AZT exerts its antitumoral effect by telomeric and non-telomeric effects in a mammary adenocarcinoma model

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Abstract. Limitless replicative potential is one of the hallmarks of cancer that is mainly due to the activity of telomerase. This holoenzyme maintains telomere length, adding TTAGGG repetitions at the end of chromosomes in each cell division. In addition to this function, there are extratelomeric roles of telomerase that are involved in cancer promoting events. It has been demonstrated that TERT, the catalytic component of telomerase, acts as a transcriptional modulator in many signaling pathways. Taking into account this evidence and our experience on the study of azidothymidine (AZT) as an inhibitor of telomerase activity, the present study analyzes the effect of AZT on some telomeric and extratelomeric activities. To carry out the present study, we evaluated the transcription of genes that are modulated by the Wnt/β-catenin pathway, such as c-Myc and cyclin-D1 (Cyc-D1) and cell processes related with their expression, such as, proliferation, modifications of the actin cytoskeleton, cell migration and cell cycle in a mammary carcinoma cell line (F3II). Results obtained after treatment with AZT (600 μM) for 15 passages confirmed the inhibitory effect on telomerase. Regarding extratelomeric activities, our results showed a decrease of 64, 38 and 25% in the transcription of c-Myc, Cyc-D1 and TERT, respectively ($p<0.05$) after AZT treatment. Furthermore, we found an effect on cell migration, reaching an inhibition of 48% ($p<0.05$) and a significant passage-dependent increase on cell doubling time during treatment. Finally, we evaluated the effect on cell cycle, obtaining a decline in G_0/G_1 in AZT-treated cells. These results allow us to postulate that AZT is not only an inhibitor of telomerase activity, but also a potential modulator of extratelomeric processes involved in cancer promotion.

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Introduction

Most tumor cells have an unlimited replicative potential, mainly due to the presence of the active telomerase holoenzyme, showing it as an attractive target for cancer therapies. Telomerase is a ribonucleoprotein complex that adds telomeric sequences at the end of the chromosome, maintaining telomere length in tumor cells (1). The human holoenzyme telomerase is a ribonucleoprotein composed by a catalytic subunit, (hTERT) and an RNA component (hTR) which acts as a template for the addition of a short repetitive sequence d(TTAGGG)n in the 3' end of the telomeric DNA and species-specific accessory proteins (2). Recently, it has been demonstrated that dyskerin binds to hTR, giving it the right conformation to ensemble with hTERT, being essential for the conformation of an active enzyme. Additionally N0P10, NHP2 and GAR1 proteins provide greater stability to the complex while pontin and reptin are two closed ATPases necessary for the stability of dyskerin and hTR *in vivo* (3).

3'-Azido-2',3'-dideoxythymidine [azidothymidine (AZT) or zidovudine] is a thymidine analogue used in the treatment of AIDS since it competitively inhibits reverse transcriptase (RT). The main component of telomerase, hTERT, is a catalytically active RT structurally similar to HIV-1 RT (4). Such similarity prompted studies on the feasibility of telomerase inhibition with a known inhibitor of viral RT such as AZT. In our laboratory, it was demonstrated for the first time that AZT is incorporated preferentially in the telomeric DNA of CHO cells (5). Few years later, we demonstrated the inhibitory action of AZT on telomere dynamics (6), and later we found that the cells of an experimental mammary carcinoma (F3II) treated for prolonged periods with no cytotoxic doses of AZT enter into senescence and programmed cell death (7).

Besides its role in telomere maintenance (canonical function), TERT has additional functions. They are related, with regulation of cell behavior, but in a telomeric-independent manner (non-canonical). For instance, TERT can function as a transcriptional modulator of the signaling pathway Wnt/β-catenin (8,9). In this manner, TERT acts as a cofactor in a transcriptional complex of β-catenin through interactions with a chromatin remodelling protein, allowing regulation of target genes of this pathway (10). Good examples are cyclin-D1 (Cyc-D1), which plays a key role in cell cycle progression (11) and c-Myc with a direct link to cell growth, differentiation and apoptosis (12).

