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In Vitro HUVECs Proliferation in the Presence of Various Types of Drugs: Experimental Analysis and Mathematical Modeling

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1. Introduction

The investigation of inhibitory/toxicity effect of drugs on cell proliferation is undoubtedly useful in drug testing and development. In vitro expansion of a cell population is in general an essential step for the systematic optimization of culture conditions. At the same time, a targeted investigation on in vitro mammalian cell expansion, may contribute to evaluate the effect of drugs in both the cell metabolism and the mitotic process. A specific study on the effect of drugs during cell proliferation can be particularly useful in oncology and toxicology. The evaluation of the intrinsic kinetics of cell proliferation may be performed using static cultivation system (i.e. Petri dishes). The interpretation and rationalization of the corresponding experiments may be achieved by means of suitable mathematical models (Mancuso et al., 2009). In the present work, along the lines of previous studies (Pisu et al., 2004; Pisu et al., 2006), we adopt PBE (Population Balance Equations) approach to interpret the kinetics of the investigated cell system in a quantitative fashion. A comprehensive collection of literature references on proliferating cultures reported in Mancuso et al. (2009) demonstrates that PBE modeling approach has not been adopted for quantitatively simulating stem cell culture experiments. Specifically, it has been clearly shown that the proposed population balance modeling approach is much more successful in predicting cell culture expansion with respect to classical generalized logistic equations which are based on phenomenological representations. A fortiori, to the best of our knowledge, no PBE models were developed to quantitatively interpret in vitro cell expansion in the presence of drugs. This approach has been followed by means of PBE "age structured", only to study in vitro/in vivo tumour cell growth and expansion in the presence of drugs (cf. Hinow et al., 2007). The purpose of this paper is therefore to formulate a PBE based mathematical model to quantitatively describe the response of human cells growth to several drugs, i.e. Captopril and Mevinolin (anti-cholesterol drugs), Clozapine and Risperidone (antipsychotic drugs).

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2. Experimental part

Human umbilical vein endothelial cells (HUVECs Clonetics, Lonza) were cultured in endothelial basal medium (EBM Clonetics, Lonza) supplemented with media-kit EGM-2 (Clonetics, Lonza). At confluence, cells were harvested with the use of 0,05% trypsin and 0,02% EDTA for six minutes at 37°C, and replated. The medium was changed every 2 days, and cells from passage 4 were used for proliferation studies. HUVECs were plated in 9,2 cm^2 petri dishes (Corning) at a density of $1x10^4$ cells/cm^2. Cells were maintained for a total of six days in culture. After one day of cultivation, cells were exposed for 24 hours to each drug selected. In particular, experiments were performed by adding 40μM Clozapine, 100μM Captopril, 1μM Mevinolin or 40μM Risperidone, respectively, all obtained from Sigma Aldrich. Each drug was dissolved in dimethyl sulfoxide (DMSO) which was used as control in all experiments. In particular, experiments were carried out by separately considering a neuropsychiatric drug and a cardiovascular one, while using the same control for both. A first set of experiments were performed by separately adding Clozapine or Captopril into culture, while in the second one cells were incubated with Risperidone or Mevinolin. At least six independent trials were performed for each investigation.

3. Mathematical modeling

Mathematical simulation, provided by suitable predictive models, represents an important tool to facilitate experiments, helping to find the optimal operating conditions and at the same time contributing to the understanding of biological mechanisms which affect cell proliferation kinetics and the effect of drug toxicity. The mathematical model proposed in the present work, on the basis of previous studies (Pisu *et al.*, 2004; Pisu *et al.*, 2006), describes cell proliferation and its size distribution during cultivation in batch systems. Details and symbols meaning is reported elsewhere (Mancuso et al., 2010). Assuming uniform spatially distribution of spherical cells and neglecting cell death by apoptosis, which is significant only in the case of apoptotic/necrotic cells, the following population balance equation for the cell density distribution, $\psi(m,t)$, may be written as (cf. Himmelblau and Bischoff, 1968):

