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Reversal of Hepatoma Cells Resistance to Anticancer Drugs is Correlated to Cell Proliferation Kinetics, Telomere Length and Telomerase Activity

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Abstract. *Background: Clinical and experimental observations indicate that resistance to anticancer drugs may be spontaneously reversible over time. Materials and Methods: This work is a mathematical and statistical analysis of the relationship, during a 9-month experiment, between the resistance of repeatedly re-seeded hepatoma cells to methotrexate (MTX) or to cisplatin (cisP) and untreated cell proliferation, telomere length and telomerase activity. Results: All variables showed complex oscillations, as previously published. In this work, cell proliferation was modelized by the logistic model, and the proliferation rates (a-values) together with their variations (va-values) were calculated. Conclusion: Significant correlations were discovered between cell resistance to treatments and a-values, va-values, telomere length and telomerase activities. These results open new insights into the handling of chemotherapy in the treatment of cancers.*

Cell resistance to anticancer drugs is a major problem in chemotherapy. Generally, the cellular toxicity of anticancer drugs is, directly or indirectly, associated with the proliferation status of the cells (1, 2). Cell sensitivity also depends both on the drug active principle and metabolism and on the permeability and other properties of the various cell types. For a long time, resistance has been considered as an invariant characteristic of the tumor and of the drug used. However, recent reports indicate that resistance to treatment, which depends on genetic alterations and epigenetic traits, may change over time. This reversibility was reported for different tumor cells, both *in vivo* and *in vitro* (3-7). Various cell functions and characteristics, including telomere length and cell growth rate, show marked fluctuations over time and may contribute to the reversibility of resistance to anticancer drugs. We previously reported the long-term oscillating growth pattern of cultured Fao hepatoma cells, repeatedly re-seeded at fixed 6-day intervals for 9 months, and the oscillations of telomere length and telomerase activity, which were measured at the beginning of each reseeding. We demonstrated that these apparently disordered oscillations of the 3 variables were regulated and probably chaotic in nature (8, 9). The resistance of these cell populations submitted to the anticancer drugs methotrexate (MTX) and cisplatin (cisP) at each passage varied markedly, with a different pattern for each drug (10). We also showed that, when MTX resistance and variations of proliferation rate were plotted *versus* telomere length (MLT), the peaks of resistance and growth accelerations were associated with specific telomere lengths, separated by 350 bp intervals, thus incriminating a periodic telomere structure (10). In the present report, the relationships between resistance to MTX or to cisP and cell proliferation rate, telomere length and telomerase activity were investigated, in order to portray the cell state at a given time-point, as a predictive tool for chemosensitivity to anticancer drugs.

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Key Words: Hepatoma, methotrexate, cisplatin, telomeres, telomerase, drug resistance, dynamics, gene amplification.

Materials and Methods

The experimental methods for obtaining the previously published data (8-10) are briefly described below.

Culture conditions. Cells of the rat hepatoma Fao clone are stably differentiated and tumorigenic (11, 12). The protocol included 43 cell reseedings over passages of 6 days each. On day 1, cells were seeded at a density of 10^5 cells/8.5 cm diameter dish in Ham F-12/Coon medium containing 5% fetal calf serum. The medium was changed every other day. On day 6, the cells were detached with trypsin, counted and re-seeded in duplicate dishes at the same initial density. The proliferation data (number of population doublings per passage) and their analysis have been reported (8).

