Telomere dynamics determine episodes of anticancer drug resistance in rat hepatoma cells

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Clinical and experimental observations indicate that resistance to anticancer drugs may be spontaneously reversible over time, but the mechanisms of this reversal are unknown. The resistance of cultured hepatoma cells to methotrexate (MTX) and cisplatin was followed for 9 months. Cells were exposed to three treatments: MTX 200 nM for 24 h or 15 nM continuously and cisplatin 50 μM for 2 h. We investigated the relation between the temporal pattern of cell resistance and the previously reported fluctuations in cell proliferation rate, telomere length and telomerase activity. Spontaneous major peaks in resistance to each drug fell in time windows of 2–3 months (60–70 population doublings) and were at different times for each drug. The frequency of the fluctuations in drug resistance was the same as that of variations in cell growth rate, but amplitudes were unrelated. By contrast, resistance was directly related to telomere length dynamics in the same cells. MTX resistance occurred when telomeres shortened and cisplatin resistance when they were elongated. Furthermore, peaks of resistance to the continuous treatment with MTX were observed at 350-bp intervals of mean telomere length (9.06, 9.41, and 9.76 kbp) during the two 2-month phases of telomere shortening. Statistical analysis demonstrates the sinusoidal relationship between intermittent MTX resistance and telomere length. Possibly, erosion of telomeres encroaches on periodically spaced nucleosomal proteins, defining the onset of resistance phases. This evidence that resistance of tumoral cells to anticancer drugs may be intermittent and that onset of resistance is dictated by telomere length has major implications for clinical practice. *Anti-Cancer Drugs* 15:671–678 © 2004 Lippincott Williams & Wilkins.

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Introduction

The occurrence of cellular resistance is a major limit for the use of anticancer drugs, requiring a change of drug or the addition of a pharmacological modulator for effective treatment. Drug resistance has generally been considered to be irreversible and a consequence of the proliferation rate of malignant cells [1–3]. Both traits have often been considered as invariant characteristics both in spontaneously arising cancers and in established tumor cell lines. However, drug resistance is spontaneously reversible over time in several tumors *in vivo* [4–7] and in tumoral cells *in vitro* [8–10]. The extent and the kinetics of such reversal of drug resistance in malignant cells are also clinically relevant issues, as they may influence treatment sequences and outcome. The transition from a resistant to a sensitive state in a population of cancer cells may be a consequence of spontaneous oscillations in proliferation rate. Such fluctuations in proliferation occur every 3–4 weeks in a variety of tumoral cell *in vivo* and many cell types *in vitro* [11–14]. Alternatively, transient resistance may be related to telomere length: telomere shortening coincides with resistance to some anticancer agents [15], but the mechanisms involved are unknown.

We studied the temporal pattern of resistance to methotrexate (MTX) and cisplatin in long-term cultured rat hepatoma cells. Both drugs are widely used in the clinic and they have different mechanisms of action. We observed alternating phases of cell sensitivity and resistance to each drug, lasting 2–3 months. We compared the kinetics of resistance to the previously reported patterns of cell proliferation [14] and telomere length [16]. Cell proliferation only partly correlated with drug resistance. By contrast, there was a direct relationship between the onset of resistance and telomere length dynamics.

Materials and methods

Culture conditions

Cells of the rat hepatoma Fao clone are stably differentiated and tumorigenic [17–19]. They were seeded at a density of 10^5 cells/8.5-cm diameter dish in Ham

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F-12/Coon medium containing 5% fetal calf serum and the medium was changed every other day. On day 6 cells were detached with trypsin, counted and re-seeded in duplicate dishes at the same initial density. The rate of proliferation during a given passage is expressed as the number of population doublings (PD). The PD is calculated thus: $PD = \log_2(N/N_0)$, where N_0 is the number of cells at seeding (10^5 cells) and N the number of cells at the end of the passage. The time series of the proliferation rate during the 43 consecutive passages and its mathematical analysis have been reported previously [14].

