Leukemia (2005) 19, 1806–1811
© 2005 Nature Publishing Group All rights reserved 0887-6924/05 $30.00
www.nature.com/leu

# Retinoid/arsenic combination therapy of promyelocytic leukemia: induction of telomerase-dependent cell death

I Tarkanyi[1,2,3], C Dudognon[1,3], J Hillion[1], F Pendino[1], M Lanotte[1], J Aradi[2] and E Ségal-Bendirdjian[1]

[1]*INSERM U685, Hôpital Saint-Louis, Institut d'Hématologie, Paris, France; and* [2]*Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary*

**Acute promyelocytic leukemia (APL) is efficiently treated with a cell differentiation inducer, all-*trans* retinoic acid (ATRA). However, a significant percentage of patients still develop resistance to this treatment. Recently, arsenic trioxide ($As_2O_3$), alone or in combination with ATRA, has been identified as an alternative therapy in patients with both ATRA-sensitive and ATRA-resistant APL. Previous investigations restricted the mechanism of this synergism to the modulation and/or degradation of PML-RARα oncoprotein through distinct pathways. In this study, using several ATRA maturation-resistant APL cell lines, we demonstrate *in vitro* that the success of ATRA/$As_2O_3$ treatment in APL pathology can be explained, at least in part, by a synergistic effect of these two drugs in triggering downregulation of telomerase efficient enough to cause telomere shortening and subsequent cell death. Such long-term low-dose combinatorial therapy strategies, developed also to avoid acute side effects, reinforce the notion that the antitelomerase strategy, based on a combination of active agents, should now be considered and evaluated not only in APL but also in other malignancies.**
*Leukemia* (2005) **19,** 1806–1811. doi:10.1038/sj.leu.2403923; published online 18 August 2005
**Keywords:** telomerase; ATRA; arsenic trioxide; APL

## Introduction

Human telomerase catalyzes the synthesis and extension of telomeric DNA. This ribonucleoprotein, usually absent or expressed at a low level in most normal somatic cells, is highly active in cancer cells, strongly indicating its key role in cell immortalization and carcinogenesis.[1] Importantly, however, recent findings indicate that telomerase confers additional functions required for tumorigenesis that do not depend on its ability to maintain telomeres.[2] As a result of its differential expression pattern in normal and tumor cells, telomerase has been proposed as a promising target for anticancer therapies.[3] Drugs have been designed to target the various molecular components of telomerase including the RNA component (hTR) containing the template for telomeric DNA extension (antisense oligonucleotides), the catalytic subunit with telomeric reverse transcriptase activity (hTERT) (reverse transcriptase inhibitors) and/or the telomeric DNA structure itself (agents stabilizing G-quadruplex formation of telomeric DNA). No anticancer strategy has so far considered the targeting of telomerase through specific control of hTERT gene expression, its mRNA stability and translation or its intracellular trafficking. These diverse mechanisms of control should prove of increasing interest since telomerase is now thought to also intervene in the regulation of proliferation and apoptosis, independently of any activity in telomere length maintenance.[2,4,5] It becomes also more and more obvious that levels of telomerase expression and function can be one of the causal mechanisms that account for the therapeutic outcomes of hormone treatment or chemotherapy in cancer.

Among myeloid leukemias, acute promyelocytic leukemia (APL) was found to be specifically sensitive to pharmacological doses of all-*trans* retinoic acid (ATRA), leading to high rates of temporary clinical remissions when combined with chemotherapy.[6–10] However, the disease may relapse with resistance to further ATRA and chemotherapy treatments. The discovery that treatment with arsenic trioxide ($As_2O_3$) induces durable remission not only in primary cases, but also in APL patients in relapse after ATRA or chemotherapy, has provided a novel therapy for APL patients.[11–13] Recently, ATRA/$As_2O_3$ combination for APL therapy was shown to bring better results than either of the two drugs used alone in terms of the quality of complete remission and status of the disease-free survival.[14] No single mechanism can explain all the effects seen with $As_2O_3$; it probably acts at multiple levels, and with various modes of action depending on the drug concentration and the cell type. It appears that at low doses ($<0.5\,\mu$M) $As_2O_3$ can induce differentiation in NB4 APL cells similar to ATRA,[12] while at higher concentrations ($>1\,\mu$M) induction of apoptosis can be observed.[15,16] Molecular investigations have provided theoretical support to the synergism between ATRA and $As_2O_3$ in that both ATRA and $As_2O_3$ induce modulation and/or degradation of PML-RARα oncoprotein through distinct pathways. Like ATRA, $As_2O_3$ triggers the degradation of the PML-RARα protein,[17,18] but conversely to ATRA, it targets PML rather than RARα in the fusion protein. It was recently found that in addition to the above actions, high doses of arsenic cause chromosomal abnormalities that can promote either genomic instability and carcinogenesis or cell death.[19–21] These effects result in most cases from the induction of an altered telomere state by oxidative stress due to the generation of reactive oxygen species (ROS) by $As_2O_3$ treatment.[22–24] An inhibition of hTERT gene transcription leading to a decrease in telomerase activity (TA) has also been reported.[25] However, whether this inhibition is sufficient to lead to cell death was not clearly demonstrated.

