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Cancer Cell Dynamics in Presence of Telomerase Inhibitors: Analysis of *In Vitro* Data

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The inhibition of telomerase activity in actively dividing cells leads to suppression of cell growth after a time delay (inhibitory delay) required to reach a threshold telomeric DNA size. We developed a mathematical model of the dynamics of telomere size distribution and cell growth in the presence of telomere inhibitors that allowed quantification of the inhibitory delay. The model based on the solution of a system of differential equations described quantitatively recent experimental data on dynamics of cultured cells in presence of telomerase inhibitors. The analysis of the data by this model suggested the existence of at least two distinct subpopulations of cells with different proliferative activity. Size distribution of telomeres, fraction of proliferating cells, and tumor doubling times are of critical importance for the dynamics of cancer cells growth in presence of telomerase inhibitors. Rapidly growing cells with large telomeric DNA heterogeneity and small proliferating fractions as well as those with very short homogeneous telomeres would be the most sensitive to telomerase inhibitors.

Introduction

Telomeres are DNA–protein complexes at the chromosome termini, which play an important role for their stability. Telomeric DNA sequences are lost after each cell division if not restored by the ribonucleoprotein enzyme telomerase (Blackburn, 1991; Greider, 1996). Telomerase is expressed in about 80–90% of human cancers but not in somatic tissue (Brocolli *et al.*, 1995; Kim *et al.*, 1994; Shay & Bacchetti, 1997). The inhibition of telomerase activity leads to inhibition of cell growth and, therefore, telomerase can be considered as an attractive target for therapy of cancer or other pathogenically proliferating cells (Hahn *et al.*, 1999; Harley, 1997; Herbert *et al.*, 1999; Kim, 1997; Morin, 1995; Shay, 1999; Zumstein & Lundblad, 1999). Recent data have demonstrated inhibition of cell division in cancer cells and shortening of their telomeres in presence of telomerase inhibitors (Hahn *et al.*, 1999; Herbert *et al.*, 1999; Shammas *et al.*, 1999). These data not only

Abbreviations: TRF, terminal restriction fragment; PBMC, peripheral blood mononuclear cell.

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provided a proof of concept for the use of telomerase inhibitors as candidate cancer drugs but also formed a basis for a quantitative analysis of their possible effects on tumor growth.

Cells would enter crisis and eventually die only after certain number of cell doublings required for the telomeric DNA to shorten to a critical size; thus, the effect of telomerase inhibitors on tumor growth would appear only after a certain time delay (Harley *et al.*, 1994; Shammas *et al.*, 1999). This inhibitory delay T can be simply estimated as $T = t_D(L-L_c)/l$, where L and L_c are the telomere lengths at the time of inhibition initiation [typically from 2 to 30 kb (Butler *et al.*, 1996; Cuthbert *et al.*, 1999; Hahn *et al.*, 1999; Shammas *et al.*, 1999)] and at crisis [about 1.5 kb, (Counter *et al.*, 1992)], respectively; l the telomere length decrease per each cell generation [from 15 to 100 bp division^{-1}, (Allsopp *et al.*, 1992; Harley *et al.*, 1990; Shammas *et al.*, 1999; Weng *et al.*, 1995; Zhang *et al.*, 1999)]; and t_D an effective cell doubling time that for different tumors could be days or longer. Therefore, it could take weeks to years before telomerase inhibition can affect tumor growth (Shay, 1999). Only for very short telomeres, the inhibitory delay would be short. This simple estimate, however, does not account for the intrinsic telomeric DNA heterogeneity and the complexity of tumor dynamics related to the existence of growing and non-growing fractions of cells. Intuitively one can argue that telomerase inhibition would lead to early suppression of dividing cells with short telomeres. Thus, heterogeneity of telomeric DNA size should be an important factor determining the dynamic of tumor growth. The effect of telomeric DNA heterogeneity is not related in a simple manner to the inhibitory delay but also depends on the fraction of proliferating cells and the operational definition of tumor growth inhibition. Therefore, a mathematical model is needed to describe the complex interplay between telomere and tumor dynamics.

