hTERT PROMOTES IMATINIB RESISTANCE IN CHRONIC MYELOID LEUKEMIA CELLS:
THERAPEUTIC IMPLICATIONS

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Running title
hTERT promotes imatinib resistance in CML cells

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Abbreviations list
CML, chronic myeloid leukemia; hTERT, human telomerase reverse transcriptase; ATRA, all-trans retinoic acid; TA, telomerase activity; PD, population doubling; WT-hTERT, wild-type hTERT; DN-hTERT, dominant negative hTERT; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labelling.

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ABSTRACT

Imatinib mesylate has demonstrated remarkable efficacy in the treatment of patients in the chronic phase of chronic myeloid leukemia (CML). However, despite an overall significant hematological and cytogenetic response, imatinib therapy may favour the emergence of drug-resistant clones, ultimately leading to relapse. Some imatinib resistance mechanisms had not been fully elucidated yet. In this study we used sensitive and resistant sublines from a Bcr-Abl positive cell line in order to investigate the putative involvement of telomerase in the promotion of imatinib resistance. We showed that sensitivity to imatinib can be partly restored in imatinib-resistant cells by targeting telomerase expression, either by the introduction of a dominant-negative form of the catalytic protein subunit of the telomerase (hTERT), or by the treatment with all-trans-retinoic acid (ATRA), a clinically used drug. Furthermore, we showed that hTERT overexpression favours the development of imatinib resistance through both its anti-apoptotic and telomere maintenance functions. Therefore, combining anti-telomerase strategies to imatinib treatment at the beginning of the treatment should be promoted in order to reduce the risk of imatinib resistance development and increase the probability of eradicating the disease.
INTRODUCTION

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder characterized by t(9;22) chromosomal translocation, which results in the generation of p210<sup>BCR/ABL</sup>, a chimeric protein endowed with constitutive active tyrosine kinase activity (1). This activity is essential for the induction of <i>in vitro</i> cellular transformation and <i>in vivo</i> leukemogenesis. The disease usually progresses in three phases: a relatively stable chronic phase, followed first by an accelerated phase and then by a blastic phase with acute leukemia characteristics, including genetic instability and acquisition of additional cytogenetic abnormalities. Imatinib mesylate, a competitive inhibitor of the ATP-binding site of the Bcr-Abl enzyme, has revolutionized the treatment of this particular type of leukemia (2). <i>In vitro</i> studies have shown that imatinib induces apoptosis in Bcr-Abl positive cell lines (3). However, despite an overall significant hematologic and cytogenetic response, 15-20% of the patients, especially in the advanced phase of the disease, develop resistance to imatinib and ultimately relapse (4-8). About half of these resistances are Bcr-Abl-dependent and are due to the reactivation of Bcr-Abl kinase activity by either gene amplification of <i>Bcr-Abl</i> or point mutations in the Abl kinase domain (9). The other half, requiring signalling pathways involved in cell proliferation and/or immortalization, remain to be characterized in order to propose additional therapies to either eradicate these Bcr-Abl positive imatinib-resistant clones or prevent the emergence of such resistances. This led us to consider telomerase as possibly involved in these resistances.

Telomerase is a ribonucleoprotein complex that is responsible for the maintenance of telomeres (10,11). The catalytic protein component of the telomerase complex, human Telomerase Reverse Transcriptase (hTERT), plays a fundamental role in cellular immortalization. Furthermore, it has been established that telomerase is endowed with additional functions that are independent of the telomere elongating activity (12-14). Its activity is undetectable or low in normal somatic cells, while it is highly expressed in approximately 85% of common cancers, supporting the notion that telomerase is a relevant target in anticancer therapy. Telomerase activity (TA) is regulated at various molecular levels, including transcription, splicing, mRNA maturation and post-translational modifications of hTERT. It has been reported that imatinib decreases TA in both Bcr-Abl positive and negative cells (15) and conversely that telomerase inhibition could enhance imatinib-induced apoptosis in K562 Bcr-Abl positive cells (16). Furthermore, it has been recently hypothesized that long-term treatment with imatinib, through the sustained suppression of Bcr-Abl activity, could induce alternative telomerase-
dependent signalling pathways, thus leading to the restoration of an abnormal proliferation of leukemic cells (17). However, no clear demonstration of this hypothesis has been provided yet. Whether telomerase could be involved in the biological effect of imatinib and whether it could contribute to the development of imatinib resistance are two unanswered questions so far.