$$\frac{\partial \psi(m,t)}{\partial t} = -\frac{\partial [v\,\psi(m,t)]}{\partial m} + 2\int_m^{\infty} \psi(m',t)\ \chi_M\ (m',C_{O_2})\, p(m,m')\ dm' - \psi(m,t)\,\chi_M(m,C_{O_2}) \quad (1)$$

along with the initial and boundary conditions:

$$\psi(m,\ t) = \psi_0(m) \qquad \text{for} \quad t=0 \quad \text{and} \quad m>0, \qquad (2)$$

$$\psi(m,t) = 0 \qquad \text{for} \quad t>0 \quad \text{and} \quad m=0. \qquad (3)$$

The left-hand-side of Equation (1) represents the temporal variation of the cell density distribution $\psi(m,t)$ while terms appearing in the right-hand-side represent the cell growth, the cell birth where two daughters cells are obtained by division of a larger mother cell, and the disappearing of mother cell due to mitosis, respectively. In Eq. (1) χ_M is the cell division function, $p(m,m')$ represents the unequal partitioning distribution of a mother cell of mass m' into daughters of mass m, and C_{O_2} is the oxygen concentration in the culture medium. In Equation (2), $\psi_0(m)$ represents the initial distribution of cells, while the physical meaning of the boundary condition for the PBE model given by Equation (3) is that there exist no cells of zero mass at any time (cf.

Mantzaris *et al*., 1999). The adopted PBE model considers the cell mass as internal coordinate since age-structured PBE models could not be validated through comparison with experimental data because of the well-known inability to easily and confidently measure age distribution within a population of cells (cf. Mantzaris *et al*., 1999). The unequal partitioning distribution of mother cell into daughters, $p(m,m')$, proposed by Hatzis *et al.* (1995), is taken into account:

$$p(m,m') = \frac{1}{\beta(q,q)}\frac{1}{m'}\left(\frac{m}{m'}\right)^{q-1}\left(1-\frac{m}{m'}\right)^{q-1} \tag{4}$$

where $\beta(q,q)$ and $\Gamma(q)$ are the symmetrical beta and the gamma function, respectively. The cell division function, χ_M, may be expressed by means of the following equation derived from Eakman *et al*. (1966):

$$\chi_M(m,C_{O_2}) = v(m,C_{O_2})\cdot\frac{f(m)}{1-\int_0^m f(m')dm'} \tag{5}$$

In particular, if it is assumed that cell division occurs only when cell reaches a critical mass, the probability density function of a cell of mass m to divide, $f(m)$ may be represented by a normal distribution of dividing mass around the average value μ:

$$f(m) = \frac{1}{\sqrt{2\pi\sigma^2}}\exp\frac{-(m-\mu)^2}{2\sigma^2} \tag{6}$$

being the variance indicated as σ (cf. 10,12). Accordingly to the postulate of von Bertalanffy, the time rate of change of cell mass, v, may be written as follows:

$$v(m,C_{O_2}) = \left(\frac{3}{d_c}\right)^{2/3}(4\,\pi)^{1/3}m^{2/3}\frac{\mu'\,C_{O_2}}{C_m+C_{O_2}}\Phi-\mu_c m \tag{7}$$

where the anabolic (positive) term for a single cell is proportional to its surface area ($m^{2/3}$), while the catabolic one (negative) is proportional to cell mass, m (cf. Himmelblau and Bischoff, 1968). The function Φ takes into account the cell "contact inhibition" whose expression is reported in detail elsewhere (cf. Mancuso *et al*., 2009; Mancuso *et al*., 2010). When a specific drug characterized by concentration C_I is added to the culture medium Equation (7) may be re-written by accounting for the drug inhibitory effect, through the introduction of the term $\left(1+\frac{C_I}{K_I}\right)^{-1}$:

$$v(m,C_{O_2}) = \left(\frac{3}{d_c}\right)^{2/3}(4\,\pi)^{1/3}m^{2/3}\left(\frac{1}{1+\frac{C_I}{K_I}}\right)\frac{\mu'\,C_{O_2}}{C_m+C_{O_2}}\Phi-\mu_c m, \tag{8}$$

where K_I is the inhibition constant. The numerical solution of partial integro-differential equation (1) along with the initial and boundary conditions represented by Equations (2) and (3) is performed by means of the method of lines (cf. Mancuso *et al*., 2010). The resulting system of ordinary differential equations in time is integrated by means of standard numerical libraries (Gear method, IMSL) as an initial value problem.