Here we propose a model that describes the dynamics of a heterogeneous population of telomeres in two cell subpopulations of distinct proliferation activities. Experimental data derived from an *in vitro* system (Herbert *et al.*, 1999) were fitted with the model which allowed to derive parameters characterizing telomere and cell dynamics. The proliferating cell fractions affected the tumor dynamics in a complex way. This analysis could help in the understanding of the tumor dynamics in presence of telomerase or other inhibitors, and the design and optimization of candidate drugs affecting conditional targets.

Model of Cell and Telomere Length Dynamics

Population of growing cancer cells consists of cells with different mean telomere lengths. Let us divide the total range of telomere length for I equal subintervals. Then the total cell population will be divided by subpopulations having telomere length from corresponding subintervals. For simplicity let us assume (as in Sidorov *et al.*, 2002a) that all cells in i-th subpopulation have the same telomere length L_i, $i = 1, \ldots, I$. In each subpopulation cells can divide with the rate a or die with the rate d when telomere length reaches the critical value L_c. It was assumed that the kinetic constants are the same for all I subpopulations of cells. The dynamics of each subpopulation can be described as

$$\frac{\mathrm{d}V_i}{\mathrm{d}t} = \begin{cases} aV_i, & L_i > L_c, \\ -dV_i, & L_i \leqslant L_c, \end{cases} \quad i = 1, \ldots, I,$$

where V_i is the volume (or number of cells) of i-th subpopulation. The total population volume can be calculated as sum of all volumes: $V = V_1 + V_2 + \cdots + V_I$.

For each subpopulation, telomere length L_i decreases by l with each population doubling (neglecting cell death) and can be calculated as follows:

$$L_i = \max(L_i^0 - alt/\ln 2, L_c),$$

where L_i^0 is the initial telomere length of cells of i-th subpopulation at the time of inhibitor application ($L_1^0 \leqslant L_2^0 \leqslant \cdots \leqslant L_I^0$). In this model, we assume that inhibitor blocks telomerase activity completely, so the inhibitory delay (time of beginning of cell growth suppression) can be calculated as a time when telomeres in first

subpopulation reach the critical value (cells in this subpopulation have the shortest telomeres at the beginning of treatment, L_I^0)

$$T = \frac{L_1^0 - L_c}{al} \ln 2.$$

This inhibitory delay corresponds to the time of the first deviation from linear increase of logarithm of total population volume. This value depends on number of subclasses (the increase of number of subclasses will lead to the decrease of L_1^0 for population with heterogeneous distribution of telomere length). The other way to introduce the inhibitory delay (T_p) is to calculate the time point when ratio between population volumes for cells treated with telomerase inhibitor and untreated cells is equal to p ($p<1$). If the distribution of telomere lengths is homogeneous (the same values for all subpopulations), inhibitory delay can be calculated easily as:

$$T_p = \frac{L_0 - L_c}{al} \ln 2 - \frac{\ln(1-p)}{d}.$$

For the model with heterogeneous distribution of telomere length, the equation is more complex and can be calculated numerically.

Let us now consider the case when each subpopulation is divided on growing and non-growing fractions $V_i = V_i^+ + V_i^-$ (Fig. 1) with telomere lengths L_i^+ and L_i^-, respectively. Let us assume that cells in the growing fraction can convert to a non-growing state with rate constants q, when $L_i^+ > L_c$ and die with the rate

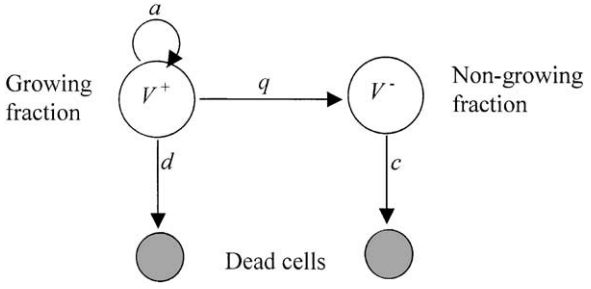

FIG. 1. Model of cell population growth with division on growing (V^+) and non-growing (V^-) fractions. Kinetic parameters: a, rate of exponential growth; d, rate of death for cells from growing fraction; c, rate of death for cells from non-growing fraction; q, rate of conversion from growing to non-growing fraction.