We therefore evaluated the consequences of imatinib treatment on telomerase expression and activity using a pair of Bcr-Abl positive, sensitive and resistant cells and investigated the putative involvement of hTERT in the establishment of imatinib resistance. We showed that imatinib sensitivity can be restored in resistant cells by anti-telomerase strategies and demonstrated that overexpression of hTERT, first, protected sensitive cells from apoptosis induced by low concentration of imatinib and second, facilitated the establishment of imatinib resistance. These observations demonstrate that telomerase is an additional factor in the mechanisms of imatinib resistance and also suggest that anti-telomerase strategies could prevent or at least delay the onset of such resistance.
MATERIALS AND METHODS

Cell cultures: LAMA84-S, a CML imatinib-sensitive cell line derived from a patient in the blast crisis stage of CML and its imatinib-resistant subline (LAMA84-R), were both kindly provided by Pr F. X. Mahon (Université Victor Ségalen Bordeaux 2, France) (18). Cells were immediately expanded, frozen upon receipt and resuscitated every 4 months, using the original frozen stock. The cells were checked for the expression of the characteristic p210
\textsuperscript{Bcr-Abl} chimeric protein by immunoblot. LAMA84-R cells are resistant to imatinib because of the amplification of the Bcr-Abl gene (18). p210
\textsuperscript{Bcr/Abl} protein expression and imatinib sensitivity were regularly tested all through the study by immunoblot and apoptosis assay (see below), respectively. Otherwise, no additional authentication was done in our laboratory. Both cell lines were cultured in RPMI-1640 medium (PAA Laboratories, Les Mureaux, France) supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), streptomycin (50 µg/ml), and L-Glutamine (2 mM; PAA Laboratories, Les Mureaux, France), and incubated at 37°C at 5% CO\textsubscript{2} atmosphere (Binder Incubators, Nanterre, France). Every two days, cell density was determined using a Coulter counter and proliferation was represented in population doublings (PD) or total cell number. Cellular morphology was evaluated using May-Grünwald-Giemsa staining on cytospin preparations. Importantly, to maintain the resistant phenotype, LAMA84-R cells were continuously cultured with imatinib (1 µM) even during the experiments. Indeed, these cells experienced a significant loss of viability and reduction of proliferation as early as 3 days after imatinib withdrawal from the medium as previously reported (19). The parental sensitive LAMA84-S cells were always maintained in parallel culture without imatinib.

Vector constructions and infections: Mig-R1-hTERT and Mig-R1-Dominant Negative (DN) form of hTERT (Mig-R1-DN-hTERT) vectors were obtained by subcloning the catalytic unit of human telomerase sequence from pBABE-puro-hTERT and pBABE-puro-DN-hTERT constructs (20) into the murine stem cell virus vector Mig-R1, containing encephalomyocarditis virus internal ribosomal entry sequence and green fluorescent protein (GFP) as a reporter gene (21), so that 59 viral long terminal repeat (LTR) promoter drives its expression as previously described (22). The Mig-R1 constructs were transfected into Phoenix retroviral packaging cell line (derived from HEK293) by using Fugene HD (Roche Diagnostics, Meylan, France), to produce (VSV-G pseudotyped) viral supernatants that were
harvested 2 days post-transfections. Mig-R1 control vector Mig-R1-hTERT and Mig-R1-DN-hTERT supernatants were harvested for further LAMA84 cell infections, which were carried out in the presence of 4 µg of Proteamine sulfate/ml (Sigma, St-Louis, MO USA). Infected LAMA84 cells were sorted several days later for GFP fluorescence.

**Drugs:** Imatinib mesylate was kindly provided by Novartis Pharmaceuticals Corporation (Basel, Switzerland). All-trans retinoic acid (ATRA) was purchased from Sigma (St Louis, MO, USA). Chemical structures are shown in Supplementary Fig. 1S.