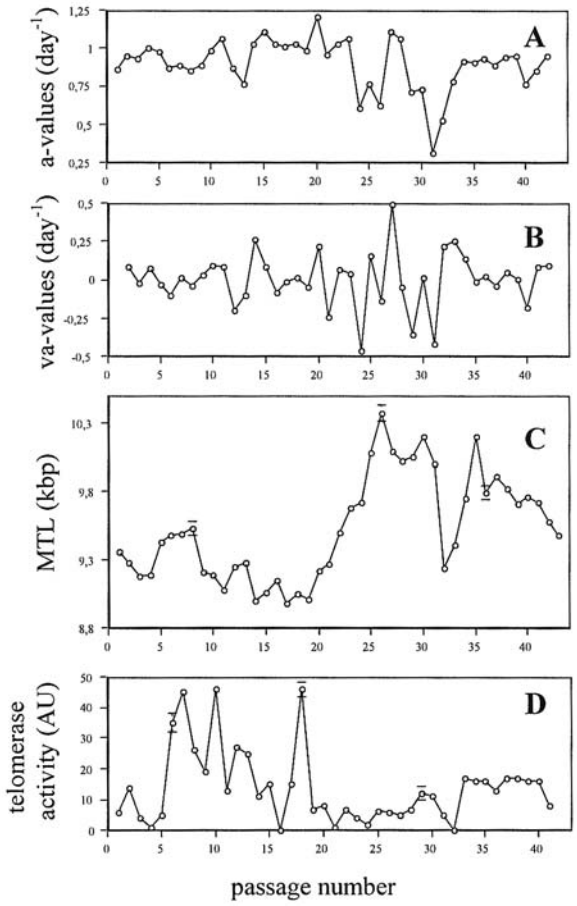

Figure 1. *Time-patterns of cell growth rate, telomere length and telomerase activity. Abcissa: passage number. Ordinates: A : proliferation rate (a-values), B : va-values, C : mean telomere length calculated as described in the text, and D : telomerase activities. Mean telomere lengths are in kilobase pairs (kbp), telomerase activity in arbitrary units and a-values and va-values are in day^{-1}. Experimental data shown are mean±SD of 3 measures.*

Treatment with anticancer drugs. At the beginning of each passage, samples of the cell population were inoculated in triplicate dishes and subjected to cisP and MTX. The cell density and drug dosage were the same for all passages. The modes of treatment were : i) short exposure to cisP and a seeding density of 10^5 cells/dish. The next day, samples were incubated for 2 h in medium containing 50 μM cisP. Cells proliferated afterwards under standard conditions (with no cisP) for 3 weeks. ii) Continuous exposure to MTX with a seeding density of 5.10^5 cells/dish. Cells were continuously cultured in medium containing 15 nM MTX for 3 weeks. Drug resistance was defined as the number of colonies observed at week 3 after treatment / number of treated cells.

Telomere length and telomerase activities. Briefly, at each passage, DNA and cell extracts were prepared for telomere length analysis by Southern blotting and telomerase assay. The mean length of telomeres (MTRL) was calculated by integrating the signal intensity above background over the entire Telomere Restriction Fragment (TRF) distribution as a function of TRF lengths:

$$MTRL=\Sigma\ CPM_i: \Sigma[CPM_i : (L_i i - X)]$$

where CPMi, Li and X are the signal (counts/minutes) at passage i, the TRF length at passage i and the mean sub-telomeric length, respectively.

Telomerase activity was determined using the TRAPeze Telomerase Detection Kit (Oncor, Gaithersburg, USA) and the Telomeric Repeat Amplification Protocol (TRAP). Three assays were performed for each sample, using from 0.25 to 1 μg of protein extract, which provided a linear range response. Controls included heat-inactivated extract, internal PCR amplification controls and PCR contamination controls.

Problems of interest; method of analysis. The apparent disorder of cell growth fluctuations, telomere length and telomerase activities over the 43 passages made it difficult to directly analyze the properties of cell resistance to anticancer drugs for the observed data. Therefore, we proceeded in several steps. First, cell proliferation within each passage was modelized by a population kinetic model and the model proliferation rate, a_i-values (see below) calculated. Changes in proliferation rate from passage to passage are discussed in the Results section. As these changes might be related to gene amplification events and drug resistance (see below), the variation of proliferation rate from one passage to the next, $va_i=a_{i-1}-a_{i-2}$, was also defined. In the second step, the relationships between a_i , va_i and the resistance to drugs, telomere length and telomerase activities were analyzed.