Using model systems of maturation-sensitive and -resistant variants,[26] we recently reported that several retinoid maturation-resistant APL cell sublines (NB4-LR1, NB4-LR1$^{SFD}$, NB4-LR2), behaved distinctly to long-term ATRA treatment.[27–29] In NB4-LR1 cells, pharmacological concentrations of ATRA exhibit antiproliferative activities through the downregulation of telomerase, leading to telomere shortening and cell death. A synergistic repression of telomerase with subsequent cell death can also be obtained using synthetic RARα and RXR selective agonists not only in NB4-LR1 but also in NB4-LR2 cells. Importantly, with the isolation of a new ATRA maturation-resistant APL subline (NB4-LR1$^{SFD}$ cell line), we have identified a novel type of resistance to retinoids, since these cells developed a defective pathway in telomerase regulation by

Correspondence: Dr E Ségal-Bendirdjian, INSERM U685, Institut d'Hématologie, Hôpital Saint-Louis, 1, avenue Claude Vellefaux 75475 Paris cedex 10, France; Fax: +33 1 42 40 95 57;
E-mail: segal@stlouis.inserm.fr
[3]These authors contributed equally to this work
Received 3 June 2005; accepted 19 July 2005; published online 18 August 2005

ATRA, RARα or RXR selective agonists (rexinoids) alone or in combination. This new type of retinoid resistance, not yet understood, could as well account for the failure of some ATRA-based therapies in the clinic.

While the efficacy of ATRA-based treatment of APL cells is due primarily to the differentiating action of ATRA, the above findings indicate that repression of telomerase by retinoids, leading to the elimination of maturation-resistant cells, could explain at least partially the success of the ATRA-based therapy of APL. Given that (a) cell death of ATRA long-term-treated NB4-LR1 cells results from telomere shortening due to downregulation of hTERT, (b) NB4-LR2 and NB4-LR1$^{SFD}$ cells resist to this death induction, and (c) ATRA/$As_2O_3$ combination represents a novel therapy for APL patients not only in primary cases but also after relapse, we wondered whether this combination could affect survival of the ATRA-resistant NB4-LR2 and NB4-LR1$^{SFD}$ cells by targeting telomerase. We show, in the present report, that in NB4-LR2 and NB4-LR1$^{SFD}$ cells, ATRA or $As_2O_3$ alone is able to regulate telomerase expression to some extent; however, cellular death response can be induced only by combined treatment of these agents.

## Materials and methods

### Reagents, cell lines and cell culture

ATRA and $As_2O_3$ were purchased from Sigma (St Louis, MO, USA). The maturation-resistant human promyelocytic leukemia cell lines NB4-LR1 and NB4-LR2,[30,31] the selected subline NB4-LR1$^{SFD}$,[28] the retrovirally infected NB4-LR1/hTERT-GFP[27] and the NB4-LR2/hTERT-GFP sublines expressing both hTERT protein and the green fluorescent protein (GFP) reporter from the same transcript and the NB4-LR1/GFP and the NB4-LR2/GFP sublines expressing only the GFP control vector were cultured as described previously.[28–30] Both NB4-LR1$^{SFD}$ and NB4-LR1/hTERT-GFP sublines escape death induced by long-term ATRA treatment.[27,28] All cells were incubated at 37°C in 5% $CO_2$ atmosphere (Binder Incubators). Cell density was determined using a Coulter counter, and proliferation was expressed as population doublings (PD). We verified that expression of GFP alone did not modify the proliferation and molecular responses of the parental cells to treatment. For this reason, only results using NB4-LR1 and NB4-LR2 were presented.

### TA assay

TA was measured using the telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit (TRAPeze Elisa telomerase detection kit, Qbiogene, Illkirch, France) according to the manufacturer's instructions. TA was expressed as a percentage of that detected in untreated cells.

### Telomere length analysis

Telomere length was measured using a nonradioactive chemiluminescent assay developed by Roche Diagnostics (Meylan, France), according to the manufacturer's instructions. The average telomere lengths can be determined by comparing the signals relative to a molecular mass standard.

### Analysis of hTERT expression by real-time PCR

Full-length hTERT mRNA expression was quantified by fluorescence real-time RT-PCR using the LightCycler® technology and the LightCycler Telo*TAGGG* hTERT kit from Roche Diagnostics (Meylan, France) according to the manufacturer's instructions. hTERT level was normalized to the expression of the housekeeping gene phosphobilinogen deaminase (PBGD).