**Real-time quantitative PCR analysis of hTERT expression:** Total cellular RNA was collected from samples using TRIzol reagent (Invitrogen, Cergy Pontoise, France) as described by the manufacturer. RNA yields and purity were determined spectrophotometrically at 260-280 nm. Reverse transcription (RT) reaction was carried out using “Transcriptor First Strand cDNA Synthesis Kit” (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions with oligo(dT)₈ primers. The expression of hTERT transcripts in leukemic cells was quantified by fluorescence real-time RT-PCR using the LightCycler® technology and the “Light Cycler FastStart DNA MasterPLUS SYBR Green” kit (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. Primer sequences were 5'-CGGAAGAGTGTCTGGAGCAA-3' for the sense primer located in exon 3 (hTERT-LCS) and 5'-CTCCCACGACGTAGTCCATG-3' for the antisense primer located in exon 4 (hTERT-LCR). hTERT level was normalized to the expression of the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH) measured in parallel.

**Telomerase activity and telomere length assay:** Telomerase activity (TA) was measured using the telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. TA was expressed as a percentage of that detected in untreated cells. Telomere lengths were determined, after isolation of genomic DNA as described previously (23), using a non-radioactive chemiluminescent “TeloTAGGG Telomere length” assay developed by Roche Diagnostics (Meylan, France). The average telomere lengths could be determined by comparing the signals relative to a molecular mass standard.
**Apoptosis assay:** Apoptosis was assessed by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technology using "In Situ Cell Death Detection Kit, TMR red" (Roche Diagnostics, Meylan, France), which labelled free 3'-OH DNA cleavage observed during apoptosis. After 3 washes with PBS solution, 2 x 10⁶ cells were fixed using a 4% paraformaldehyde solution in PBS pH 7.4 for 30 min. They were washed again once and could be stored at −20°C during 5 days in 70% ethanol solution or directly permeabilized using 0.1% Triton -0.1% sodium citrate freshly prepared, for 2 min on ice. After 2 washes, cells were incubated in TUNEL reaction mixture (containing Terminal deoxynucleotidyl transferase which catalyses polymerization of labelled nucleotides to free 3'OH-DNA ends of DNA stand breaks, mixed to label solution 1:50) in humid atmosphere at 37°C during one hour. After 3 final washes, TMR red labelled nucleotides, incorporated in nucleotide polymers, were detected and quantified by flow cytometry.

**Clonogenic assay:** LAMA84-S and LAMA84-R cells were seeded in MethoCult TM H4230 (StemCell Technologies Inc., Grenoble, France) at a concentration of 60 cells/cm² and 250 cells/cm², respectively. This semi-solid medium contains methylcellulose (1%), fetal bovine serum (30%), bovine serum albumin (1%), L-glutamine (2 mM) and 2-mercaptoethanol (10⁻⁴ M). The clonogenic assay was performed by adding the cell suspension directly to methylcellulose medium in a 1:10 (v/v) ratio. Finally the methylcellulose cell suspension was seeded in the dishes and the cultures incubated at 37°C in 5% CO₂ for 20 days. For LAMA84-R cells, imatinib (1 µM) was added to the methylcellulose medium.
RESULTS AND DISCUSSION

**Imatinib induces a transcriptional repression of hTERT in imatinib-sensitive, not in imatinib-resistant, CML cells.** In order to find new targets and effective strategies for imatinib-resistant cells, we investigated whether telomerase could be involved in the biological effect of imatinib and therefore used a pair of sensitive and resistant blast crisis CML cells.

We first treated the imatinib-sensitive Bcr-Abl positive LAMA84-S cells with increasing concentrations of imatinib (from 0.125 to 1.0 µM) for 48 h. For each dose tested, we determined hTERT mRNA levels by real-time quantitative PCR, telomerase activity (TA) by TRAP assay, and apoptosis by TUNEL assay. Fig. 1A shows that treatment with imatinib resulted in a dose-dependent apoptotic response with ED50 between 0.250 and 0.5 µM. hTERT mRNA level and TA were not significantly modified by imatinib treatment at low doses (0.125-0.25 µM), whereas they were significantly decreased in cells treated at high doses (0.5 and 1.0 µM).

We then performed a kinetic analysis in order to evaluate whether the effects of imatinib at high concentrations on hTERT expression could result from apoptosis induction (Fig. 1B). LAMA84-S cells showed a clear transcriptional repression of hTERT as early as 4 h after treatment while no significant apoptosis was detected at this time. This effect is clearly not a mere consequence of apoptosis that was clearly detected only after 36 h of imatinib treatment as telomerase activity decreased.