The classic Pearson correlation, r-values, were calculated. To test the statistical significance of the r-values, two methods were employed. The first method read the *p*-values in standard statistical tables. As the variables implied in the analyses, *i.e.* the resistances to drugs, cell proliferation rate, cell genetic amplification, telomerase length and telomerase activities, are not all normally distributed, some transformations of these variables were first performed to obtain variables that are normally distributed (see details below). The second method used was the Monte Carlo simulation. Briefly, to estimate the dependence between 2 variables x and y, the Pearson correlation coefficient r(1) between the observed data x and y was calculated first. The order of the variables was randomly perturbed over 1, 2,..., n-1 times and the correlation coefficients r(2), r(3), ...r(n) of the randomized series were calculated. In the actual calculations, perturbations were repeated up to 4999 times to get a total of 5000 r-values. Let n1 be the cases where the r-values are more extreme than r(1) [larger than r(1), if r(1)>0 and lower than r(1), if r(1) <0]. If n1 is small (n1/5000<0.05), the probability that random series can give an r-value more extreme than the observed value r(1) is low; we thus conclude that the observed data are significantly correlated.

Calculations of the cell proliferation rate and the determination of the transformations to normalize the statistical distribution of an observed variable are presented below.

Model of the proliferation dynamics. We assumed that the hepatoma cell dynamics in each 6-day period obey the classical logistic model, $dN(t)/dt=aN(t)(1-N(t)/K)$, where N(t) is the number of cells at time t, and a and K are constants. Constant a is the rate of cell proliferation, while K reflects the maximum capacity of the culture medium to support the cell population. From the culture conditions, we assume that K is the same for all 43 passages. The solution of the logistic equation is $N(t)=K/(1+(K/N_0-1)\exp(-at))$, where N_0 is the number of initial cells. We used 10^6 as the unit for the cell

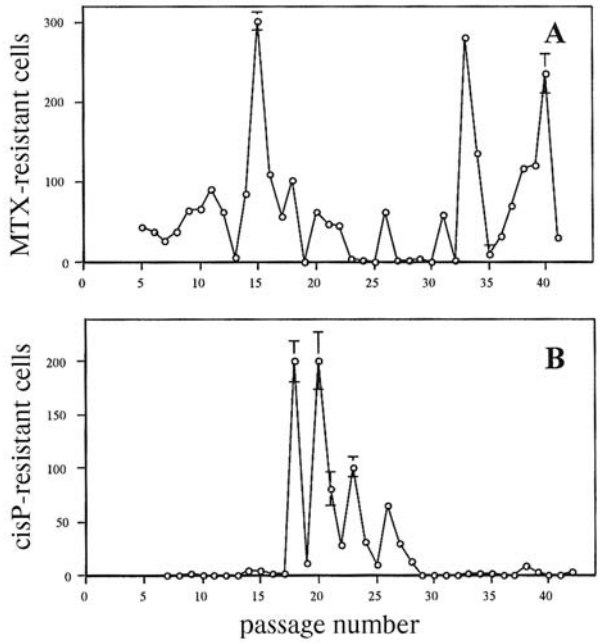

Figure 2. *Time-pattern of resistance to MTX and cisP. At each passage, aliquots of the cells were seeded in a series of dishes and subjected to various drug treatments. Abcissa: passage number. Ordinates: A: number of cells resistant to the permanent treatment with MTX per 5 10^5 cells tested; B: number of cells resistant to the short treatment with cisP per 10^4 cells tested. Data are means and standard deviations for 3 dishes. The tests started at passage (p) 5 for the MTX series and at p7 for the cisP series.*

population, and day as the time unit. The 43 passages were indexed by the letter i, and the model constant a in passage i was denoted by a_i. At day t=0, we started with N_0=0.1 unit, then $N_1(t)$ increases following the logistic model, with constants K and a_1, until day t=6 where $N_1(6)$ was measured. On day 6, we took a portion N_0=0.1 of $N_1(6)$ and recommenced the same procedure until the next sixth day. The dynamic $N_2(t)$ again follows the model equation, with constants K and a_2. The protocol continued until passage 43. From the knowledge of N_0 and Ni(6), the constant a_i can be determined if K is known. Since the highest observed Ni(6) is about 13.5, we first chose the level K=15 for K, and with this K-value, we calculated the a_i.