4. Results and discussion

Three unknown parameters, i.e. the proportionality constant, μ', of cell mass rate, the parameter α_P for inhibition contact, and σ, the value of the variance of the normal distribution of dividing mass appearing in Equation (6), have been determined using a nonlinear fitting procedure. Model parameters used in simulation run are reported elsewhere (Mancuso *et al*., 2010). The comparison between model results and experimental data (in absence of drugs, CTRL) in terms of total cell number as a function of cultivation time is depicted in Figure 1, while in Figure 2 a typical comparison in terms of cell percentage distributions (histograms) as a function of cell diameter is reported for t=2 days of cultivation time. The good agreement between model results and experimental data demonstrate the validity of the proposed modeling approach. From Figure 1 it may be also observed that the model well interprets the exponential growth which occurs during the first days of the cultivation (up to 2-3 days) and properly simulates the effect of the contact inhibition which arises after to about 3 days of cell expansion. The confluence is not fully reached after six days of culture and therefore the expansion curve doesn't display the typical plateau. As it is shown in Figure 2 the agreement between model results and experimental data is also good in terms of cell percentage distributions (histograms) as a function of cell diameter. In Figure 1 the comparison between model results and experimental data in terms of total cell count as a function of time when selected drugs are added (i.e. Clozapine 40 μM and Captopril 100 μM, respectively) is also reported. In this case, for each drug, the parameter K_I has been estimated by a specific fitting procedure (Mancuso *et al*., 2010). From Figure 1 it is possible to observe that there is a good agreement between model results and experimental data. Clozapine and Captopril both inhibit cell expansion and this is particularly true for the first one which reduces the total cell count of about 40% after 6 days. The model predictive capability is displayed in Figure 3 and 4 in terms of cell distribution (percentage as a function of cell diameter) for experimental runs performed with Clozapine and Captopril, respectively. The agreement between model results and experimental data is acceptable for the cultivation time considered.
The same approach has been followed to investigate the effect of Mevinolin and Risperidone on cell proliferation and growth. In this case, the experimental data in absence of drugs (CTRL) are entirely predicted through our model. The corresponding comparison between model predictions and experimental data (in absence of drugs, CTRL) in terms of total cell number as a function of cultivation time is depicted in Figure 5. The good agreement between model results and experimental data confirms the validity of the proposed model. As for the experimental run previously described, the confluence is not reached after 6 days of cell expansion but the effect of cell contact inhibition appears evident since 2-3 days of cultivation. Figure 5 also shows the comparison between model results and experimental data in terms of total cell count as a function of time when 1 μM Mevinolin or 40 μM Risperidone are added after 1 day of cultivation. Both drugs inhibit cell expansion while the inhibition effect is enhanced when Risperidone is used. The predictive capability of the model is demonstrated in Figure 6 where the comparison between model results and experimental data is shown in terms of cell distribution (percentage) as a function of cell diameter when Mevinolin is added during cell cultivation. Similar result has been obtained for the case of Risperidone. The agreement between model results and experimental data, as illustrated in Figure 6 seems to be acceptable. It is worth noting that the effect of selected drugs on HUVECs proliferation quantitatively described in this work (cf. Figures 1 and 5) has a

sound biological justification. Antipsychotic drugs have significant effect on cell proliferation due to dopamine D2 receptor antagonism and serotonin 5-HT2 blockade. This fact may explain the inhibition effect of antipsychotic drugs such as Clozapine (Figure 1) and Risperidone (Figure 5) on HUVECs proliferation.