d when $L_i^+ \leqslant L_c$. Non-growing fraction can only die with death rate constant c. Thus,

$$\frac{dV_i^+}{dt} = \begin{cases} (a-q)V_i^+, & L_i^+ > L_c, \\ -dV_i^+, & L_i^+ \leqslant L_c, \end{cases}$$

$$\frac{dV_i^-}{dt} = \begin{cases} qV_i^+ - cV_i^-, & L_i^+ > L_c, \\ -cV_i^-, & L_i^+ \leqslant L_c. \end{cases}$$

Let us assume also that when $L_i^+ > L_c$ for any time t (including $t=0$) the ratio δ between growing and non-growing fraction of cells does not change

$$\delta = V_i^+/V_i = 1 - V_i^-/V_i.$$

It is easy to show that in this case the following equation must hold

$$(1-\delta)/\delta = q/(a-q+c).$$

The dynamics of L_i^+ can be calculated by using the same formula as for L_i: $L_i^+ = \max(L_i^0 - alt/\ln, L_c)$. For the non-growing fraction, it was assumed that the mean telomere length is determined by the decreasing telomere length of cells from the growing fraction passing to non-growing state with time

$$\frac{dL_i^-}{dt} = (L_i^+ - L_i^-)\frac{qV_i^+}{V_i^-}.$$

For simplicity, it was assumed that initial conditions for telomere lengths for both fractions are the same: $L_i^+(0) = L_i^-(0) = L_i^0$.

The proportion of cells in apoptosis (A) was calculated as

$$A = \sum_{i=1}^{I} A_i / \sum_{i=1}^{I} (V_i + A_i),$$

where

$$\frac{dA_i}{dt} = \begin{cases} cV_i^-, & L_i > L_c, \\ dV_i^+ + cV_i^-, & L_i \leqslant L_c, \end{cases} \quad \text{and } A_i(0) = 0.$$

With each cell division the heterogeneity of telomere length in subpopulation increases. One of the reasons of this increase is the existence of intracellular distribution of telomere length. Let us assume that telomeres in cell are distributed

normally for each subpopulation with mean value L_i and standard deviation $\sigma : N(L_i, \sigma)$. To calculate the number of cells in each subpopulation having the telomere length ranged from l_- to l_+ the following formula can be used

$$V_i(l_-, l_+) = V_i \int_{l_-}^{l_+} N(L_i, \sigma)\, \mathrm{d}x.$$

So, the total number of cells having this range of telomere length (including cells from growing and non-growing fractions) can be calculated as

$$V_P = \sum_{i=1}^{I} \left(V_i^+ \int_{l_-}^{l_+} N(L_i^+, \sigma)\, \mathrm{d}x + V_i^- \int_{l_-}^{l_+} N(L_i^-, \sigma)\, \mathrm{d}x \right),$$

The value of σ was interpreted as standard deviation of intracellular telomere length distribution and $\sigma = 0.3$ kb (calculated using the data in Lansdorp *et al.*, 1996) was used during the numerical simulations.

The solution of the initial-value problem for all differential equation was performed using DIFSUB_DDE code described previously in (Marchuck *et al.*, 1991). To fit the model solution to experimental data the least squares method was used. For each set of experimental data [see Fig. 2(a–c)] simultaneous fitting of model solution to experimental values of population doublings, percent of cells in apoptosis and number of cells having particular range of telomere length was used. Only three parameters were changed during the fitting: the critical value of telomere length, L_c; rate of death in non-growing fraction, c; and ratio between growing and non-growing fractions, δ. Parameter values after fitting are shown in Table 1.

Results

Recently, several groups successfully demonstrated the specific shortening of telomeres and cell death in the presence of telomerase inhibitors (Hahn *et al.*, 1999; Herbert *et al.*, 1999; Shammas *et al.*, 1999). These experiments provided data for well-characterized tissue culture systems, which could serve as a basis for development of quantitative theoretical models of tumor dynamics in presence of telomerase inhibitors. Here we analyse in detail the data reported by D. Corey, J. Shay and their associates (Herbert *et al.*, 1999). Three types of cells (human mammary epithelial cells spontaneously immortalized from an epithelial culture, HME-50-5E; prostate tumor derived cells, DU145; and HME50 cells containing the gene encoding human telomerase reverse transcriptase component, HME50-hTERT) were used in these experiments. Cells were transfected with 2′-*O*-MeRNA oligonucleotides (matched and mismatched). The number of population doublings, proportion of cells in apoptosis, and distribution of TRF lengths were measured in untreated and treated cells. To calculate the distribution of TRF distribution lengths, we used a formulae described in Sidorov *et al.* (2002b).