The a-values are the rate of cell proliferation at the beginning of the passage. The variation of a-values was defined by taking the change in proliferation rates in the preceding passages: $va_i=a_{i-1}-a_{i-2}$. The a-values (and therefore the va-values) depend on the choice of K.

Transformation of variables. There are many possibilities for transforming the variables. The most commonly used are y=exp(x) and y=log(b+x), where x is the observed data and b is a constant. The first equation is used when the histogram of x is shifted to the right, and the second equation is used when the histogram of x is shifted to the left. When the logarithm is taken, we looked for constant b to ensure a normal distribution of y. The normalcy of y was tested by the Chi-square test. A positive constant b was used to avoid calculating the logarithm of zero (when some x values were zero).

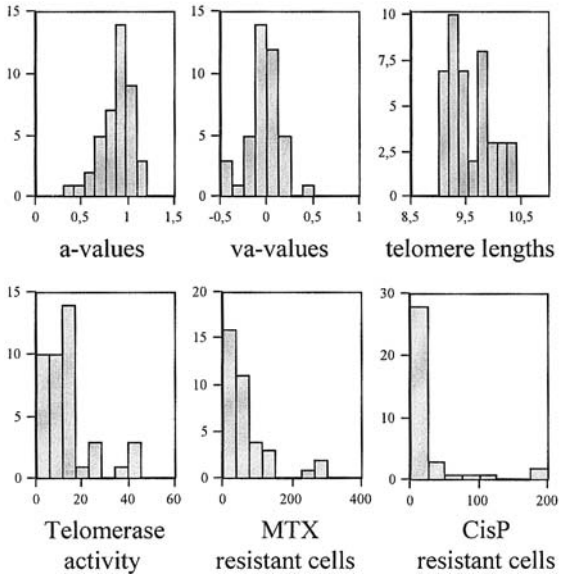

Figure 3. *Histograms of the a-values, va-values, telomere length, telomerase activity, MTX and cisP resistance. Units of the variables are indicated in Figures 1 and 2.*

Results

Proliferation of kinetic, telomere length and telomerase activities. The a-values of cell proliferation, va-values, telomere length and telomerase activities over the 43 passages of cell cultures are provided in Figure 1A-D. The 4 series show quite complex variations. It should be noted that the va-values include positive and negative figures. The data in parts C and D have been previously reported and discussed (9). The complex relationships between the dynamics of cell proliferation, telomere length and telomerase activities will be modelized and discussed elsewhere. Here, we focused on the relationships between cell dynamics and resistance to drugs.

Resistance to drugs. Drug resistance is shown in Figure 2. The resistance varied along the passages with quite different patterns for the 2 drugs (note that the 2 treatments are also different). After continuous exposure to MTX, resistant cells presented several persistent, ungrouped potent peaks. After short exposure to cisP, resistant cells developed a grouped bundle of consecutive peaks. The outcome of the patterns after the 43rd passage is unknown.

Transformation of the variables. The histograms of the 6 variables are provided in Figure 3. The histogram corresponding to a-values was slightly shifted to the right, while the va-values had an apparently normal histogram. The histogram of telomere length was between normal and exponential, while the 3 remaining histograms were clearly