Resistance of Fao cells to MTX and cisplatin

Cells in triplicate dishes were subjected to three different treatments at each of the 43 consecutive passages. Cell seeding density and doses of drugs were chosen to ensure severe toxicity but allow accurate counting of colonies after 3 weeks of growth. (i) Short exposure to cisplatin. The seeding density was 10^5 cells/dish. On day 1, samples were incubated for 2 h in medium containing 50 μM cisplatin and then grown under standard conditions (with no cisplatin) for 3 weeks. (ii) Short exposure to MTX. The seeding density was 2×10^3 cells/dish. On day 1 the medium was changed to medium containing 200 nM MTX and the samples incubated for 24 h. Cultures were then grown under standard conditions (with no MTX) for 3 weeks. (iii) Continuous exposure to MTX. The seeding density was 5×10^5 cells/dish. Cells were continuously cultured in medium containing 15 nM MTX for 3 weeks from day 1.

Stability of resistance following short exposure to MTX and cisplatin

After 3 weeks of growth, resistant cells from three dishes were harvested, counted and re-seeded into six new dishes at the same initial density. Three of these subcultures were treated again (with either 50 μM cisplatin for 2 h or 200 nM MTX for 24 h) on day 1. The other three were not treated and served as controls. The number of colonies was recorded 3 weeks later.

Stability of resistance following continuous exposure to MTX

Four resistant clones and two pools of 50 colonies resistant to selection at p12 and p22 were cultured under normal conditions for about 70 more PD. Aliquots were then seeded at low density (200 cells/dish) in 15 nM MTX and in normal medium. The number of colonies generated under each condition was recorded.

Analysis of the heterogeneity of the Fao cell population

We used three criteria to determine whether the observed fluctuations in growth, telomere length and resistance to MTX resulted from transient predominance of a subset of cells or from variations in the whole cell population.

(i) Histograms of the colony sizes were established: a series of three passages was performed and the growth rate measured as described above. In parallel, dishes were inoculated with 1000 cells and the diameter of each of 200 clones was measured 6 days later. Histograms of the colony sizes were analyzed to determine the mono-, bi- or pluri-modal distribution of the clone sets.
(ii) Profiles of the telomeric smear were examined (see method for telomere length analysis below).
(iii) We examined if variations in the frequencies of resistant cells reflected the presence of independent subsets with different levels of resistance. A separate 20-passage culture was performed. At each passage, cell cultures were tested in duplicate for resistance to two different drug concentrations: continuous treatment of 5×10^5 cells with 7.5 and 15 nM MTX; 24 h treatment of 10^4 cells with 200 or 400 nM MTX; 2 h treatment of 10^4 cells with 25 or 50 μM cisplatin. The frequencies of resistant cells were recorded as described above.

Telomere length analysis

Telomere lengths analysis and details of the methods were described previously [16]. Briefly, at each passage, DNA and cell extracts were prepared for telomere length analysis by Southern blotting. 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:

$$\mathrm{MTRL} = \Sigma \mathrm{CPM}_i : \Sigma[\mathrm{CPM}_i : (L_i - X)]$$

where CPM_i, L_i and X are the signal (counts/min) at passage i, the TRF length at passage i and the mean subtelomeric length, respectively.