## Results and discussion

### ATRA and $As_2O_3$ cooperate to induce cell death of retinoid maturation-resistant leukemia cells

It has been previously shown that treatment of NB4-LR1 and NB4-LR2 cells with high concentrations (>0.5 $\mu$M) of $As_2O_3$ alone is responsible for massive apoptosis.[32] In these conditions, the assessment of a synergistic effect of a long-term treatment with ATRA would be difficult. Furthermore, one critical factor that limits the utility of $As_2O_3$, especially in the case of extended treatments, will be its cytotoxicity to normal tissues; thus, one of our objectives was the substantial reduction of its dose. For this reason, NB4-LR1, NB4-LR1$^{SFD}$ and NB4-LR2 cells were treated with $As_2O_3$ at low concentration (0.2 $\mu$M) in the presence or absence of ATRA (1 $\mu$M).

Figure 1a shows that long-term treatment of NB4-LR1 cells with 1 $\mu$M ATRA induced a reproducible cell growth arrest followed by cell death at day 75. In a similar study, NB4-LR1$^{SFD}$ and NB4-LR2 cells continued to proliferate even after 3 or 4 months of continuous treatment with ATRA (data not shown). Long-term treatment of the three cell lines with 0.2 $\mu$M $As_2O_3$, alone had no effect either on proliferation or viability. However, the combination of both drugs greatly accelerated cell death in NB4-LR1 cells compared to cells treated with ATRA alone, and reduced cell growth and viability in both NB4-LR2 and NB4-LR1$^{SFD}$ cells.

### ATRA/$As_2O_3$ combination induces hTERT downregulation, TA decrease and telomere shortening

As we have previously shown that in the NB4-LR1 cells long-term treatment with ATRA led to cell death, which resulted from the downregulation of the telomerase catalytic subunit hTERT,[27] we evaluated hTERT expression and measured TA as well as telomere length in all three cell lines after long-term treatment with ATRA and $As_2O_3$, alone or in combination. As shown in Figure 1b, the level of hTERT mRNA measured by quantitative RT-PCR was greatly reduced in the ATRA-treated cell lines. However, only in NB4-LR1 cells was this decrease (95%) associated with an efficient diminution of TA (Figure 1c) and telomere shortening (Figure 2a) resulting in cell death (Figure 1a). Interestingly, NB4-LR1 cells under ATRA alone proliferate at day 32, with telomeres as short as those in cells that are practically blocked by the combination treatment. This observation suggests that additional mechanisms may affect cell growth. As $As_2O_3$ treatment may result in the generation of ROS that can directly damage telomeres,[22–24] it is possible that such an oxidative stress, negligible at low concentrations of $As_2O_3$ used as a single drug, can be enhanced by the addition of ATRA in the combined treatment. Furthermore, as it has been recently shown that not only the overall telomere length but also the length of the telomeric 3′ single-strand overhang are important factors in triggering cell death,[33] it cannot be excluded that