In order to increase insight into the mechanism of action of AZT as an anticancer drug, the present study analyzed the possible effects of AZT treatment on the transcription of genes involved in the signaling pathway Wnt/β-catenin (TERT, cMyc and Cyc-D1) its effect on telomerase activity, cytoskeleton structure, tumor cell migration and cell cycle in a murine mammary adenocarcinoma cell model (F3II).

Materials and methods

Cell line and culture conditions. The mammary carcinoma cell line F3II is a highly aggressive and metastatic variant, established from a clonal subpopulation of a spontaneous hormone-independent BALB/c mouse mammary tumor (13). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 80 μg/ml gentamicin at 37˚C in 5% CO_2 atmosphere. Cell cultures were routinely subculture by trypsinization using standard procedures.

Cell proliferation assay. Cells ($1x10^4$) were plated in 96-well plates, and then treated with different concentration of AZT (50-2,000 μM) for 72 h. Cell growth was measured by colorimetric MTT assay (Sigma). The concentration producing 50% inhibition (IC_{50}) was determined by non-linear regression function of GraphPad Prism 6®. Results shown correspond to the average of three individual experiments. Furthermore, in order to demonstrate that AZT was not toxic at this dose, another control with AZT in parallel with the untreated control cells was added on the experiments on doubling time, telomerase activity and cell migration.

RNA extraction and cDNA synthesis. For each treatment, total RNA was isolated by extraction with TRIzol reagent (Life Technologies) according to the manufacturer's specifications. cDNA synthesis was performed using SuperScript III First-Strand Synthesis System (Life Technologies) from ~1 μg of total RNA with oligonucleotide dT18 (Life Technologies) according to the manufacturer's instructions. All cDNA samples were normalized by quantification at 260 nm in NanoDrop 1000 (Thermo).

Analysis of Cyc-D1, c-Myc and TERT transcription by real-time PCR. Specific primers were designed for each target using the Primer Express® Software Version 3.0 (Life Technologies). All primers designed presented efficiency values between 90 and 100%, and suitable dynamic ranges, allowing its proper use. The assays were carried out on One-Step Real-Time PCR System (Life Technologies) using SYBR-Green detection reagent (Life Technologies). β-actin transcript was used as a loading control. Once optimized all the parameters for RT-qPCR, the analysis of transcriptional expression was carried out by the ΔΔCt method.

Determination of telomerase activity. Telomerase activity was determined by TRAP assay, using RT-qPCR method with SYBR-Green (StepOne™ System equipment). The growing tumor cells were harvested and washed once with phosphate-buffered saline (PBS). Cells ($2x10^6$) were transferred to 1.5 ml conical tubes and centrifuged for 8 min at 450 x g. The pellet was lysed with 200 μl of buffer CHAPS (RNaseOUT + Protease Inhibitor), quantified and stored at -20˚C until use.

Actin staining. Control and AZT-treated cells were grown in glass coverslips in serum-free DMEM. Cells were fixed in 4% formaldehyde in PBS and stained with Alexa Fluor 555-conjugated phalloidin (Molecular Probes, Life Technologies) following the manufacturer's instructions. Images were recorded by an inverted fluorescence microscope (Nikon Eclipse T2000). Alternatively, a quantitative analysis in which the intensity of emitted fluorescence of the same reagent was determined by measurement in a fluorometer Synergy HT (BioTek).

Cell migration. Cell migration was measured using an *in vitro* wound healing assay as previously described (14). Briefly, *in vitro* 'scratch' wounds were created by scraping confluent F3II (controls and 5, 10 and 15 AZT passages) monolayers with a sterile pipette tip. After 16 h incubation in DMEM with 10% FBS in the presence or absence of AZT, cells were fixed and stained. Ten random micrographs/well were obtained and migration area was quantified using NIS-Elements 3.0 (Nikon) software.