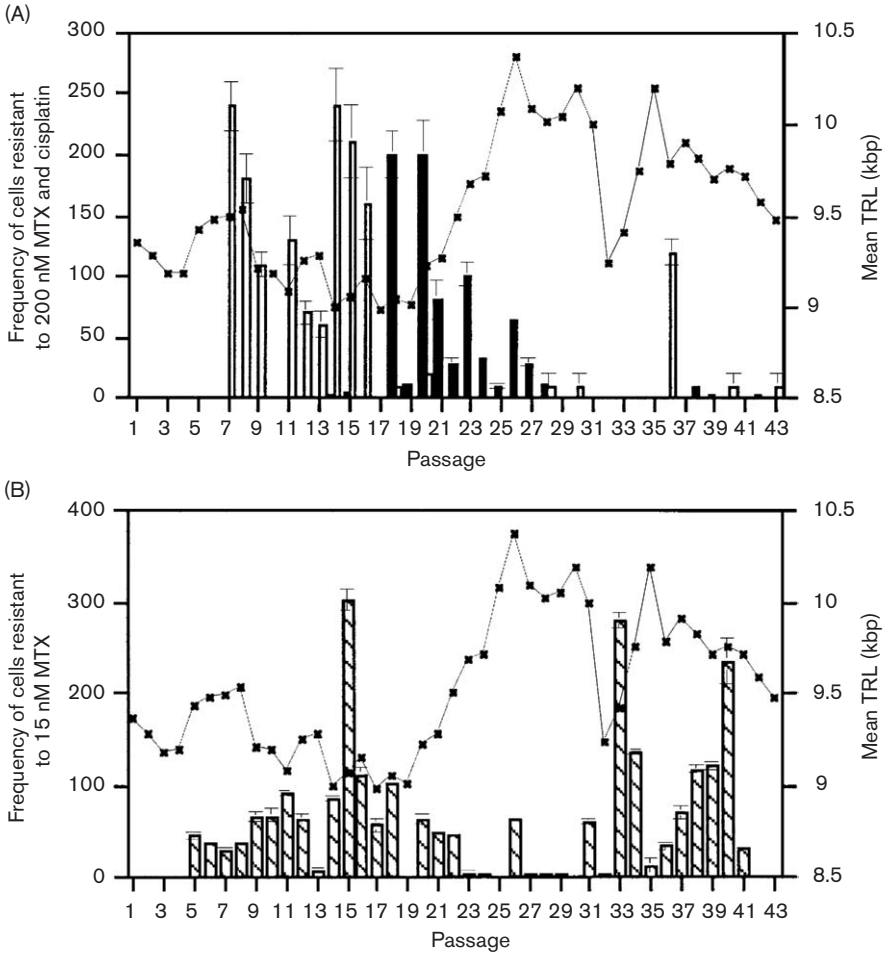
Results

Resistance of Fao cells to MTX and cisplatin

There were spontaneous abrupt rises in the frequency of cells resistant to short treatments with 50 μM cisplatin or 200 nM MTX. These rises were from $< 10^{-5}$ to 2×10^{-2} for cisplatin and from $< 5 \times 10^{-4}$ to 1.2×10^{-1} for MTX. In each case the rise was followed by a progressive decrease (Fig. 1A). The stability of resistance to the drugs, as assessed following a second round of treatment at the next passage, was about 25 and 30%, respectively.

Similarly, marked peaks in the frequency (from $< 2 \times 10^{-6}$ to 6×10^{-4}) of cells resistant to chronic treatment with 15 nM MTX were observed (Fig. 1B). Four resistant clones and two pools of 50 colonies resistant to selection at p12 and p22 were cultured under normal conditions for

Fig. 1



Resistance of cells to MTX and cisplatin. At each passage, aliquots of the cells were seeded in a series of dishes and subjected to various drug treatments. (A) Short treatments with MTX and cisplatin. The test was initiated at p7. Open columns: number of cells resistant to MTX (200 nM; 24 h) among 2000 cells tested. Filled columns: frequency of cells resistant to cisplatin (50 μM; 2 h) per 10^4 cells tested. Data are means + SD for 10 dishes for MTX and four dishes for cisplatin. (B) Chronic treatment with 15 nM MTX. The test was initiated at p5. Filled columns: number of cells resistant to MTX (15 nM) per 5×10^5 cells tested. On both diagrams, the line corresponds to mean telomere length [16].

about 70 more PD. They were then seeded at low density (200 cells/dish) in 15 nM MTX and in medium without MTX. The clonal efficiency was very similar (70–80%) under the two conditions demonstrating the stability of this resistant phenotype. Southern Blot analysis, using a dihydrofolate reductase (dhfr) cDNA probe, revealed a 3- to 4-fold amplification of the *dhfr* gene in two of the four resistant clones and in the two pools (data not shown).

These bursts of genesis of resistant cells were brief (lasting less than a 6-day passage) and synchronized for the two drugs. The amplitudes of resistance were, however, very different, leading to mutually exclusive time windows of resistance to each drug. The peaks of resistance to MTX were observed in two windows of 2.5 and 2 months, separated by a 3-month period of cell sensitivity. Cisplatin resistance occurred during this 3-month window of MTX sensitivity.

