \((k_c) = 2\ \text{mL}\cdot\text{cells}^{-1}\).

Systematic structure of model residuals across experimental conditions suggests this hypothesis is not compatible with the growth data (Fig. 2ii); in particular the model under-represented the growth advantage of the partial media change in scenario A3 and overestimated the growth potential from a lower density in A1. This suggested a consideration of media exhaustion was required. The simplest hypothesis for media exhaustion is either toxification of media or consumption of a media component at a constant cell specific rate. Based on prior work indicating the likely role of an inhibitory mediator a decay parameter for the inhibition was included to accommodate this potential phenomenon (Model 2):

Model 2

\[ \frac{d\rho}{dt} = r_\rho (1 + e^{k_c (\rho - \kappa)})^{-1} \] (3.2)
\[ \frac{dI}{dt} = r_I \rho - r_d I \] (3.3)

Before analysis, the model expression is first transformed without loss of generality to express the sigmoidal \(k_c \rho^{*} = r_c (\rho | k, c ; k_c | s \gamma = r_p | p | k, c ; k_c | s \gamma = r_p | p^{-1} \) \(k, s\) dependence on the inhibitor concentration.

Fig. 3. The parameter optimisation was improved by fitting a second series of data sets including a cell density reduction (Table 1: B1, B2, B3) simultaneously with the first (A1, A2, A3). A common set of parameters were found for Model 3 to fit i, ii) data series A and iii, iv) data series B whilst maintaining high \(R^2\) values and minimal structure in residual vs. time plots.
A validation exercise was conducted to test the predictive capability of the model over a wider range of phenotypic maturities and with new operational conditions including cell density reduction and full or partial medium exchanges. New input cells required refitting of the growth rate parameter. However when this was done: i, ii) cells of different erythroid maturity had similar growth inflection points that were well predicted by the model; however the model was parameterised to represent a secondary phase of growth that only occurred in the more mature cells (as can be seen from the systematic increase in residual values after 20 h in less mature cells Day 5 and Day 7). The cell phenotype that conformed most closely to the model (day 8) was phenotypically most similar to the day 7 cells used to parameterise the model in the initial work (Table 2) iii) using day 8 cells the model could accurately predict the effect on cell growth of a complete medium exchange combined with a cell density adjustment (Table 1: C1, C2). iv) validation of partial medium exchanges showed an initially accurate model prediction of growth rate followed by some over-prediction of growth (Table 1: D1, D2, D3). v-viii) growth curves of day 7 erythroblasts from four further different donors were well predicted by the model if only the growth rate parameter was refitted for each donor (bioreactor replicates from the same donor are shown as different symbols). Dotted lines (vi, vii) show the model predicted growth trajectories obtained if using an estimate of model parameter \( r_g \) obtained from an exponential fit to four sequential data points in a manner that could potentially be applied to use early growth characteristics to predict future growth inhibition.

\[
\frac{dl^*}{dt} = \rho - r_d l^*
\]  
(3.5)  

Best fit: Growth rate \( (r_g) = 0.058 \text{ h}^{-1} \), inhibitor threshold value \( (k_c^*) = 0.04 \), inhibitor sensitivity \( (k_s^*) = 80 \) and inhibitor decay \( (r_d) = 0.004 \text{ h}^{-1} \)

(3.4)

\[
d\rho = r_g \rho \left( 1 + e^{(k_c^* - 1)} \right)^{-1}
\]
Table 2
The expression of phenotypic markers (population% positive) indicative of erythroid maturation on cell populations used for initial model development and testing/validation of different maturities and process operations. DB phenotype validation (shaded) conformed most closely to the developed model (Fig. 4) and also most closely shared the phenotype of cells used for model development (D7 shaded).