If cells in subpopulation grow exponentially, the number of subpopulation doublings can be calculated as $n_i = at/\ln 2$. Experimental data in Fig. 2 show that the number of doubling is increased nonlinearly for cells untreated and treated with mismatch oligonucleotides. To include this nonlinear behavior in the model we used the following approach. The solution of equation

$$\frac{\mathrm{d}V}{\mathrm{d}t} = a(t)V, V(0) = V_0,$$

where growth rate depends on time

$$a(t) = (p_0 + p_1 t + p_2 t^2) \ln 2$$

can be calculated as

$$V = V_0 \exp\left(\left(p_0 t + \frac{p_1}{2} t^2 + \frac{p_2}{3} t^3 \right) \ln 2 \right)$$

and the formula for the number of population doublings is

$$n = p_0 t + \frac{p_1}{2} t^2 + \frac{p_2}{3} t^3.$$

The values of p_0, p_1, and p_2 are shown in Table 1. So, to account for the influence of non-specific effects on cell growth the growth rate a for each subpopulation was calculated as: $a = a(t) = (p_1 + p_2 t + p_3 t^2) \ln 2$. The same dependence of a was used for cells treated with match

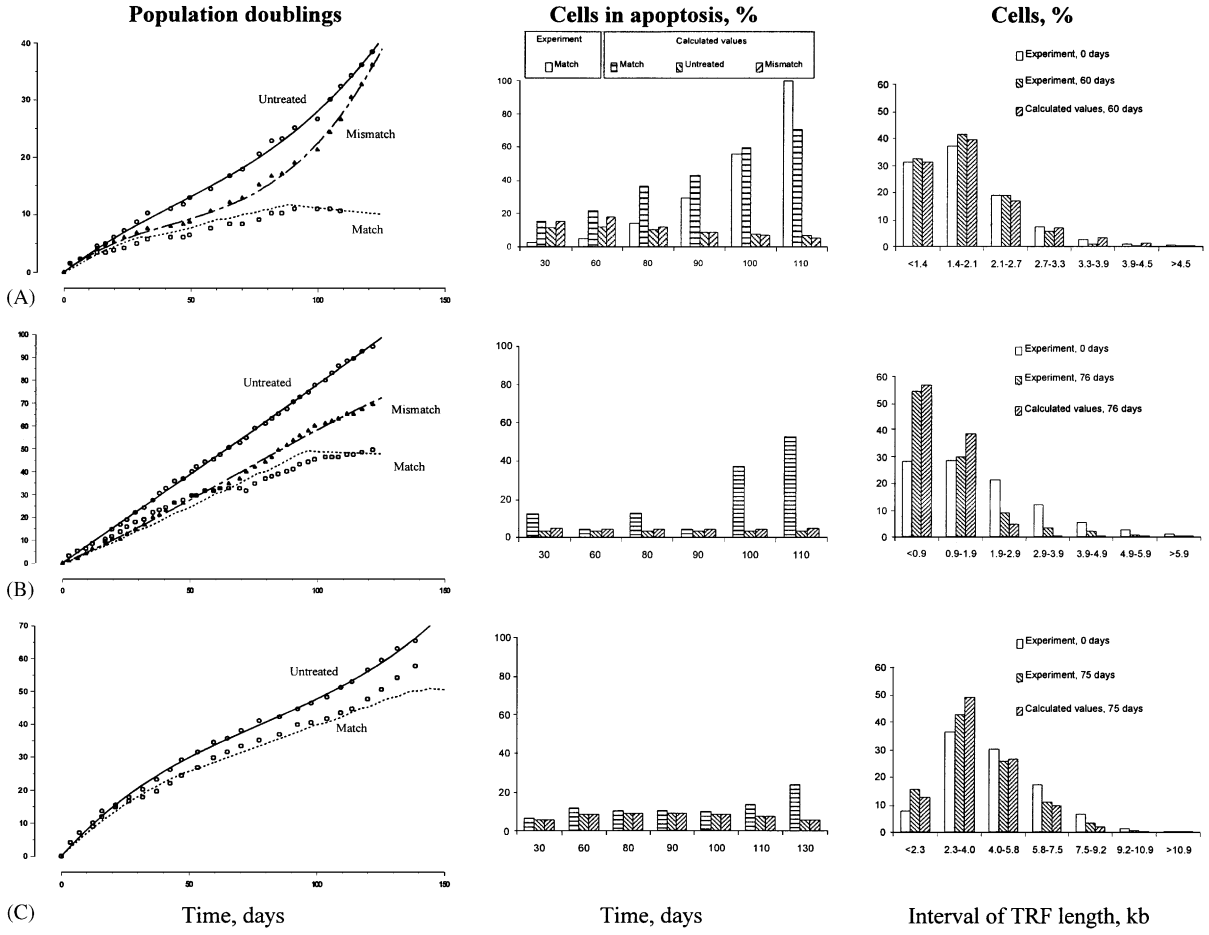

FIG. 2. Dependence of population doublings and percentage of cells in apoptosis on time and TRF length distribution for different types of cells (data from Herbert *et al.* (1999)): (a) HME50-5E; (b) DU145; and (c) HME50-hTERT. Cells were untreated (○,——) or treated with mismatch 2′-*O*-MeRNA (△,–·–·–·–), and match 2′-*O*-MeRNA (□, --------). Exponential model with dividing of cell population on growing and non-growing fractions was used for simulations. Number of doublings was calculated using the formula $n = \ln(V/V^0)/\ln 2$. The initial values of cell subpopulation having the different mean TRF length were calculated using gel images from Herbert *et al.* (1999). Parameter values are in Table 1.