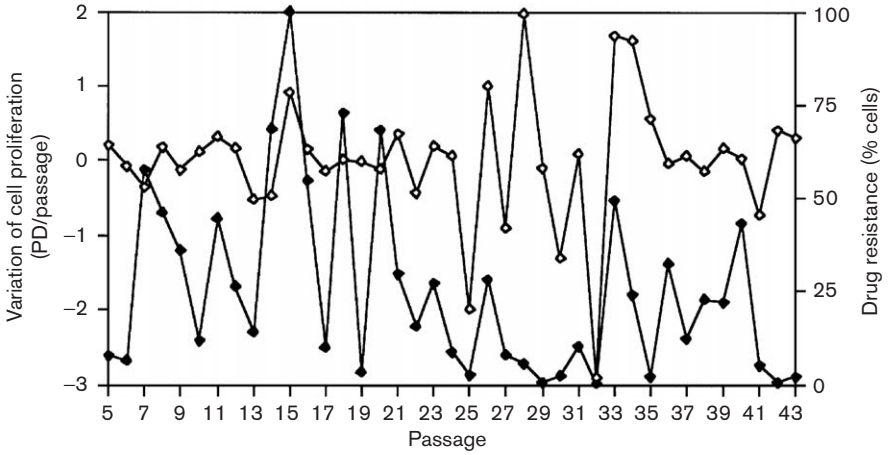
Drugs resistance and proliferation

The rate of cell proliferation displays persistent aperiodic oscillations which are deterministic and presumably chaotic [14]. The frequency of the peaks of resistance was similar to that of the variations in cell proliferation (Fig. 2). However, only 13 of the 17 peaks in resistance coincided with increases in cell growth rate. Furthermore, the clustering of the peaks of resistance over 2 months was not related to the proliferation pattern.

Telomere dynamics and drug resistance

Fluctuations of MTRL, and their analysis, have been reported elsewhere [16]. Briefly, the range of MTRL was

Fig. 2



Variations in cell proliferation and resistance to MTX and cisplatin. Data for resistance to short and permanent treatment to MTX and to cisplatin were combined, and are expressed as percentages of the highest value (dark diamonds). Variations of cell proliferation (VCP) (white diamonds) were calculated as: $VCP_i = CP_i - CP_{i-1}$.

8.98–10.37 kbp, with an oscillating pattern with three phases (Fig. 1A). First, MTRL decreased from passage 1 (p1) to p19 in rebounds. In the second phase (p20–p26), it rapidly increased from 8.98 to 10.37 kb (net lengthening of 29 bp/PD), from the minimal to the maximal observed MTRL, respectively. Third, from p27 to p43, MTRL shortened again in rebounds.

These three phases of the MTRL time series corresponded to drug resistance phases: the time windows of resistance to MTX (transient or permanent treatment) were observed during the phases of telomere shortening, whereas resistance to cisplatin coincided with short and re- lengthening telomeres (Fig. 1A and B). The frequency of cells resistant to the permanent treatment with 15 nM MTX was plotted against MTRL (Fig. 3A). The relationship between the two variables is complex and sinusoidal (see the statistical analysis below). The three major peaks of MTX resistance coincided with telomere lengths of 9.06, 9.41 and 9.76 kbp, and shoulders with lengths of 9.24, 9.59 and 9.94, thus with 350-bp intervals. Plotting the variations of cell growth against MTRL revealed the same structure: the five highest values were recorded at MTRL 9.06, 9.41, 9.75, 10.02 and 10.37 kbp. Figure 3(B) illustrates this periodic organization of both resistance to 15 nM MTX and variations of cell proliferation, and the 1.4-kbp length delineated by the minimal and maximal MTRL observed during the time series.

Statistical analysis

We used two methods for the statistical analysis of the relationship between telomere length and MTX resistance: (i) Single Cosinor [20] and (ii) TSA Seriel Cosinor (Software 'Time Series Analysis Seriel Cosinor 5.1', © by L. Gouthière; 'Expert Soft Technologie').