For what concern the experiments performed by adding Mevinolin in the culture medium, our results demonstrate that this drug inhibits endothelial cells proliferation (Figure 5), according to the well known evidence that statins give rise to cell proliferation inhibitions.

About the effect of Captopril on HUVECs cultivation, no considerations can be made since to the best of our knowledge no information concerning this drug is available when used at low concentration level as in our experiments.

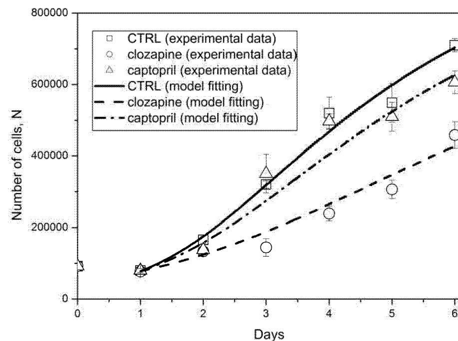

Figure 1: Comparison between model results and experimental data in terms of total cell count cultivated in Petri dish.

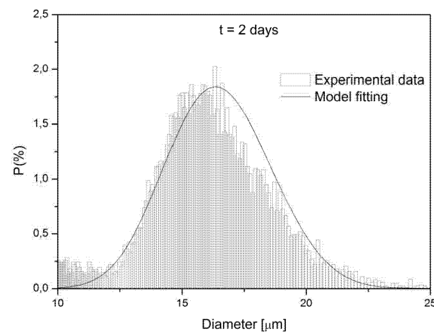

Figure 2: Comparison between model results and experimental data in terms of cell number percentage (CTRL run).

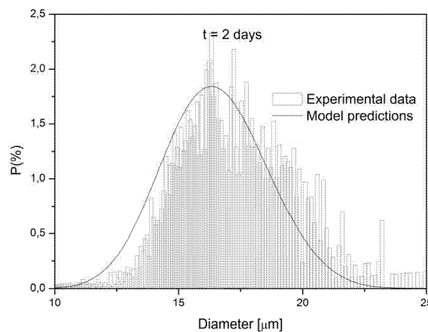

Figure 3: Comparison between model results and experimental data in terms of cell number percentage (Clozapine run).

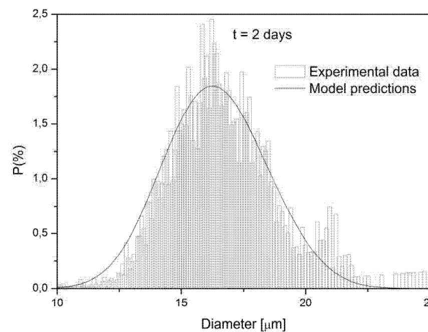

Figure 4: Comparison between model results and experimental data in terms of cell number percentage (Captopril run).

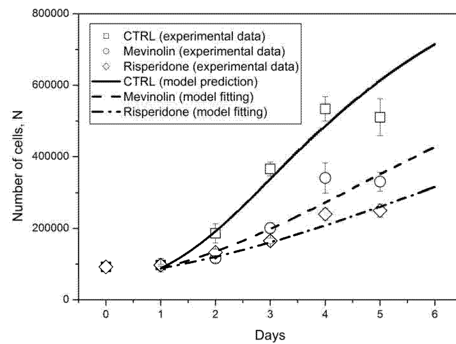

Figure 5: Comparison between model results and experimental data in terms of total cell count cultivated in Petri dish.

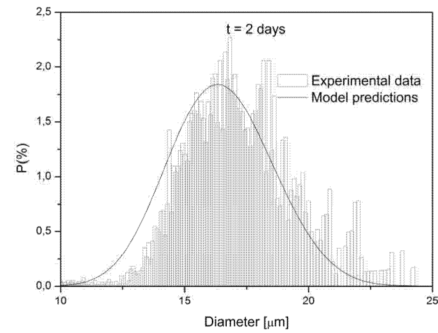

Figure 6: Comparison between model results and experimental data in terms of cell number percentage (Mevinolin run).

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