TABLE 1
*Parameter values after data fitting**

Cells Experiment	L_c (kb)	c (day^{-1})	δ —	p_1 (day^{-1})	$p_2 \times 10^2$ (day^{-2})	$p_3 \times 10^4$ (day^{-3})	$T_{0.5}$ (day)
HME50-5E							
Untreated†	—	0.022	0.217	0.348	−0.524	0.589	—
Match 2′-*O*-MeRNA‡	1.16	0.022	0.217	0.341	−0.961	1.10	31
Du145							
Untreated†	—	0.000	0.215	0.776	−0.019	0.050	—
Match 2′-*O*-MeRNA	0.971	0.000	0.215	0.464	−0.465	−0.338	25
HME50-hTERT							
Untreated†	—	0.022	0.217	0.857	−1.31	0.827	—
Match 2′-*O*-MeRNA	1.16	0.022	0.217	0.769	−1.25	0.806	98

* For all the experiments: $l = 0.025$ kb division^{-1}; $d = 1\ day^{-1}$; $h = 0\ day^{-1}$; $q = (a-h+c)(1-\delta)$.
† $l = 0$ kb division^{-1}.
‡ Parameter values for mismatch 2′-*O*-MeRNA and match 2′-*O*-MeRNA are the same except $l = 0$ kb division^{-1} for mismatch case.

2′-*O*-MeRNA and mismatch 2′-*O*-MeRNA assuming that this component has no toxic effect for cells. Each cell subpopulation was divided on growing and non-growing fractions. For growing fraction, the rate of growth was increased

$$\hat{a} = \frac{a + c(1-\delta)}{\delta}$$

to compensate the existence of non-growing fraction. For simplicity, we assumed that death rate d is equal to $1\,\text{day}^{-1}$. The rate of telomere shortening per doubling $l = 0.025\,\text{kb}$ division^{-1} was used for all cell types during the calculations.