Imatinib-resistant LAMA84-R cells maintained continuously under the selective pressure of 1 µM imatinib showed similar hTERT mRNA levels and TA (data not shown) as LAMA84-S cells cultured in absence of the drug, even though they displayed shorter telomeres (see below). In contrast to their sensitive counterparts, LAMA84-R cells remained resistant to imatinib-induced repression of hTERT, even at imatinib doses of 2.5 µM (Fig. 1C). These results support a relationship between regulation of hTERT and imatinib sensitivity.

**Imatinib is able to induce apoptosis in imatinib-sensitive LAMA84-S cells through both telomerase-dependent and -independent mechanisms.** In order to clarify further whether telomerase was involved or not in imatinib-induced apoptosis, LAMA84-S cells were transduced with hTERT (hTERT-LAMA84-S) and were then sorted on the basis of GFP expression. The expression of...
the transgene was confirmed both by mRNA hTERT expression and TA analysis compared to the
GFP-control cells (see below). Apoptosis induction by imatinib was then compared in hTERT
overexpressing and in mock transduced cells. Fig. 2 shows that hTERT overexpression was able to
significantly protect LAMA84-S cells from apoptosis induced by 0.250 µM imatinib. In contrast, it did
not protect them from the strong and rapid apoptotic effect induced by higher concentrations of this
drug. This result indicates that even though imatinib at high concentrations is able to down-regulate
hTERT expression and activity, apoptosis is likely not the consequences of this down-regulation. Note
that the ectopic expression of hTERT expression and TA remained unaltered by imatinib treatment
(data not shown).

Altogether, these data suggest that imatinib could induce apoptosis through both telomerase-
dependent and -independent mechanisms.

*Long-term ATRA treatment decreases telomerase activity and slows down proliferation
of imatinib-resistant LAMA84 cells.* Since LAMA84-R cells resist to imatinib-induced apoptosis, we
wondered whether targeting hTERT expression could be a means to overcome imatinib resistance.
We have previously shown that, in myeloid leukemic cells isolated from patients, ATRA may exert its
anti-proliferative action independently of differentiation through down-regulation of telomerase activity,
which develops slowly during long-term treatment and leads to telomere shortening and subsequent
cell death (24). Hence, LAMA84-R cells were incubated in the presence of 1 µM ATRA for a long-term
treatment. As shown on Fig. 3A, hTERT mRNA levels measured by quantitative RT-PCR decreased
as early as after 3 days of ATRA treatment and remained at 60% of control expression as early as day
7. This decrease was associated with a clear reduction of TA and a slowdown of proliferation. As a
consequence of the decrease of TA, a small but significant reduction of the mean length of telomeres
could be noticed throughout ATRA treatment of the LAMA84-R cells (Fig. 3B). However this decrease
of hTERT expression was not associated with increased apoptosis (data not shown). This observation
is in agreement with the notion of a threshold determined by the level of enzyme activity, which has to
be reached in order to induce sufficient shortening of telomeres and cell death, as previously reported
(25). Importantly, in contrast to LAMA84-R cells, the sensitive LAMA84-S counterpart cells were
neither responsive to the anti-proliferative effect of ATRA (Fig. 3C) nor to its action on hTERT
expression and activity (data not shown). This observation reinforces the notion that hTERT represent an important actor in imatinib resistance.

The dominant-negative form of hTERT (DN-hTERT) overcomes imatinib resistance in LAMA84-R cells. Even though ATRA treatment slowed cellular proliferation of imatinib-resistant cells, it failed to restore fully imatinib sensitivity, especially as regards cell death induction, because of a limited inhibition of TA. The fact that ectopic expression of a dominant-negative (DN)-hTERT variant is able to sensitize K562 CML cells to imatinib-induced cell death has already been established (16,26). Therefore, we wondered whether this strategy could be more efficient than ATRA treatment to overcome imatinib resistance in LAMA84-R cells. Both the LAMA84-R and the sensitive LAMA84-S counterpart cells were then transduced with DN-hTERT, WT-hTERT and the control vector. DN-hTERT and WT-hTERT expressions were confirmed by real-time quantitative PCR (Fig. 4, first panel). TRAP assay showed that TA was dramatically reduced in DN-hTERT transduced cells (Fig. 4, second panel) compared to control LAMA84 cells (vect LAMA84-S and vect LAMA84-R), indicating that the expression of the DN-hTERT results in the disruption of TA in these cells. The extinction of TA was equivalent in both sensitive and resistant LAMA84 cell lines.