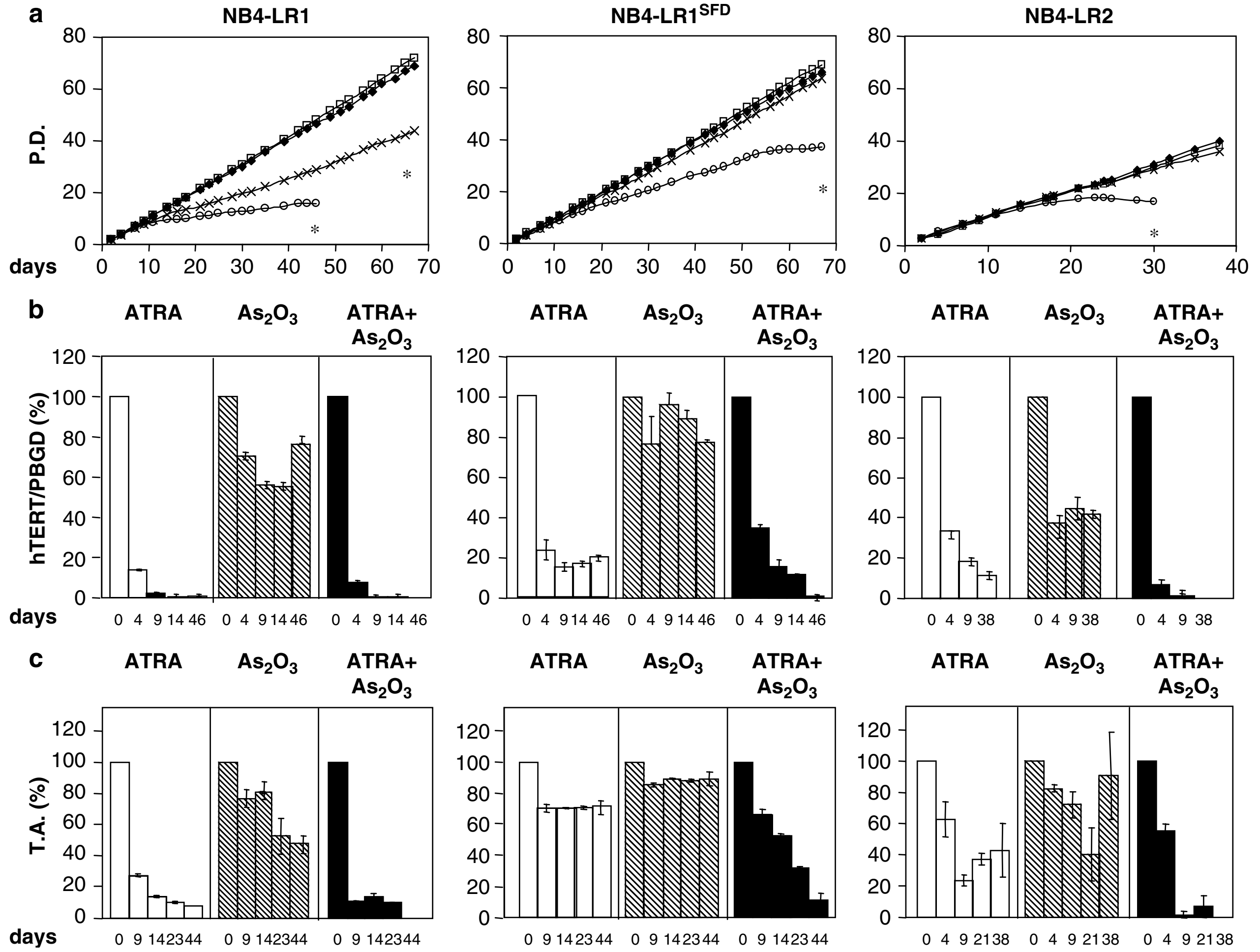


**Figure 1** ATRA and $As_2O_3$ synergize to induce growth arrest, hTERT downregulation, inhibition of telomerase activity, and subsequent cell death. Cells were cultured in the presence of medium alone (□), in the continuous presence of ATRA (1 $\mu$M; X), $As_2O_3$ (0.2 $\mu$M; ♦), or the combination of both drugs at these concentrations (○). Cell proliferation was assessed as population doublings (PD) (a). *Cultures that died after prolonged treatment. At the indicated time, hTERT mRNA expression was quantified by fluorescence-real-time RT-PCR, the LightCycler® technology and the LightCycler Telo*TAGGG* hTERT kit from Roche Diagnostics (Meylan, France) according to the manufacturer's instructions. hTERT level was normalized to the expression of the housekeeping gene phosphobilinogen deaminase (PBGD) (b). At the indicated time, protein extracts were prepared and TA was measured using the telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit (TRAPeze Elisa telomerase detection kit, Qbiogene, Illkirch, France) according to the manufacturer's instructions. TA was expressed as a percentage of that detected in untreated cells (c); ATRA (1 $\mu$M; □), $As_2O_3$ (0.2 $\mu$M; ▧), ATRA (1 $\mu$M) and $As_2O_3$ (0.2 $\mu$M) in combination (■).

$As_2O_3$ in combination with ATRA induces some kind of telomere dysfunction by the erosion of the 3′ telomeric overhang whose integrity cannot be determined in the telomere length assay used here. Besides, we have to keep in mind that telomerase is endowed with survival functions independent of telomere maintenance; thus, the diminution in hTERT protein level, by eliminating it as a survival factor, can also contribute to the above observed effect. However, the duration of the lag period before the induction of cell death suggests that telomere shortening is actually an important factor in this process. In the ATRA-treated NB4-LR2, TA stabilized around 50% of the untreated cells, and in the NB4-LR1$^{SFD}$, TA remained even higher (80% of the untreated cells). In these cases, no telomere shortening was observed (Figure 2a and b). In all the three cell lines, $As_2O_3$ alone induced much less modifications in the level of hTERT expression and TA stabilized above 50% of the control cells without any consequences on either telomere length or cell proliferation. The absence of phenotypic effect, in contrast with already published studies,[25] can be explained by the very low doses used in our studies (0.2 $\mu$M) and the fact that the cell lines used in our study are of different nature. In sharp contrast, the combination of the two compounds greatly reduced hTERT mRNA and TA below the levels needed to prevent the reduction of telomere length and cell death. It should be noted that modulation of TA and induction of cell death in NB4-LR1 cells occurred faster in cells treated with ATRA/$As_2O_3$ than in cells treated with ATRA alone. The decrease in hTERT transcription and activity is rather progressive in NB4-LR1$^{SFD}$ cells compared to that in the other cell lines. Although we have no clear explanation for this observation, it is likely that hTERT regulation and perhaps also the properties of hTERT protein are altered in this cell line. This is supported by our previous study showing that in NB4-LR1$^{SFD}$ cells ATRA long-term treatment fails to downregulate hTERT, whereas during their differentiation with appropriate treatments, this downregulation can be observed as in the parental NB4 cells.[28] The faster cell death observed in ATRA/$As_2O_3$-treated NB4-LR2 cells can be explained by the fact that this cell line carried shorter telomeres than the two others