Figure 2 shows the fitting of the experimental data with our model by using the parameter values presented in Table 1. A notable feature of the fitting was that with the same parameters we were able to describe the dynamics of three independently measured variables: number of population doublings, proportion of cells in apoptosis and the dynamics of TRF length distribution. This suggests internal consistency and good representation of the cell and telomere dynamics by the theoretical model. An essential finding was the existence of two (at least) fractions of cells: growing and non-growing. It was not possible to fit the data within the framework of our model without using this assumption. The cell dynamics represented by the number of doublings was described quantitatively. The percentage of cells in apoptosis was about 2–12% (except two values for HME50-5E cells) for cells untreated and treated with 2′-*O*-meRNA containing mismatch bases. This theoretical values correspond to the values obtained experimentally in Herbert *et al.* (1999): 2–3%. The theoretical dynamics of apoptotic cells corresponds quantitatively to the experimental results [HME50-5E, Fig. 2(a)] and has the same behavior for DU145 and HME50-hTERT cells [Fig. 2(b) and (c)]. The dynamics of TRF length distribution for all types of cells were also adequately fitted by the model solutions. All distributions are non-symmetrical and display a shift to smaller values of the mean TRF length with time; histogram values increase for small values of TRF length and decrease for the large ones with time. The inhibitory delays $T_{0.5}$ for each type of cells were also calculated (Table 1). These delays correspond to the 50% decrease of the total cell volume for experiment when match 2′-*O*-MeRNA oligonucleotides were used with respect to the volume of cells growing exponentially without inhibitor. One can see that the inhibitory delays for HME50-5E and DU145 cells are close (31 and 25 days, respectively), and it is more than 3 times higher (98 days) for the cells containing the gene encoding human telomerase reverse transcriptase component (HME50-hTERT cells).

Discussion

Recent advances in inhibition of telomerase activity (Hahn *et al.*, 1999; Herbert *et al.*, 1999; Norton *et al.*, 1996; Shammas *et al.*, 1999; Zhang *et al.*, 1999) provided a quantitative basis for development of mathematical models which may help in the development of novel approaches for cancer treatment *in vivo*. Previous mathematical models (Arino *et al.*, 1995; De Boer & Noest, 1998; Kowald, 1997; Levy *et al.*, 1992; Olofsson & Kimmel, 1999; Pilyugin *et al.*, 1997; Rubelj & Vondracek, 1999; Wolthers *et al.*, 1999) have examined various aspects of telomere shortening but have not analysed tumor growth in presence of telomerase inhibitors partly because of lack of appropriate experimental data. The model presented in this article describes quantitatively and simultaneously important dynamic features of dividing populations of cells in presence of telomerase inhibitors obtained under well-defined conditions *in vitro* by independent methods: number of cell doublings, proportion of dead cells and telomere length distribution dynamics. The data can be fitted by the model, which allowed derivation of biologically reasonable parameters and suggested the existence of growing and non-growing fractions of cells.

Two major assumptions were essential for this fitting: heterogeneous telomere length distribution in the cell populations and existence of at least two fractions of cell (growing and non-growing one). The first assumption is supported from experimental data and the second one is inferred by fitting of the data. Figure 3 shows population dynamics for varying degrees of telomere length heterogeneity and ratio between

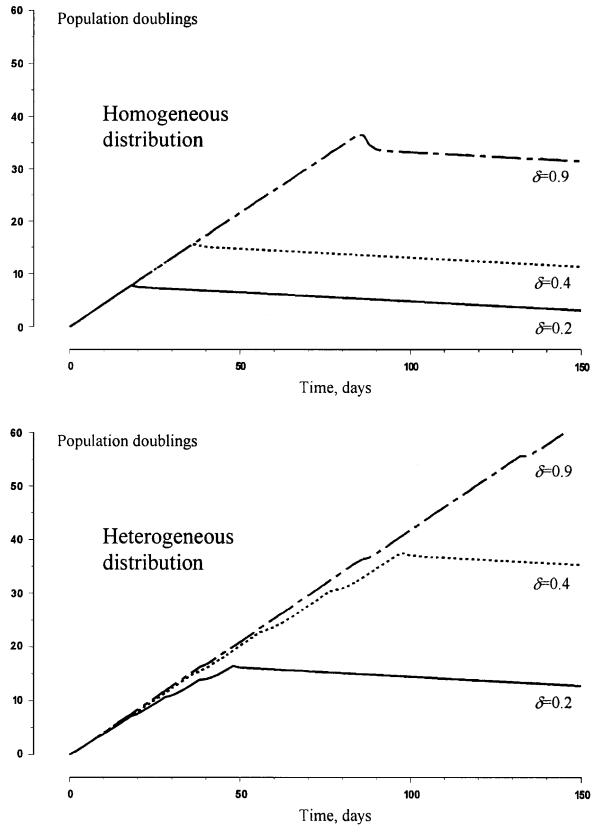

FIG. 3. Dependence of population doublings on ratio between growing and non-growing fractions of cells for homogeneous (all cells have the same telomere lengths) and heterogeneous telomere lengths distribution. Parameter values are in Table 1, rate of cell growth a ($0.3\,\mathrm{day}^{-1}$) does not depend on time.