Table I. *Correlation coefficient for the relationship between (i) drug resistance to MTX or to cisP and (ii) cell proliferation rate (a-values), cell proliferation rate variations (va-values), telomere length and telomerase activities. P-values read from tables are given in parentheses. The p_{sim} are p-values calculated by the simulation method (see text for details)*

	a-values	va-values	telomere length	telomerase activities
MTX resistance	0.20 (NS)	0.40 ($p<0.001$)	–0.36 ($p<0.05$)	0.35 ($p<0.05$)
p_{sim}	(0.23)	(0.016)	(0.034)	(0.033)
cisP resistance	0.42 ($p<0.01$)	0.15 (NS)	0.07 (NS)	–0.39 ($p<0.01$)
p_{sim}	(0.012)	(0.39)	(0.67)	(0.020)

decreasing exponentials, with low and comparable slopes for telomerase activity and resistance to MTX, and a very high slope for cisP resistance. The va-values were kept unchanged, an exponential transformation was tested for the a-values and a logarithmic transformation was tested for the 3 other cases. No logarithmic transformation could improve the histogram of telomere length and, thus, this variable was not transformed. After some tests, we used the equation y=log(b+x), with b=6 for telomerase activity, b=2 for the resistance to MTX and b=0.01 for the resistance to cisP. We have verified, by Chi-square, that the distributions of va-values, telomere length and the 4 transformed variables are not significantly different from a Gaussian variable.

Resistance to treatment and cell characteristics. The relationships between drug resistances and cell proliferation, telomere length and telomerase activities are shown in Table I, where Pearson r-values are displayed, with probability levels obtained from statistical tables given in the parentheses. As expected, the relationships are quite different for the 2 drugs. MTX resistance was not correlated to a-values, but was correlated to va-values, while resistance to cisP was correlated to a-values, but not to va-values. In addition, MTX resistance was correlated to both telomere length and telomerase actitivies, while resistance to cisP was correlated only to telomerase activities. Some scatter plots are given in Figure 4. It can be observed that the data points are regularly displaced along the regression lines, and the significant relationships are rather convincing.

By using the Monte Carlo simulation, the probability *p*-values of the 8 above relationships ($p=n1/5000$, see Methods) were obtained (see Table I, p_{sim} values). It is clear that the results of the simulation method are entirely consistent with the Pearson method.

Note on the role of the constant K used in the kinetic modelization. In the Methods section (modelization of the proliferation dynamic), we indicated that the calculation of the proliferation rate, a_i, and hence, the va_i, depends on the choice of the constant K. We verified that, by using K values from 13.5 up to values as large as 20, all a_i's were only very slightly modified and this did not change the statistical significance of the correlation study, implying the a-series or va-series in Table I.

Discussion

Our data indicate that cell resistances to MTX and cisP are not constant, but rather exhibit time fluctuations with large amplitudes. Furthermore, the time-pattern of these fluctuations define different windows of resistance for the 2 drugs (10). The present analysis disclosed several significant relationships between cell resistance to MTX and cisP and some characteristics of the cell kinetics.

Although the Pearson statistic is commonly used, the normalcy of the variables involved is not always discussed. It can be seen in Figure 3 that some variables included in the proposed correlation analysis were not normally distributed. Of course, the Pearson r-value between variables that are not Gaussian can be calculated, but the corresponding *p*-value (to assess the so-called H0 hypothesis of no correlation) must be calculated by a specific method corresponding to the given distribution of the variables. Standard tables of statistical significance (*p*-values) have been constructed for normal variables, and should not be used when the variables are not distributed normally. In addition, the data used in our study are time-series and may be auto-correlated. It is difficult to isolate the part due to such auto-correlation in the meaning of the *p*-value read from statistical tables. Therefore, we have used the simulation method to estimate the significance of the correlation. In the randomly perturbed series, not only the pairing between the two variables, but also the serial correlation in the variables was destroyed. The consistency of the 2 methods suggests that auto-correlation in the data, if any, may not play a significant role in our relationships. This consistency, together with the scatter plot of the data in Figure 4, should be convincing to the reader.