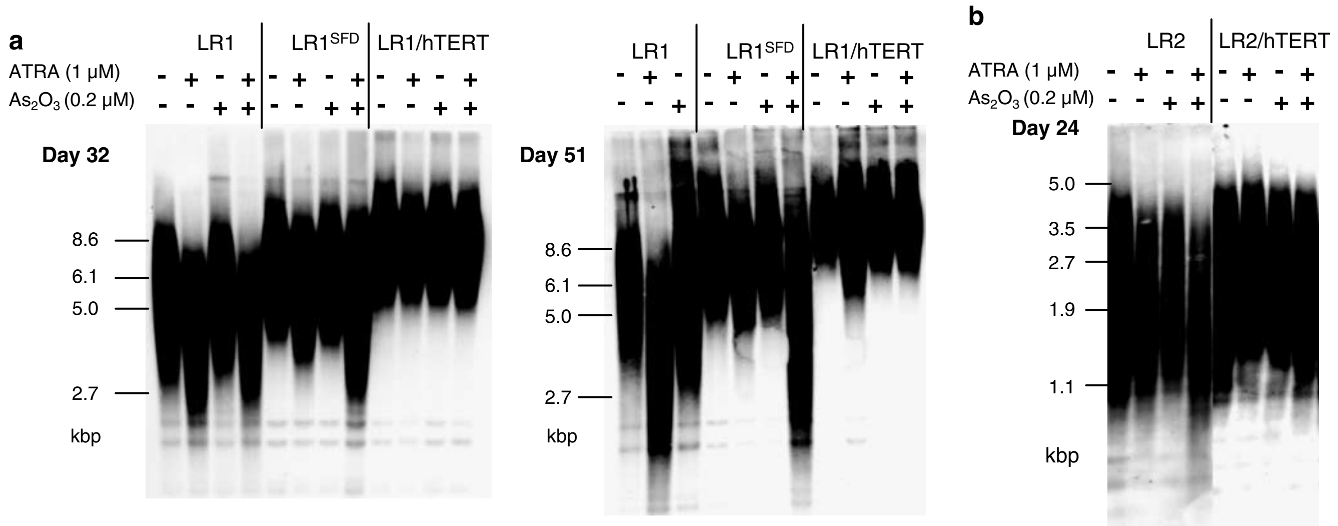


**Figure 2** ATRA and $As_2O_3$ synergize to induce telomere shortening. NB4-LR1, NB4-LR1$^{SFD}$ and NB4-LR2 cells were cultured as in Figure 1. Telomere restriction fragment lengths, measured using a nonradioactive chemiluminescent assay developed by Roche Diagnostics (Meylan, France), were determined after 32 and 51 days (NB4-LR1 and NB4-LR1$^{SFD}$ cells (a) and after 24 days of treatment (NB4-LR2 cells (b). Size markers are shown on the left.

(see mean of telomere lengths comparatively in Figure 2a and b). As a significant portion of the telomeres of the NB4-LR2 cells is expected to be at critical stage of shortening, even a small erosion (compared to what was observed in NB4-LR1- and NB4-LR1$^{SFD}$-treated cells, using this terminal restriction fragment assay) could be enough to trigger crisis and death of these cells. These results indicate that cell death induced by ATRA/$As_2O_3$ combination is due to the decrease in TA, which is itself the consequence of hTERT downregulation. Although causal relation between degradation of PML-RARα and changes in gene expression followed by ATRA/$As_2O_3$ administration cannot be doubted, it is clear that it cannot solely account for the presented downregulation of hTERT, since the breakdown of the chimeric protein following ATRA treatment alone is already rapid and occurs to the same extent in NB4-LR1 and NB4-LR1$^{SFD}$ cells, despite different biological responses (cell death and survival, respectively[28]).

Our observations also support the notion of two distinct thresholds necessary for telomere shortening and cell death. The first one features to what extent hTERT mRNA expression has to be decreased to obtain an efficient reduction of TA. The second one identifies the level of enzyme activity for which telomerase inhibition resulted in a sufficient shortening of telomeres to cause cell death. The biological significance of these two distinct thresholds has to be taken into account for the development of telomerase inhibitors targeting either hTERT mRNA expression or telomerase itself. In fact, focusing solely on a relative inhibition of telomerase may overestimate the therapeutic efficacy of telomerase inhibitors. Indeed, tumor cells can be responsive to a drug in terms of downregulation of hTERT and decrease in TA, without demonstrating any modification in proliferation and viability. Importantly, we also show in this report that these thresholds, as well as the outcome of telomerase inhibition, may vary depending on the physiology of the targeted cancer cells. Cellular response of NB4-LR1$^{SFD}$ is also of further interest. In these cells, long-term treatment with ATRA or $As_2O_3$ alone was not able to induce any significant decrease in TA. In contrast, the combination of these two drugs induced an efficient decrease in TA leading to telomere shortening and subsequent death. Although it cannot be excluded that some nontelomeric effects induced by each drug can participate in the cellular response to the combined treatment, this observation provides strong evidence of two distinct but convergent mechanisms of action for ATRA and $As_2O_3$ on telomerase regulation. For these reasons, the NB4-LR1$^{SFD}$ cell line provides an efficient tool for further screening and evaluation of the therapeutic benefit of drug combinations.