<table>
<thead>
<tr>
<th>CD34</th>
<th>CD36</th>
<th>CD117</th>
<th>CD71</th>
<th>CD235a</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7 (Model development)</td>
<td>0.9</td>
<td>91</td>
<td>59</td>
<td>97</td>
</tr>
<tr>
<td>Day 5 phenotype validation</td>
<td>10</td>
<td>62</td>
<td>79</td>
<td>90</td>
</tr>
<tr>
<td>Day 7 phenotype validation</td>
<td>1</td>
<td>84</td>
<td>75</td>
<td>93</td>
</tr>
<tr>
<td>Day 8 phenotype validation</td>
<td>0.5</td>
<td>90</td>
<td>62</td>
<td>95</td>
</tr>
<tr>
<td>Day 9 phenotype validation</td>
<td>0.1</td>
<td>92</td>
<td>38</td>
<td>95</td>
</tr>
<tr>
<td>D9 batch and partial medium exchange validation</td>
<td>0.6</td>
<td>95</td>
<td>60</td>
<td>96</td>
</tr>
</tbody>
</table>

Although an improvement on Model 1, the best fit for Model 2 still exhibited significantly structured residuals during the secondary phase of growth (Fig. 2iii, iv); the model was unable to reproduce continued relatively high growth rate late in culture. We hypothesised that this could be due to the media exhaustion or toxification occurring in a manner dependent on the cell population growth rate. This was formalised in Model 3:

Model 3

\[ \frac{dp}{dt} = r_g \rho \left( 1 + e^{(k_1^*(t - t_p))} \right)^{-1} \]  

\[ \frac{dt^*}{dt} = \frac{dp}{dt} - r_d dt^* \]  

As for Model 2, this model was partially non-dimensionalised prior to solution.

Best fit: Growth rate \( r_g = 0.0541 \) h\(^{-1}\), Inhibitor Threshold \( k_1^* = 3.4 \), Inhibitor sensitivity \( k_1^* = 5.6 \), Inhibitor decay \( r_d = 0.014 \) h\(^{-1}\).

A relatively good fit was achieved across all three growth curves with Model 3 (Fig. 2v, vi). The inhibitor decay dynamic was retained in Model 3 as this gave a minor improvement in the quality of the fit (i.e. reduced structure in residuals).

3.2. Model validation

3.2.1. Validation of predictive capability

If the proposed phenomena underpinning the developed model accurately represent dominant dynamics in the real-life system, the model should be capable of predicting cell growth through a range of operational scenarios. Certain non-intuitive outcomes would be anticipated. For example, if the cell density were reset to a lower level after growth had become substantially inhibited we would potentially observe an increased growth rate dependent on the new balance of inhibitor production and decay. If the model were inadequate, e.g. the medium was simply terminally exhausted through depletion, growth would not recover.

To test the predictive capability of the model we ran a further series of cultures (Table 1: B1, B2, B3) with cells reset to lower densities in the same medium at the predicted point of growth inhibition. The second phase growth data showed qualitative agreement with the behaviours predicted by the model i.e. an increase in growth rate when cells were reset to a lower density. However systematic lack of fit was observed with cell growth increasingly underperforming that predicted by the model the lower the experimental cell density. This would be consistent with imprecise optimisation of the inhibitor decay parameter; to improve the model the parameters were optimised including the new data set (Fig. 3).

Best fit: Growth rate \( r_g = 0.057 \) h\(^{-1}\), Inhibitor Threshold \( k_1^* = 3.4 \), Inhibitor sensitivity \( k_1^* = 2.6 \), Inhibitor decay \( r_d = 0.005 \) h\(^{-1}\).

A validation exercise was conducted to test the predictive capability of the model over a wider range of phenotypic maturities. Growth curves of cells at day 5, 7, 8 and 9 of culture (post CD34+ isolation) were tested against the model prediction (Table 2 shows the expression of key maturation markers at each time point). The cells exhibited a significantly slower growth rate than those used to build the model. However, when the growth rate was re-estimated \( (r_g = 0.037) \), with all other parameters held constant, the model reasonably predicted the growth inflexion point of all cell maturities (Fig. 4i). However, only the more mature cells (day 8 and day 9) showed a secondary growth period that agreed with the model prediction after the inflexion point. Although the model was originally developed with cells at day 7 of culture, Table 2 shows the phenotype of the day 8 cells in this validation most closely match those of the original cell population. The performance of the original model with altered growth rate cells is potentially a powerful tool as it presents the opportunity to predict process performance, and therefore adapt operation, in the event of input material driven variation.