We next determined whether inhibition of TA by DN-hTERT expression influenced telomere lengths in the transduced cells. We assessed telomere lengths in LAMA84 and LAMA84-R cells expressing either DN-hTERT or WT-hTERT or control vector (Fig. 4, third panel). Telomere length analysis revealed shorter telomere lengths in DN-hTERT-LAMA84-R compared to the control vector. Note that although TA was similarly reduced in both DN-hTERT-LAMA84-R and DN-hTERT-LAMA84-S cells, telomeres remained significantly longer in DN-hTERT-LAMA84-S (~ 3.2 kb) than in DN-hTERT-LAMA84-R (~ 2.0 kb). As expected from their higher telomerase activity, telomere lengths of WT-hTERT-LAMA84-S and WT-hTERT-LAMA84-R cells were significantly longer than those of their respective controls.

We then characterized the growth properties of LAMA84-S and LAMA84-R cells expressing either DN-hTERT or WT-hTERT or the control vector. The DN-hTERT transduced LAMA84-R cells exhibited a reduced clonogenic proliferation compared to the control vector or WT-hTERT-transduced cells (Fig. 4, fourth panel). This decrease in proliferation was associated with the induction of apoptosis (data not shown). Altogether, these results demonstrate that the repression of telomerase...
activity by the expression of a dominant-negative form of hTERT was able to restore imatinib sensitivity in an imatinib-resistant cell line. No effect on cell proliferation was observed in DN-hTERT-LAMA84-S cells. It can be speculated that this differential effect of imatinib results from the difference observed in the telomere lengths between the two cell lines.

It can be noticed that the anti-telomerase strategy through the expression of DN-hTERT was more efficient than ATRA treatment at restoring imatinib sensitivity. As previously pointed out, this can be explained by a higher inhibition of telomerase activity by DN-hTERT than by ATRA, leading to a sufficient telomere shortening and consequently an efficient induction of cell death. This observation supports the notion that telomere length could represent an important parameter to measure in CML patients, especially to evaluate the efficiency of such anti-telomerase treatment.

Altogether, these results indicate that the extinction of hTERT expression or activity could alter specifically the viability of imatinib-resistant cells. However, it is important to keep in mind that although LAMA84-R cells were resistant to the toxic action of imatinib, their viability relies on the continuous presence of this drug (as already mentioned, see Materials and Methods). Therefore, it cannot be excluded that the specific action of anti-telomerase strategies on imatinib-resistant cells requires cooperation with imatinib signalling pathways.

**hTERT overexpression facilitates imatinib-resistance establishment.** Altogether, the above results indicate that the extinction of hTERT expression or activity could modulate specifically imatinib sensitivity of CML cells and they point to a possible involvement of hTERT in the emergence of imatinib resistance. To check this hypothesis we investigated whether the ectopic expression of WT-hTERT in the LAMA84-S cells could facilitate the development of imatinib resistant clones compared to non-transduced LAMA84-S cells. For this purpose we proceeded as already described for the generation of the imatinib-resistant LAMA84-R cells (18). hTERT-LAMA84-S and their non-transduced LAMA84-S counterpart cells were exposed to successive dose escalations, from 0.125 µM imatinib (first dose-step) to 0.250 µM about 2 weeks later, when cells stopped dying (second dose-step). Proliferation curves (Fig. 5A), cytospin slides (Fig. 5B) stained with May-Grünwald Giemsa and clonogenic assays (Fig. 5C) clearly showed that hTERT-LAMA84-S cells recovered more rapidly from imatinib-induced cell death than the non-transduced LAMA84-S cells did, allowing a more rapid emergence of resistant clones. Out of the many attempts to generate imatinib-resistant clones,
resistant cells emerged reproducibly faster from WT-hTERT-LAMA84-S than from LAMA84-S cells. These observations indicate that hTERT expression is able to give cells a growth advantage during the establishment of imatinib-resistance. The median telomere length of imatinib treated cells shortened during the establishment of the resistance over an expansion time of 15 weeks. In contrast, hTERT expression stabilized telomere length in the hTERT-LAMA84-S cells suggesting that the telomere maintenance function of hTERT is likely to participate in the emergence of resistant cells.

**Concluding remarks.** Imatinib discovery represents a major breakthrough in the therapy of CML. However, despite satisfactory remission rates, resistance to this drug is an important issue for therapy since it is now clear that a significant proportion of patients in advanced disease phases display reduction of imatinib sensitivity over time and undergo relapse. Activation or up-regulation of telomerase is believed to play an important role in the progression of most human malignancies. While implication of telomerase in the development of imatinib resistance has already been suggested (27,28), no direct obvious demonstration has been given. Furthermore some contradictory studies can be found in the literature.