### *Ectopic expression of hTERT durably protects retinoid maturation-resistant cells from ATRA/$As_2O_3$-induced cell death*

Altogether, our results suggest that, after long-term treatment of maturation-resistant cells with ATRA/$As_2O_3$ combination, loss of TA and subsequent telomere shortening cause cell death. To substantiate this result, we investigated whether ectopic expression of hTERT could rescue NB4-LR1 and NB4-LR2 cells from death. These retrovirally infected sublines have already been described.[27,29] In similar long-term treatments with ATRA and $As_2O_3$ alone or in combination, NB4-LR1/hTERT-GFP and NB4-LR2/hTERT-GFP continued to proliferate without any change in cell viability (Figure 3). Even though NB4-LR1/hTERT-GFP cells demonstrated a reduced cell proliferation upon 40 days of ATRA/$As_2O_3$ treatment, no loss of viability was detected. It indicates that some effects of this combination on cell growth might occur in a telomerase-independent mechanism. Importantly, we demonstrate that ectopic hTERT expression is sufficient to prevent cell death induced by ATRA/$As_2O_3$ combination, further supporting the contention that the therapeutic action of this combination of drugs relies on a telomerase-dependent mechanism.

### *Concluding remarks*

The mechanisms underlying the improved efficacy of ATRA/$As_2O_3$ compared to single drug treatments among APL patients

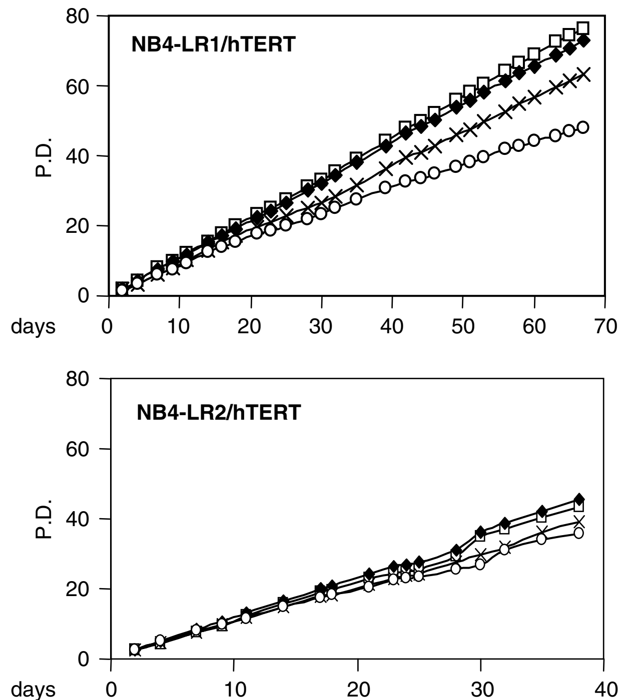


**Figure 3** hTERT ectopic expression protects against cell death induced by ATRA/$As_2O_3$ combination. NB4-LR1/hTERT and NB4-LR2/hTERT cells were cultured in the same conditions as in Figure 1 in the presence of medium alone (□), the continuous presence of ATRA (1 $\mu$M; X), $As_2O_3$ (0.2 $\mu$M; ♦), or the combination of both drugs at these concentrations (○). Cell proliferation was assessed as PD.

are still unknown, although the synergistic effects of ATRA and $As_2O_3$ on apoptosis and degradation of PML-RAR$\alpha$ have been suggested as possible mechanisms.[17,18] Although the molecular mechanisms of action remain to be determined, our findings provide direct evidence that telomerase targeting can represent a likely new mechanism by which ATRA/$As_2O_3$ therapy can exert its action in APL patients. The synergism between these two agents highlights important aspects of their possible integration into the future clinical protocols (for a review see Douer and Tallman[34]). Although benefit of $As_2O_3$ was shown first in APL patients in the first or subsequent relapse after ATRA and chemotherapy, a recent study[14] showed the clear benefit of arsenic–ATRA combination in newly diagnosed APL, when applied in induction and following maintenance therapy. We suggest that the success of the latter trial can be also explained by the fact that the administration of the combination was sustained over the period required for complete remission, which is around 30–40 days in general. In light of our results presented here, the time span of induction therapy seems to be not always enough to obtain critical telomere shortening; thus resistant clones could escape, while sustaining the therapy with the combination would also allow the eradication of multiple kinds of resistant cells that would emerge. It seems that long-term low-dose combinational treatments could lead to a favorable outcome in APL patients, with the enhanced benefit of minimal toxicity, as $As_2O_3$ concentration used in our study (0.2 $\mu$M) is much lower than the therapeutic serum levels of arsenic during standard and alternative therapeutic protocols, which are settled to yield a concentration around 1 $\mu$M, with peak concentrations of 3–6 $\mu$M.[35] As $As_2O_3$ as postremission therapy seems to be favorable in patients receiving this drug after relapse, our experiments indicate that it might be used as well in low dose in combination with ATRA. The fact that deregulation of hTERT expression is a mechanism that is not restricted to APL clearly indicates that the eradication of the residual cells will likely require pharmacological treatment targeting TA. Thus, it is worth considering that this combinational treatment could be involved in the clinical applications targeting other malignancies, and raises the idea of a more general concept to investigate other, clinically approved, combinations in order to know whether their extended administration, influencing telomerase in a convergent manner, could not be of therapeutic benefit. Although the complexity of telomerase regulation and malignant transformation, as well as the individuality in the clinical response, show clearly that a treatment protocol, similar to our experimental layout, may not be effective in all cases, and not applicable as the first and only treatment, our data encourage its integration into extended trials performed *in vitro* and in the clinic.