A further validation was conducted to determine the ability of the model to predict cell growth in response to new operational conditions including cell density reduction and full or partial medium exchanges. Day 8 cells (from the same donor as above) were subject to the operational conditions specified in Table 1 and growth curves tested against model predictions. In the event of a complete medium change (Table 1: C1, C2) the model accurately predicted onward growth rates after cell density reduction, including the inflexion point in the second growth curve (Fig. 4iii). However, in the event of a partial medium change (Table 1: D1, D2, D3) an initially accurate model prediction of growth rate was followed by some over prediction of growth (Fig. 4iv). This suggests further model development may be required for accurate optimisation involving partial medium change or perfusion scenarios.

A set of historical data of erythroblast growth (day 7) from four different donors was analysed to provide insight into autologous variation (Fig. 4v–viii). Again, growth rate variability was significant requiring the growth rate parameter \( (but only the growth rate parameter) \) to be refitted to provide a predictive model of growth and inhibition in each case. A mean growth rate of 0.047hr\(^{-1}\) and standard deviation of 0.008hr\(^{-1}\) was observed. Although this variability could also be attributed to other run specific factors (e.g. growth factor batches) the predictive point remains valid; once growth rate is established the remainder of the model is robust for prediction of growth inhibition. This provides an opportunity (in the current absence of any initial measurable attribute to predict growth performance) to assess growth rate early in process and predict subsequent critical operational timings. An example of this is shown in vi and vii in which the temporal resolution of the data is sufficiently consistent to allow early assessment of growth rate. Growth rate was estimated through an exponential fit to four sequential data points. A rolling 4 data point growth rate was calculated until 2 consecutive values were less than 10% different (after approximately 8 h in both cases). This was intended to avoid any noise or deviation in the culture initiation process influencing the growth rate estimate. The model was applied to re-predict the full culture period using this early estimate of growth rate as the model parameter \( r_g \). In both cases (vi and vii) there was an 11% discrepancy between the value of \( r_g \) obtained from early data compared to the value obtained from parameterisation with the full data set and model. The model predicted growth trajectories obtained using the early estimate of \( r_g \) have a marginally lower R-squared (96% and 93% reduced from 98% and 97% respectively) compared to the prediction if parameterised with the full dataset and model. However the early estimate still offers a good prediction of growth performance. In industrial applications a focus on
accuracy of assessment of early growth rate would be anticipated to offer further improvements to this method.

3.2.2. Validation of modelled biological behaviour

The model describes inhibition of cell growth; a potential alternative mechanism with the same net growth outcome would be increased cell death. To verify the modelled biological behaviour cells cultured under condition A1 (Table 1) were sampled during the exponential growth phase (time 0), as they became inhibited (17–19 h), and several hours into the inhibition phase (26–28 h). Sampled cells were assessed for necrosis, apoptosis and phase of cell cycle [16] (Fig. 5). Although a small increase in dead or apoptotic cells was observed this was insufficient to explain the reduced growth rate through death alone and therefore supports the modelled growth inhibition mechanism. A reduced number of cells in M, G2, G1, and an increase in cells in S phase were observed to occur with growth inhibition; this supports previous reports that growth inhibition is unlikely to be growth factor related where it would be anticipated cells would accumulate in G1 [14,19]. Some metabolic restrictions, such as amino acids, have been reported to lead to prevention of cells exiting S phase; however, prior work has also indicated that simple amino acid supplementation cannot overcome growth inhibition [19].