- Solution model: 3.97E + 01cos(2πt/0.35) – 1.58E + 01sin (2πt/0.35) + 6.03E + 01
- Model (ANOVA) is valid at the probability level: 0.9000

Chronobiometric test

- Ellipse test (H0: $\beta = 0$ and $\gamma = 0$) for T = 3.5000000E −01 is significant at the probability level: 0.9000
- Area of the confidence ellipse: 4.45E + 03

Residuals distribution—goodness of fit

- Adjusted r^2: 4.86E – 01
- Residual sums of squares: 1.65E + 05
- K–S test (H0: normal residuals distribution) H0 accepted: 0.9500. Average test (H0: RS average = 0) H0 accepted: 0.9500. Q test (H0: independent residues) H0 accepted: 0.9500

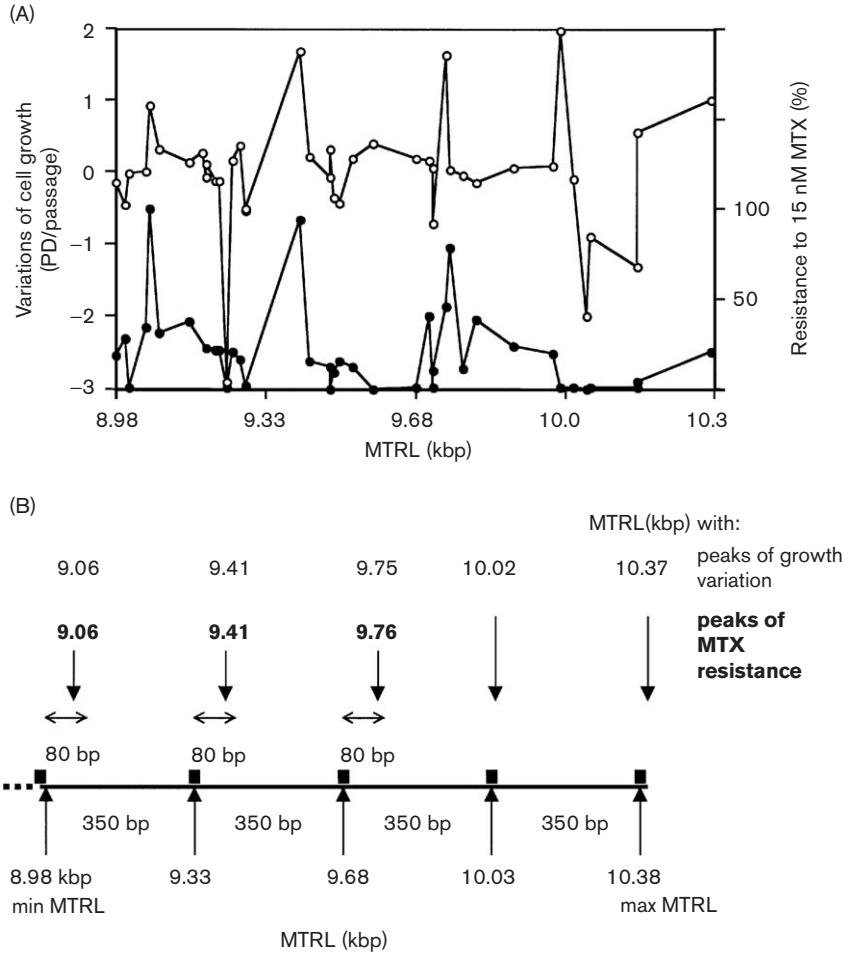
Conclusion

(i) Cosinor: period at 0.350 kbp (probability 0.90).
(ii) Elliptic inverse spectral plot: periods at 0.112, 0.181 and 0.358 kbp (probability 0.95).

Heterogeneity of the Fao cell population

(i) Histograms of size of colonies: the mean size of the clones was proportional to the growth rate at each

Fig. 3



Telomere structure and resistance to the permanent treatment with MTX. (A) Resistance to 15 nM MTX (dark dots) and variations of cell proliferation (white dots) were plotted against MTRL (abscissa). (B) Schematic representation of the 1.4-kbp telomere fragment showing the dynamic evolution of this structure during the 8 months of the experiment. Localization of the peaks of MTX resistance and growth variation are indicated on the upper part of the scheme.

passage. Histograms of the data gave no evidence of different subpopulations with different proliferation rates (Fig. 4A).