## Acknowledgements

This work was supported by INSERM, the Ligue contre le Cancer, the Association pour la Recherche contre le Cancer (ARC nos. 4513 and 3416), and OTKA T-038163 (Hungary) research grant, BIO-00032/2001 Biotechnology Grant and F-1/03 French–Hungarian Bilateral Intergovernmental S&T Cooperation. FP was funded by the Fondation pour la Recherche Médicale (FRM). CD is a research fellow of Association pour la Recherche contre le Cancer. IT is supported by EMBO, the Société Française du Cancer and Société Française d'Hématologie.

## References

1 Hahn WC. Role of telomeres and telomerase in the pathogenesis of human cancer. *J Clin Oncol* 2003; **21**: 2034–2043.
2 Stewart SA, Hahn WC, O'Connor BF, Banner EN, Lundberg AS, Modha P *et al.* Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc Natl Acad Sci USA* 2002; **99**: 12606–12611.
3 Mergny JL, Riou JF, Mailliet P, Teulade-Fichou MP, Gilson E. Natural and pharmacological regulation of telomerase. *Nucleic Acids Res* 2002; **30**: 839–865.
4 Fu W, Begley JG, Killen MW, Mattson MP. Anti-apoptotic role of telomerase in pheochromocytoma cells. *J Biol Chem* 1999; **274**: 7264–7271.
5 Dudognon C, Pendino F, Hillion J, Saumet A, Lanotte M, Segal-Bendirdjian E. Death receptor signaling regulatory function for telomerase: hTERT abolishes TRAIL-induced apoptosis, independently of telomere maintenance. *Oncogene* 2004; **23**: 7469–7474.
6 Tallman MS, Nabhan C, Feusner JH, Rowe JM. Acute promyelocytic leukemia: evolving therapeutic strategies. *Blood* 2002; **99**: 759–767.
7 Mistry AR, Pedersen EW, Solomon E, Grimwade D. The molecular pathogenesis of acute promyelocytic leukaemia: implications for the clinical management of the disease. *Blood Rev* 2003; **17**: 71–97.
8 Puccetti E, Ruthardt M. Acute promyelocytic leukemia: PML/RARalpha and the leukemic stem cell. *Leukemia* 2004; **18**: 1169–1175.
9 Gallagher RE. Retinoic acid resistance in acute promyelocytic leukemia. *Leukemia* 2002; **16**: 1940–1958.
10 Ohno R, Asou N, Ohnishi K. Treatment of acute promyelocytic leukemia: strategy toward further increase of cure rate. *Leukemia* 2003; **17**: 1454–1463.
11 Zhang P. The use of arsenic trioxide ($As_2O_3$) in the treatment of acute promyelocytic leukemia. *J Biol Regul Homeost Agents* 1999; **13**: 195–200.
12 Chen GQ, Shi XG, Tang W, Xiong SM, Zhu J, Cai X *et al.* Use of arsenic trioxide ($As_2O_3$) in the treatment of acute promyelocytic