3.3. Manufacturing operational consequence of the model

The phenomena leading to the growth behaviour have potentially complex consequences for the intensification and hence efficient manufacture of erythroblasts. The validation suggests the model will support manufacturing optimisation for a given cost (input material, time) scenario for a phenotypically bounded erythroblast population characterised by a 2-phase growth curve. Control of the medium exchange timing and cell density (based on modelled inhibitor accumulation and consequent growth) will determine production efficiency (cells per cost). The optimum media exchange time (i.e. time at which inhibitor should be diluted) for a given growth model is determined by the ratio of media cost to operational time cost irrespective of absolute values. The absolute level of optimal production efficiency will depend on the specific cost to the manufacturer of production time and medium volume.

As an example, an optimal exchange time and cells per cost is calculated for running a 1L or 100L bioreactor from a starting density of 3 × 10^6/mL with hypothetical facility and equipment operating costs of $150/hr and cell culture medium costs of $10/L (applying the original model with a 0.057 h^-1 growth rate). In the 100 L scenario, where media is more expensive relative to facility, a 16hr medium exchange gives the optimal cells per cost of 1.1 × 10^8 cells/$. As medium cost reduces as a proportion of total operating costs optimal exchange time is earlier, and the severity of consequence of missing the optimum by a given time, represented by the sharpness of the inflexion point, also changes (Fig. 6i ). This reflects the highest observed growth rate (and therefore presumably close to best cell/$ achievable); a slower growth rate would decrease cell/$ realised with the impact being greater in scenarios where time cost constituted a larger proportion of overall costs. The model can also be used to inform investment decisions by evaluating the benefit of reducing facility time cost, reduced medium cost or fundamentally engineering the cell culture system to be less self-inhibitory. The impact of a change in medium cost at various facility operating time costs (Fig. 6i) or a change in facility time cost at various medium costs (Fig. 6ii) is shown. The sensitivity to either input cost varies significantly dependent on the level of the
other; the range of potential efficiencies described varies over an order of magnitude between $3.2 \times 10^7$ to $4.7 \times 10^8$ cells/$\$. A unit of red blood cells contains $2 \times 10^{12}$ cells. As an example of the scale of the intensification challenge, to produce $2 \times 10^{12}$ erythroblasts for $5000$ under the growth restrictions identified by the model would require $54/L$ cell culture medium and a $30/hr$ operational cost or a similarly efficient combination. Medium costs will scale 1:1 with production volume whereas operational costs will scale at a lower proportion.

The model describes the initial phases of rapid growth and inhibited growth. It does not include terms that would accommodate reduction of viability and cell loss. This clearly limits extrapolation as there will be a finite time cells can stay in inhibited growth (i.e. after the growth inflexion point) without damage. We observed that after extended periods of the substantially inhibited growth phase (approximately 35–40 h) cells lose viability and absolute numbers drop. The model optimisation was therefore bounded to durations less than 30 h. Considering this limit, the maximum tolerable medium cost to achieve $5000/unit production is $12/L$ (running at a scale where medium cost is entirely dominant to time cost).

3.4. Further optimisation opportunities

Beyond reducing cost through operational improvement such as decreasing time costs or medium costs, a process developer could seek to fundamentally alter the modelled properties of the system to improve efficiency. Presuming that the fitted growth rate ($0.057 \text{ h}^{-1}, 12$ hr doubling time) is close to the biological maximum for the cells, the most plausible optimisation opportunities come from decreasing the production rate of the growth inhibitor, increasing the decay rate of the inhibitor, or reducing the sensitivity of cell growth rate to the inhibitor. Understanding the benefit of manipulating these could be used to justify a hypothesis driven
research programme. By way of example, the predicted effect on production efficiency of altering the threshold of the inhibitor \(k_{c}^{*}\) from 0.25 to 4-fold the fitted value is shown (Fig. 4iv).