Our results demonstrate *first*, that imatinib could induce apoptosis in imatinib sensitive cells through both telomerase-dependent and -independent mechanisms; *second*, that imatinib induces a transcriptional repression of hTERT associated with the induction of apoptosis only in imatinib-sensitive cells and not in their imatinib-resistant counterparts. These observations suggest that telomerase represents an additional factor in imatinib resistance in blast crisis CML cells. This hypothesis has been validated by showing that the emergence of resistance occurred faster in cells overexpressing hTERT compared to control cells. It has been shown that telomerase contributes to installing an immortal cell phenotype by preventing apoptosis, and thus plays a role in cellular resistance to anticancer drugs (12,13,29-31). In keeping with this notion, we showed that hTERT overexpression significantly protects these cells from apoptosis induced by 0.250 µM imatinib. Therefore the emergence of imatinib-resistant cells could result from both the anti-apoptotic and telomere-maintenance functions of hTERT.

Hence, targeting this enzyme could be beneficial for the elimination of resistant clones. We therefore submitted imatinib-resistant LAMA84-R cells to two anti-telomerase strategies known to use different mechanisms: the expression of a dominant-negative form of hTERT and ATRA treatment.
leading to a transcriptional repression of hTERT. These two anti-telomerase strategies resulted in either cell death (DN-hTERT transduction) or anti-proliferative effects (ATRA treatment). Intriguingly, although constitutive hTERT expression and activity and Bcr-Abl expression did not differ between imatinib-resistant LAMA84-R and imatinib sensitive LAMA84-S cells, these effects were observed only in the imatinib-resistant and not in the imatinib-sensitive cells. It can be speculated that this specific effect on LAMA84-R cells could be partly explained by the fact that telomeres in these cells are shorter than those in the sensitive parental ones and therefore more prone to reach the telomere length threshold ultimately leading to cell death induction. If so, telomere length measurement in newly diagnosed CML patients might have a predictive value for the evaluation of the efficiency of anti-telomerase treatments. Alternatively, since LAMA84-R cells need to be maintained in the continuous presence of imatinib it cannot also be excluded that this specific effect of antitelomerase strategies on imatinib-resistant cells requires cooperation with imatinib signalling pathways.

The complexity of telomerase regulation, which can differ between various cell lines as well as between patient cells, makes it obvious that our treatment protocol, performed on a single cell line, could not be effective in all cases. In keeping with this notion, a recent study reports that the success of anti-telomerase strategies could depend on the presence of a functional p53 pathway (32). Nevertheless, our strategy, i.e., combining anti-telomerase agents to imatinib at the beginning of the therapy, should be considered as an attractive approach to be integrated into extended trials in order to reduce the risk of imatinib resistance development and ultimately increase the probability to eradicate the disease.

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REFERENCES


LEGENDS TO FIGURES

Figure 1: Effect of imatinib treatment of sensitive LAMA84-S and resistant LAMA84-R cells on telomerase expression, activity, and apoptosis. LAMA84-S, a cell line derived from a patient in the accelerated stage of CML and its imatinib-resistant LAMA84-R subline were kindly provided by Pr F. X. Mahon (Bordeaux, France) (18). (A) LAMA84-S cells were cultured for 48 h with various concentrations of imatinib (Im). (B) LAMA84-S cells were treated with high concentrations of imatinib (0.5 and 1.0 µM). (C) Imatinib-resistant LAMA84-R cells maintained continuously under the selective pressure of 1µM imatinib were treated with 2.5µM imatinib. At the indicated time, apoptosis was measured (upper panel) using Tunel technology using "In Situ Cell Death Detection Kit, TMR red" (Roche Diagnostics, Meylan, France). At the indicated time, hTERT mRNA expression was quantified by fluorescence-real-time RT-PCR (middle panel). hTERT level was normalized to the expression of the housekeeping gene glyceraldehyde 3 phosphate deshydrogenase (GAPDH) and expressed as a percentage of that detected in control cells. At the indicated time, protein extracts were prepared and telomerase activity (TA) was measured using the telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Meylan, France) (lower panel). TA was expressed as a percentage of that detected in untreated cells.