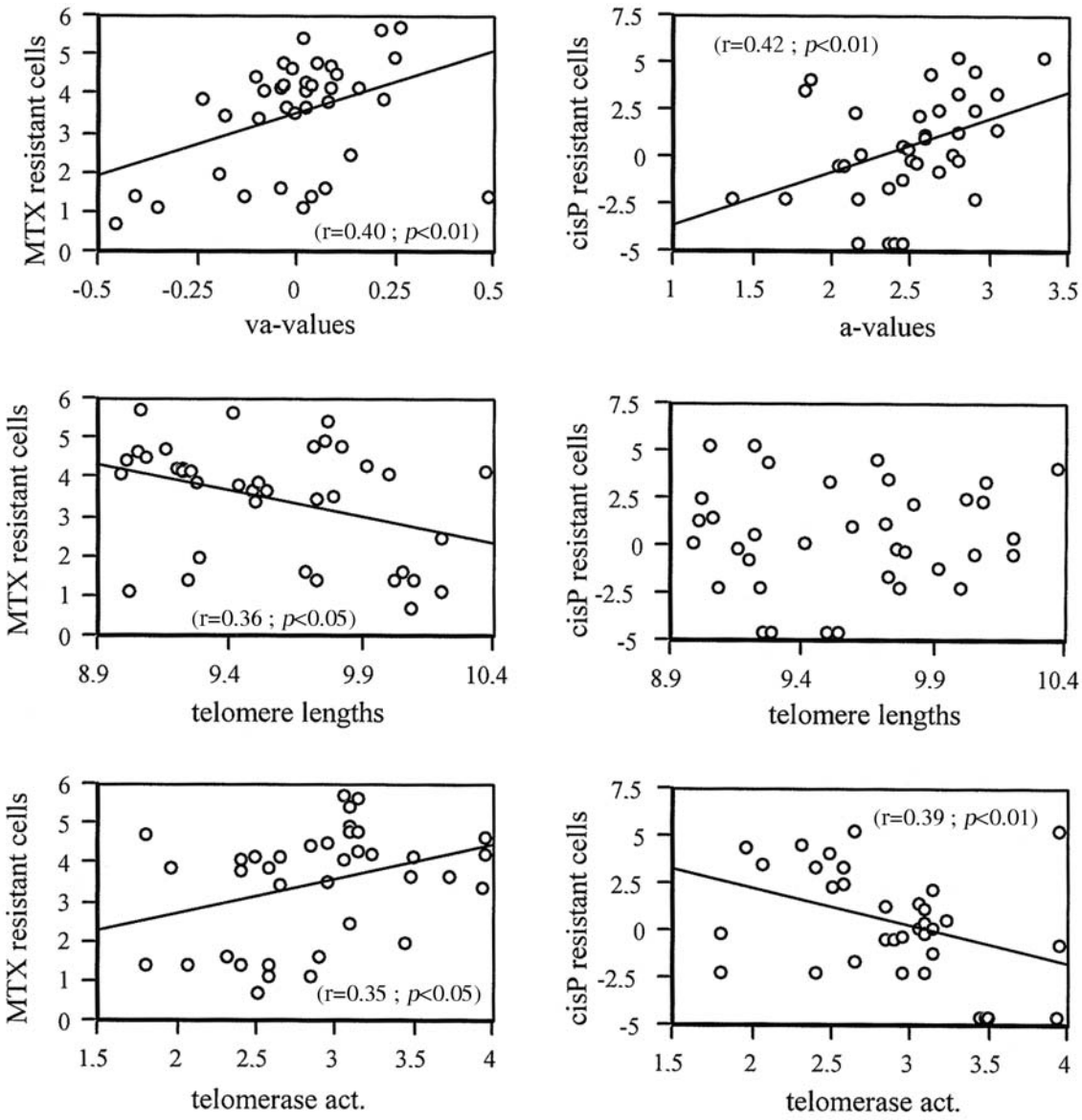

Figure 4. *Correlations between resistance to MTX and cisP versus cell growth rate, variations of growth rate, telomere length and telomerase activity. Left: MTX resistance. Right: cisP resistance.*

It is not surprising that cell resistance is not the same against MTX and cisP. First, the two modes of treatment are different. Permanent and short treatments with cisP were also performed on other shorter series. The results indicate that the fluctuations of the frequency of resistant cells evolved in parallel (data not shown). Therefore, the different time-patterns of resistance to MTX and cisP reported herein are not relevant to the protocols. Second, the modes of action are not the same. MTX inhibits the dihydrofolate reductase (*dhfr*) enzymatic activity and thus the replicative process (S-phase of the cell cycle). CisP interacts with DNA and arrests cells in G2/M. Resistance to cisP has been associated with drug efflux, overexpression of topoisomerase, mismatch repair system, glutathione mediated detoxification or amplification of a series of genes (including ABC-transporter encoding genes) (see ref. 13 for a review).