Cell cycle. For cell cycle analysis by flow cytometry, cells were washed and incubated in serum-free DMEM for synchronization for 24 h. Cells, both control and treated with AZT 600 μM for passages 5, 10 and 15 were cultured, trypsinized and centrifuged at 450 x g. Cells were fixed in 70% methanol in PBS and stained with propidium bromide (1 mg/ml) (Life Technologies). Cell cycle progression was analyzed in a BD FACSCalibur™ (BD Biosciences) flow cytometer. Before recording 10,000 events, the verification of the doublet discrimination function of the flow cytometer was performed using DNA QC Particles kit (BD Biosciences).

Doubling time assay. Cells ($1,5x10^4$) treated with AZT 600 μM for 5, 10 and 15 passages were plated in 12-well plates. After incubation with DMEM 10% FBS and AZT treatment for 24, 48, 72 and 96 h, each plate was stained and fixed by 0.5% violet crystal 20% methanol, resuspended in 500 μl of ethanol: acetic acid (3:1) and measured at 570 nm. A growth curve was built, where the 100% was defined as the 24 h value for each condition.

Statistical analysis. All data are presented as the mean ± SD. Significant differences were determined by one-way ANOVA with Dunnett contrast, and two-way ANOVA with a Bonferroni's multiple comparisons test. The analyses were performed by GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). The criterion for a statistical significance is $p<0.05$.

Results

Effect of AZT on cell viability. In order to confirm the effect of AZT on cell viability, we determined the IC_{50} of this molecule for F3II cell line, obtaining a value of 1,195±37 μM. Regarding the data, we determined 600 μM as the treatment dose, equivalent to an IC_{25} value.

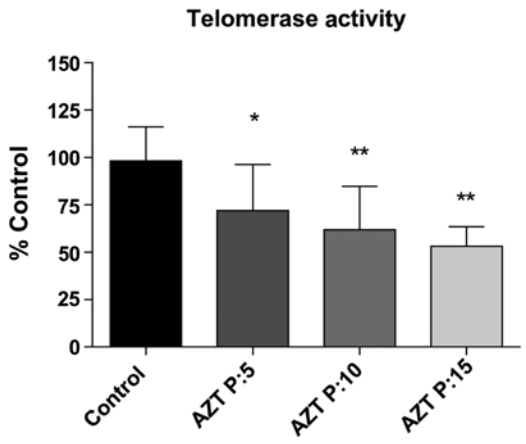

Figure 1. Determination of telomerase activity by qPCR. Quantification was performed by real-time PCR with specific primers using as template protein lysates obtained from the treated cells, at different passages (P). Values represent mean ± SEM (n=6, *p<0.05 and **p<0.01 vs. the control) (ANOVA with Dunnett contrasted).

Determination of telomerase activity. Telomerase activity significantly decreased and was evaluated progressively during the period. Telomerase activity dropped 26.22% (p<0.05), 36.27% (p<0.01) and 45.08% (p<0.001) after 5, 10 and 15 passages with AZT, respectively (Fig. 1). In this manner, corroborating the inhibitory function of AZT on telomerase activity.

Analysis of TERT, c-Myc and Cyc-D1 transcription. We quantified relative expression by ΔΔCt method of the expression of TERT, Cyc-D1 and c-Myc genes, where β-actin gene was used as endogenous loading control.

We determined the relative expression of TERT and confirmed its expression was influenced by the treatment, in a passage-dependent manner. After 5 passages TERT expression decreased by 17.1% (p<0.05), at 10 passages, expression reduced by 20.28% (p<0.05) and at the end of treatment had declined by 30.91% (p<0.05) (Fig. 2C).

Regarding the expression of Cyc-D1 in cells treated with AZT, we found a passage-dependent decrease. After 5, 10 and 15 passages, we observed a decline of 24.52% (p<0.05), 30.87% (p<0.05) and 38.24% (p<0.01), respectively (Fig. 2A).