leukemia (APL): I. $As_2O_3$ exerts dose-dependent dual effects on APL cells. *Blood* 1997; **89**: 3345–3353.
13 Au WY, Chim CS, Lie AK, Liang R, Kwong YL. Combined arsenic trioxide and all-*trans* retinoic acid treatment for acute promyelocytic leukaemia recurring from previous relapses successfully treated using arsenic trioxide. *Br J Haematol* 2002; **117**: 130–132.
14 Shen ZX, Shi ZZ, Fang J, Gu BW, Li JM, Zhu YM *et al*. All-*trans* retinoic acid/$As_2O_3$ combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. *Proc Natl Acad Sci USA* 2004; **101**: 5328–5335.
15 Chen GQ, Zhu J, Shi XG, Ni JH, Zhong HJ, Si GY *et al*. *In vitro* studies on cellular and molecular mechanisms of arsenic trioxide ($As_2O_3$) in the treatment of acute promyelocytic leukemia: $As_2O_3$ induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood* 1996; **88**: 1052–1061.
16 Huang X. [Regulation of arsenic trioxide-inducing apoptosis]. *Zhonghua Xue Ye Xue Za Zhi* 1999; **20**: 258–260.
17 Zhu J, Lallemand-Breitenbach V, de The H. Pathways of retinoic acid- or arsenic trioxide-induced PML/RARalpha catabolism, role of oncogene degradation in disease remission. *Oncogene* 2001; **20**: 7257–7265.
18 Zhu J, Koken MH, Quignon F, Chelbi-Alix MK, Degos L, Wang ZY *et al*. Arsenic-induced PML targeting onto nuclear bodies: implications for the treatment of acute promyelocytic leukemia. *Proc Natl Acad Sci USA* 1997; **94**: 3978–3983.
19 Zhang Y, Cao EH, Liang XQ, Qin JF. Increasing sensitivity to arsenic trioxide-induced apoptosis by altered telomere state. *Eur J Pharmacol* 2003; **474**: 141–147.
20 Zhang TC, Schmitt MT, Mumford JL. Effects of arsenic on telomerase and telomeres in relation to cell proliferation and apoptosis in human keratinocytes and leukemia cells *in vitro*. *Carcinogenesis* 2003; **24**: 1811–1817.
21 Liu L, Trimarchi JR, Navarro P, Blasco MA, Keefe DL. Oxidative stress contributes to arsenic-induced telomere attrition, chromosome instability, and apoptosis. *J Biol Chem* 2003; **278**: 31998–32004.
22 Chen YC, Lin-Shiau SY, Lin JK. Involvement of reactive oxygen species and caspase 3 activation in arsenite-induced apoptosis. *J Cell Physiol* 1998; **177**: 324–333.
23 Hei TK, Liu SX, Waldren C. Mutagenicity of arsenic in mammalian cells: role of reactive oxygen species. *Proc Natl Acad Sci USA* 1998; **95**: 8103–8107.
24 Liu SX, Athar M, Lippai I, Waldren C, Hei TK. Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proc Natl Acad Sci USA* 2001; **98**: 1643–1648.
25 Chou WC, Hawkins AL, Barrett JF, Griffin CA, Dang CV. Arsenic inhibition of telomerase transcription leads to genetic instability. *J Clin Invest* 2001; **108**: 1541–1547.
26 Roussel MJ, Lanotte M. Maturation sensitive and resistant t(15;17) NB4 cell lines as tools for APL physiopathology: nomenclature of cells and repertory of their known genetic alterations and phenotypes. *Oncogene* 2001; **20**: 7287–7291.
27 Pendino F, Flexor M, Delhommeau F, Buet D, Lanotte M, Segal-Bendirdjian E. Retinoids downregulate telomerase and telomere length in a pathway distinct from leukemia cell differentiation. *Proc Natl Acad Sci USA* 2001; **98**: 6662–6667.
28 Pendino F, Sahraoui T, Lanotte M, Segal-Bendirdjian E. A novel mechanism of retinoic acid resistance in acute promyelocytic leukemia cells through a defective pathway in telomerase regulation. *Leukemia* 2002; **16**: 826–832.
29 Pendino F, Dudognon C, Delhommeau F, Sahraoui T, Flexor M, Bennaceur-Griscelli A *et al*. Retinoic acid receptor alpha and retinoid-X receptor-specific agonists synergistically target telomerase expression and induce tumor cell death. *Oncogene* 2003; **22**: 9142–9150.
30 Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood* 1991; **77**: 1080–1086.
31 Duprez E, Ruchaud S, Houge G, Martin-Thouvenin V, Valensi F, Kastner P *et al*. A retinoid acid 'resistant' t(15;17) acute promyelocytic leukemia cell line: isolation, morphological, immunological, and molecular features. *Leukemia* 1992; **6**: 1281–1287.
32 Gianni M, Koken MH, Chelbi-Alix MK, Benoit G, Lanotte M, Chen Z *et al*. Combined arsenic and retinoic acid treatment enhances differentiation and apoptosis in arsenic-resistant NB4 cells. *Blood* 1998; **91**: 4300–4310.
33 Stewart SA, Ben-Porath I, Carey VJ, O'Connor BF, Hahn WC, Weinberg RA. Erosion of the telomeric single-strand overhang at replicative senescence. *Nat Genet* 2003; **33**: 492–496.
34 Douer D, Tallman MS. Arsenic trioxide: new clinical experience with an old medication in hematologic malignancies. *J Clin Oncol* 2005; **23**: 2396–2410.
35 Zhou J, Meng R, Yang BF. Comparing two arsenic trioxide administration methods in APL therapy. *Chin Med J (Engl)* 2004; **117**: 1411–1413.