Resistance to MTX has been associated with mutations which affect cell permeability to the drug, mutant alleles of the *dhfr* gene encoding an enzyme with a lower affinity for the drug and overexpression of *dhfr* associated with amplification of the gene (see ref. 14 and 15 for reviews). In fact, during the experimental procedure, 2- to 3-fold

amplification of the *dhfr* gene was observed for 3 clones and 2 pools of resistant cells selected at different passages (10). The correlation between va-values and MTX resistance involves the amplification of the *dhfr* gene as a major mechanism of resistance to the drug. In this context, our results may be compared with a series of studies which demonstrate that resumption of cell growth after an inhibition enhances the frequency of amplification events including the *dhfr* gene (16-20).

Cell resistance belongs to a complex system involving several factors, including telomere length regulation. No clear cut, highly significant relationships should be expected from our qualitative observations of such a complex system. We have demonstrated a negative correlation of MTX resistance with MTL, and a positive correlation with telomerase activity. This must be compared to our previous analysis, which indicated that peaks of MTX resistance are associated with periodic telomere structures of 350 bp and 180 bp, presumably linked to the nucleosomic organization (10). In a preceding report (9), we showed that the fluctuations of telomere length and telomerase activities along the whole protocol have an inverse timing. Telomeric structure affects sub-telomeric and other gene transcription rates in yeast and human cells (21-23). This may set up a cascade of regulatory effects, ultimately leading to the genesis of resistant cells. Furthermore, telomerase modulates the expression of genes controlling cell proliferation (24, 25), and *c-myc*, a transcription factor involved in the control of cell proliferation, activates telomerase expression (26, 27).

Integrating our results leads to the following interpretation of the connection between telomeres and MTX resistance. The replicative telomere erosion alters nucleosomes, and nucleosomal content affects the transcription of genes as a result of cis or trans effects (21-23). For instance, the promoter of *c-myc*, which includes *myb* sites, may be up-regulated by *myb* domains of telomeric proteins (28). Intermittent telomere repair would, thus, lead to fluctuations in cell proliferation and, ultimately, to gene amplification events.

The search for a correlation between telomerase activity and cisP resistance in different tumoral cell systems has been the topic of a series of reports with contradictory results. Some data reveal a positive correlation between enzymatic activity and cell resistance (29-31). Authors interpreted this link by a better restoration of cell cycle progression and cell recovery from drug-induced damage by fast growing cells with telomerase activity (29). The inverse correlation has also been reported (32, 33). However, these correlations were established either by comparing different tumor cell lines, or cell lines with inhibition or trans-genesis restoration of telomerase activity. Our own analysis established positive and negative correlations between cisP resistance and cell growth rate and telomerase activity, respectively, in the same cells in fixed conditions over a long period of time.

It is important to note that the data was considered as a set of interrelated time-series. Thus, the dynamic aspect of the whole system must be studied by an appropriate modeling of the 5 sequences (cell proliferation over the 43 passages, telomere length, telomerase activities and effects of the 2 treatments) as components of a dynamic system in 5 dimensions. Our present results reveal some correlations between cell growth rate, telomere length, telomerase activity and resistance to the drugs. In this complex system, telomerase activity presents characteristics that deserve further investigation, such as: i) positive and negative correlations, respectively, with cisP and MTX sensitivity that give this cell trait a potential predictive value for cell response to the drugs, and ii) a relatively simple and fast assay.

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