4. Discussion

We have shown that a relatively parsimonious deterministic mechanistic model based on dominant phenomena can predict erythroblast growth behaviour as a consequence of medium provision and cell density strategy. The developed model was consistent with the hypothesis of an unidentified autocrine growth inhibitor and supported previous observations that mass transfer constraints or nutrient depletion are not the primary limitations of erythroblast culture productivity [14]. It further enabled cost optimisation of culture operation dependent on simple division of resource cost between operating time and input material. Of particular value, it enables rapid evaluation of the potential benefit of improving operational costs vs. changing the fundamental biological characteristics of the system through cell or medium engineering.

Several clinical opportunities for manufactured red cells have been discussed including transfusion medicine and drug delivery vehicles; both have challenging production scale requirements due to high cell doses [12, 13]. The model suggests that the magnitude of economic benefit achieved by manipulating inhibitor accumulation or reducing operational costs within a plausible range will be limited; orders of magnitude changes in model terms would be required to achieve similar benefits in productivity. The most plausible way to achieve more economic manufacturing for very high dose applications would be by identifying and subsequently blocking or neutralising the implicated inhibitory factor.

A mechanistic approach to modelling culture systems provides benefits in terms of its value in risk analysis and optimisation. The erythroblast growth behaviour within the system bounds was sufficiently simple for a deterministic and relatively parsimonious model to predict, and hence optimise, growth consequences of alternative media supply and cell density maintenance strategies. However, there are a number of important considerations for model utilisation and further development. Firstly, it is possible that several alternative mechanistic phenomena may be represented by the same model. For example, adaptive tolerance to an inhibitor or depleted factor manifests similarly to decaying inhibitor and may warrant further investigation [20, 21]. Although model based optimisation would be correct in this instance, it is an important consideration to maintain when deriving hypothesis testing experiments for further model development where such distinctions may become consequential. Secondly, it is possible that the model does not describe the underlying phenomena but still fits the test data sets. The likelihood of this is reduced via model fitting with a range of operationally distinct data sets as well as testing predictive capability and experimentally confirming growth inhibition. Confidence will increase with incorporation of further datasets, but any lack of fit should be evaluated for this underlying cause. Thirdly, any model will become inadequate if extended over a sufficiently wide operational range where non-modelled mechanisms become influential, for example cell death.

The model currently targets short term efficiency and does not consider any long-term effect that may be incurred from relative time spent in fast growth or inhibited growth phases. Differentiation trajectory and rate may be altered by feedback signalling, with the result that “optimal” operation for economic erythroblast production over a short period is sub-optimal when considering proliferative capacity and economics over a whole process [22]. However, our approach provides a platform that can be incrementally augmented as required with additional mechanisms functioning over different timeframes or that have relevance in a wider operational context. For example, incorporation of phenotypic maturity into the model, and quantitation of autocrine signalling effects on maturation in addition to growth inhibition, would enable any necessary balancing of short term and whole process production efficiency.

We note that reported proliferation rates are generally lower than those predicted by the model. Lower proliferation rates are erroneously calculated when inhibited growth phases are included (or death, where significant, is unaccounted). Growth rate is typically calculated as an average over a time period; unless the cells remain in optimal growth conditions for the duration of this period, the reported average will always be less than the maximum the cells can achieve. Generating a model that accounts for inhibitory phenomena enables more accurate determination of potential growth rate; given the importance of exponential growth to optimal productivity understanding uninhibited growth and mechanisms of reduction is essential for an informed route to optimisation. It is particularly important with the advent of immortalised progenitor lines as cell stocks to underpin CBTs [23]; these progenitors are currently predominantly selected on phenotypic characteristics for therapeutic target, however they could equally vary on growth properties underpinning manufacturing feasibility. A model framework(s) to define this would be valuable if added to early selection criteria.

In conclusion we have described an experimentally efficient approach by which key cell growth behaviour and operational consequences for manufacturing can be modelled. Such an approach and the discussed evolutions would have general relevance across therapeutic cell culture systems where feedback signals are prevalent. The approach supports a high degree of confidence in manufacturing control due to mechanistic underpinnings and is complementary and supportive of a hypothesis-driven approach to further understand influences of cell growth and commitment.

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References