growing and non-growing fractions [note that the following values of rate of cell growth, $\hat{a} = (a + c(1-\delta))/\delta$ were used for different values of the ratio δ]. The heterogeneous distribution of telomere length is necessary for describing the very early decrease in population doublings (existence of cells with shorter than the average telomere length) as well as the delay in reaching of critical value of telomere length (existence of cells with longer that the average telomere length). The time of reaching the critical value of telomere length was also dependent on the ratio δ both for homogeneous and heterogeneous distribution of telomere lengths. The first attempts to fit the data without dividing on growing and non-growing fractions led to increased values of rate of telomere length shortening up to $100\,\mathrm{bp\,division}^{-1}$ and lack of fitting of the heterogeneous telomere length distribution. As it is shown in Fig. 3, the effect of telomerase inhibitors is stronger for the low values of the ratio δ. So, rapidly growing cells with large telomeric DNA heterogeneity and small proliferating fractions as well as those with very short homogeneous telomeres would be the most sensitive to telomerase inhibitors. The rapid cell cycling for growing cells can be a strategy for compensation for high apoptotic rate (Shackney & Shankey, 1999).

Telomeres in tumors can be longer, shorter or of the same length as in normal cells and their length varies from patient to patient (Adamson *et al.*, 1992; Butler *et al.*, 1996; Hiyama *et al.*, 1995; Huang *et al.*, 1998; Nurnberg *et al.*, 1993; Remes *et al.*, 2000). In some cases telomere lengths are negatively correlated to telomerase activity (Bechter *et al.*, 1998) but in general such correlation has not been observed. For tumor-derived and *in vitro* immortalized cell lines it was found that both telomere lengths and telomerase activity vary significantly and levels of telomerase components or telomerase activity were not predictive of telomere length (Savre-Train *et al.*, 2000). Chemotherapy can reduce the level of telomerase in tumor cells whereas telomerase activity was detected in most tumors after surgery (Albanell *et al.*, 1997). Therefore, the effects of telomerase inhibitors *in vivo* are expected to be highly variable. Our model, however, allows of making general predictions that may help in the design of clinical protocols and could raise the optimism for the use of telomerase inhibitors in the clinic.

First and most important, estimations based on the model suggest that simple calculations based on the assumption of homogeneous telomere length distribution may lead to gross overestimation of the length of the inhibitory delay.

Second, the proliferating fraction is of critical importance for the effect of telomerase inhibitors. Smaller proliferating fractions would require more cell divisions and therefore would be more sensitive to the effect of the telomerase inhibitors. The quantitative evaluation of the proliferating fraction by the model confirms this intuitive reasoning. However, it also suggests that the two effects, telomere heterogeneity and proliferating fractions, are somewhat synergetic.

A note of caution is that some of the assumptions of the model which are valid for the relatively simple *in vitro* system could not be relevant for the more complex *in vivo* system, e.g. the possibility for changes of the proportion of proliferating fraction of cells with time. In addition, the efficacy of these inhibitors is still under question. Our model assumes complete inhibition of the telomerase which may not be the case; however, this effect could be easily taken into account by the model by simply renormalizing the rate of telomere shortening per cell division and introducing an effective rate dependent on the inhibitor efficacy. A variety of other effects, which are not taken into account by this model, could also affect tumor growth. For example, the influence of telomerase inhibitors on immune system is also still under investigation. This effect can be controversial: reverse transcriptase inhibitors such as azidothymidine and 3′-deoxy-2:3′-didehydrothymidine decrease the growth of leukemic cell lines and PBMC while dideoxyinosine has no effect on Jurkat cells but increases growth of PBMC (Beltz *et al.*, 1999). The reverse transcriptase inhibitors azidothymidine and carbovir can block telomerase function in various cells, whereas dideoxycytidine does not exhibit such activity (Yegorov *et al.*, 1999).

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