(ii) Telomere smears each exhibited a single main zone and thus gave no indication of subpopulations (Fig. 4B).

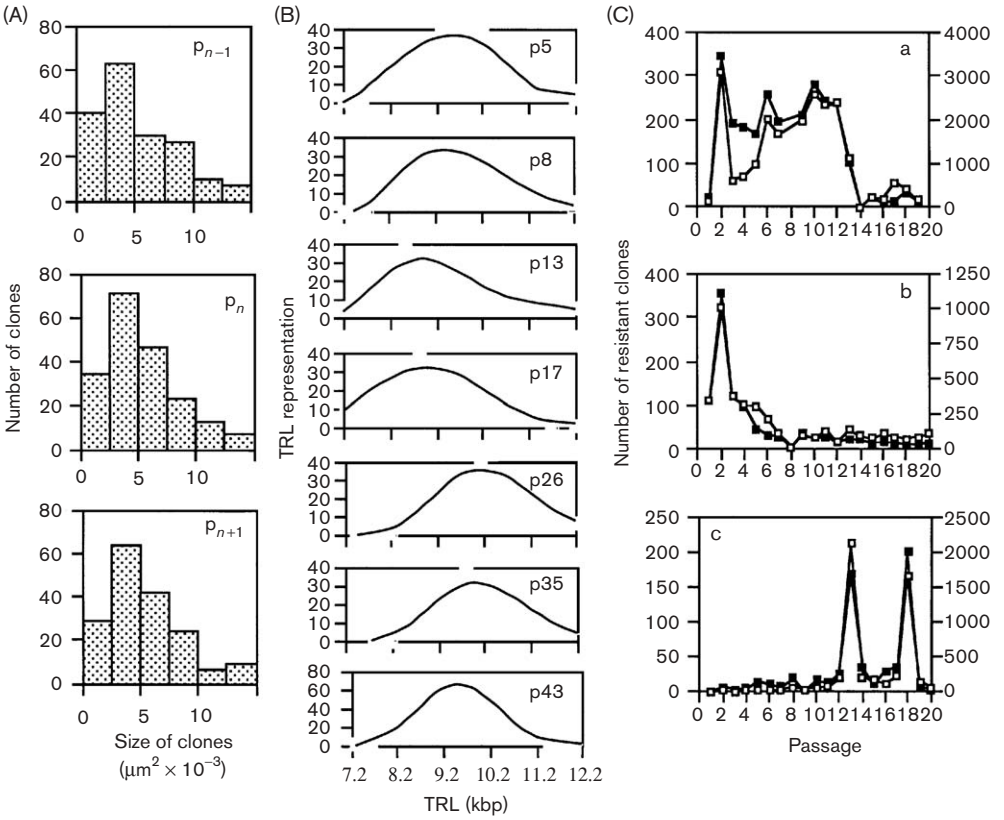
(iii) We examined if the fluctuations in the frequency of resistant cells could be due to the succession of cell subsets with different levels of resistance. The resistance to two drug concentrations was recorded during 20 additional passages. The frequencies of resistant colonies were very diverse (3- to 10-fold) depending on the two drug concentrations but time courses were strictly identical indicating that the whole cell population was involved (Fig. 4C).

Discussion

Our finding that the peaks of resistance to anticancer drugs are clustered in time windows about 2.5 months long, separated by periods of high cellular sensitivity, is consistent with several *in vivo* observations. In particular, a review of spontaneous reversal of resistance in patients led to the prediction that the time to allow for reversal was between 1 and 4 months [4].