Figure 2: hTERT overexpression rescues LAMA84-S cells from apoptosis induced by low concentrations of imatinib. Imatinib-sensitive LAMA84-S cells transfected with either hTERT or GFP-vector construct were cultured in the absence or in the presence of imatinib for 48h. Apoptosis was quantified using the TUNEL assay as described in Fig. 1.

Figure 3: Long-term ATRA treatment decreases telomerase activity and slows down proliferation of imatinib-resistant LAMA84-R cells. (A) LAMA84-R cells were cultured in medium supplemented with imatinib (1 µM) in the absence or in the continuous presence of ATRA (1 µM). Cell proliferation was assessed as population doublings. At the indicated time, hTERT mRNA expression, TA and apoptosis were measured as described in Figure 1. (B) Telomere length of LAMA84-R cells was measured during a long-term treatment with ATRA (1 µM). (C) The parental LAMA-S cells were
cultured in the absence or in the presence of ATRA (1µM). Cell proliferation was assessed as population doublings.

**Figure 4: The dominant-negative form of hTERT (DN-hTERT) overcomes imatinib resistance in LAMA84-R cells.** After transduction with hTERT, DN-hTERT (DN) or GFP-control (vect.) retroviral vectors, LAMA84 and LAMA84-R cells were sorted on the basis of GFP expression and expanded. All the experiments were performed between 20 and 30 days after infections. Transduced cells were analyzed for hTERT mRNA level by real-time quantitative PCR and TA by TRAP assay as in Fig. 1 (first and second panels). hTERT expression and TA are expressed as a percentage of that detected in vector transduced LAMA84-S cells. Telomere lengths of transduced LAMA84-S and LAMA84-R cells were measured (third panel). The clonogenic assay was performed in methylcellulose medium in (lower panel). Note that Imatinib resistant LAMA84 cells (LAMA84-R) were continuously cultured with imatinib (1 µM) to maintain the resistant phenotype thus imatinib (1 µM) was also added to the methyl cellulose medium.

**Figure 5: hTERT overexpression facilitates imatinib-resistance establishment.** (A) Imatinib-sensitive LAMA84-S cells transfected with either hTERT or GFP-vector construct were cultured in the absence or in the presence of imatinib. Cell number was determined during the generation of imatinib-resistant cells by stepwise culture with increasing concentrations of imatinib. The 2 first steps of dose escalation corresponding to 0.125 and 0.250 µM imatinib, respectively, are shown. (B) Morphology analyses of LAMA84 and hTERT-LAMA84 cells during the establishment of imatinib resistance. Cytospin slides were stained by May-Grunwald Giemsa. Days are counted from the beginning of the second dose escalation. (C) hTERT-LAMA84-S and vector transduced LAMA84-S cells were cultured in the absence or in the presence of imatinib. Clonogenic assays were performed after a 72 h exposure of the cells to imatinib. (D) Telomere lengths were measured at each step of dose escalation in both control LAMA84-S and hTERT-LAMA-S cells.
Figure 1

(A) Apoptosis (%)

(B) hTERT/GAPDH (% control)

(C) TA (%)

LAMA 84-S

LAMA 84-R

control

Im 0.125 µM

Im 0.250 µM

Im 0.50 µM

Im 1.0 µM

Im 2.50 µM

control (Im 1.0 µM)

Im 2.50 µM

control

Im 0.50 µM

Im 1.0 µM

Im 0.50 µM

Im 1.0 µM

control

Im 0.125 µM

Im 0.250 µM

Im 0.50 µM

Im 1.0 µM

Im 1.0 µM

0 24 48 h

0 24 48 h

0 24 48 h

0 24 48 h
Figure 2

![Graph showing apoptosis levels for LAMA84-S and hTERT-LAMA84-S.](image)

- LAMA84-S
- hTERT-LAMA84-S

Apoptosis (%) vs. Im (µM): 0.0, 0.125, 0.25, 1.0
Figure 3

A) LAMA84-R vs. LAMA84-R + ATRA (1µM)

- hTERT/GAPDH (% of control)
- TA (% of control)

B) LAMA84-R

- Telomere length (kbp)

C) LAMA84-S vs. LAMA84-S + ATRA (1µM)

- Population Doubling
Figure 4
hTERT PROMOTES IMATINIB RESISTANCE IN CHRONIC MYELOID LEUKEMIA CELLS: THERAPEUTICAL IMPLICATIONS
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