The coincidence between the temporal fluctuations in cell proliferation and drug resistance is consistent with the well-documented relationship between the two variables [1–3]. This may be relevant to the direct toxicity of anticancer drugs on DNA replication. Alternatively, genetic controls shared by proliferation and metabolism of these drugs may account for this

Fig. 4



Analysis of the heterogeneity of the Fao cell population. (A) Histograms of colony size. At three consecutive passages, the sizes of 200 colonies were measured 7 days after inoculation. (B) Profiles of the telomere signals in Southern blots. Seven of the 43 samples of the time series are shown. The corresponding passages are indicated on each diagram. (C) Cells were subjected to two concentrations of MTX or cisplatin: (a) permanent treatment (5×10^5 cells) with 7.5 nM and 15 nM MTX (white and dark dots, respectively), (b) 24 h treatment (10^4 cells) with 200 and 400 nM MTX (white and dark dots, respectively), and (c) 2 h treatment (10^4 cells) with 25 and 50 μM cisplatin (white and dark dots, respectively).

observation, e.g. c-*myc* stimulates cell growth and regulates expression of the gene encoding dhfr, the target for MTX cytotoxicity [21–23]. However, peaks in drug resistance did not always coincide with increased cell growth and the amplitudes of the temporal variations in proliferation and resistance were unrelated. Furthermore, the peaks of resistance to MTX and cisplatin did not occur simultaneously, which indicates the involvement of other regulating factor(s).

Telomere length exhibited a succession of phases of oscillatory shortening and elongation. Our data indicate that this regulation of telomere length is a key mechanism for the switch from cell resistance to cell sensitivity to each drug. First, the timing of resistance to MTX (both short and permanent exposure) coincides with telomere shortening and that of resistance to cisplatin with telomere lengthening. Second, a periodic telomere structure of 350 bp was significantly associated with the peaks of MTX resistance and of cell proliferation, with an additional periodicity of about 180 bp for MTX resistance.

MTRL evolution ranged from a minimum of 8.98 kbp to a maximum of 10.37 kbp over the eight month time series, which corresponds to a 1.4-kbp telomere structure containing four 350-bp subunits. Rat liver telomeres contain arrays of tightly packed nucleosomes, each being 155–160 bp long with short linkers [24]. We propose that the replicative telomere erosion alters nucleosomes and that nucleosomal content affects the transcription of genes as a result of *cis* or *trans* effects. For instance, the promoter of c-*myc*, which includes myb sites, may be up-regulated by myb domains of telomeric proteins [25]. The involvement of telomere shortening has been increasingly evoked in the repression or activation mechanisms of various genes in yeast [26,27] and in human cells [28,29]. Sensitivity to cisplatin was observed during the two phases of MTRL shortening (p1–p18 and p 26–p41), whereas resistance to cisplatin occurred in the

intermediate phase when very short telomeres started re-lengthening (p18–p24). These dynamics reconcile apparently contradictory reports of a correlation between telomere shortening and cell sensitization to cisplatin [15], on the one hand, and a correlation between short MTRL and resistance to cisplatin [30,31], on the other.

In conclusion, we demonstrated that resistance to anticancer drugs is episodic in hepatoma cells and that the onset of resistance is determined by the dynamics of telomeres. The homogeneity of the profiles of growth rate of cell clones, of the profiles of telomere length and of drug resistance indicate that the variations of these variables involve the cell population as a whole rather than cell subpopulations. Our prediction, from this cell model, is that all tumoral cell populations with progressive telomere erosion and episodic re-lengthening, which is a common pattern, should display an episodic pattern for various cell functions, including growth rate and resistance to anticancer drugs such as MTX and cisplatin. However, because of the irregular pattern of telomere erosion-repair, these 2- to 3-month long windows are not strictly periodic. Non-linear, complex systems combine great flexibility and strong control of their evolution [32,33]. We showed previously that telomeres and telomerase activity behave as a complex system in hepatoma cells [16], which accounts for the maintenance of telomere length within a determined corridor of values. This is consistent with both the persistent proliferation and the recurrent episodes of drug resistance of these cancer cells. These non-linear dynamics in telomere length and in cell resistance to anticancer drugs raise the question of the molecular mechanisms involved. We will try to identify these mechanisms by two approaches: (i) time series of expression of genes that may control cell growth, telomere length and drug resistance will be established, and (ii) the system will be modified by introducing inducible *trans*-genes (i.e. *tert*, c-*myc*) and the consequences will be studied. The present work could well provide new grounds for chemotherapy schedules, including the possibility of re-treatment with the same drug.

Acknowledgments

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