Analyzing c-Myc we found a more pronounced decline, reaching 34.14% (p<0.01), 43.85% (p<0.001) and 64.2% (p<0.01) of inhibition at 5, 10 and 15 treated passages, respectively (Fig. 2B).

Anti-migratory effect of AZT. Due to the changes in the transcription of c-Myc, we expected an effect on cell migration. Using the wound assay, we analyzed this effect on cells treated with 600 μM AZT for 5, 10 and 15 passages. After 5 passages significant effects on cell migration were not seen. However, after 10 and 15 passages, the invaded area decreased 31% (p<0.01) and 47.79% (p<0.01) (Fig. 3).

Evaluation of the actin cytoskeleton. Control cells showed normal actin fibers in most cells with intense fluorescence on the whole cell area (Fig. 4A). At passage 5 treated cells exhibited some fluorescence in the cell periphery and also actin fibers like the untreated ones (Fig. 4B). Changes in the cytoskeleton were evident after passage 10. Cells had less actin fibers whereas a greater amount of cortical fluorescence was observed (Fig. 4C). Similar effect was observed at passage 15, where the fluorescence was much more diffuse, actin fibers were no longer present, and low fluorescence intensity was observed in the cytoplasm (Fig. 4D).

Effect of AZT on growth and the cell cycle. Cell populations were without significant differences throughout all phases of the cell cycle (Fig. 5). There were slight differences in the cell proportions in each cell cycle stage, but along the treatment, cells growth rate was not the same between control and treated cells. Once discarded effects on cell cycle arrest, we proceeded to determine doubling times of cells during treatment with AZT. Doubling time determination was performed by cell counting after 24, 48, 72 and 96 h. The F3II cell line in normal growth presented a doubling time of 22.82 h. At passage 5 after treatment, the doubling time was 43.13 h, at 10 passages was 56.21 h, and finally after 15 passages was 67.22 h (Fig. 6). This information demonstrates a clear and progressive increase in the doubling time, in relation with the number of passages treated with AZT.

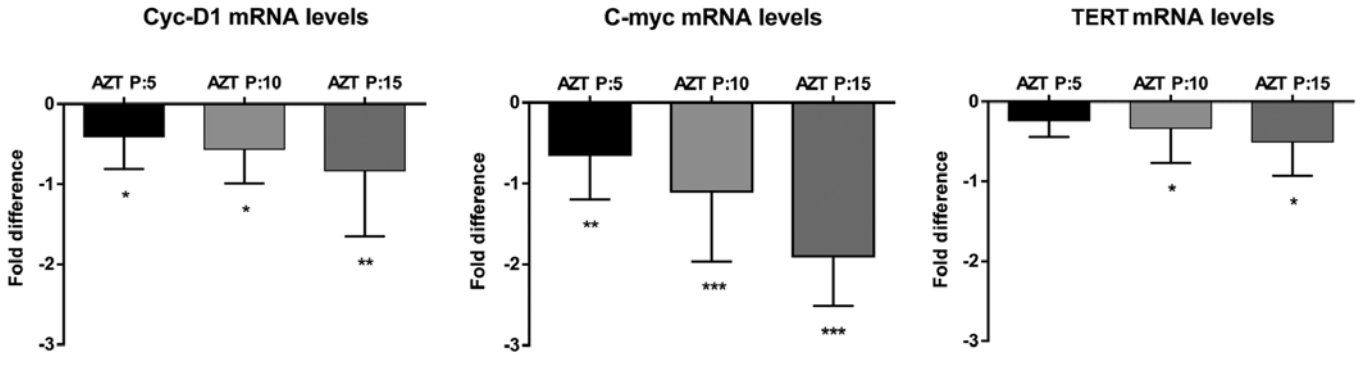

Figure 2. Determination of gene expression of Cyc-D1, c-Myc and TERT. Relative quantitation was performed by qPCR of the target genes using as charge control gene β-actin. Values represent mean ± SEM (n=6; *p<0.05 and **p<0.01 vs. the control) (ANOVA with Dunnett contrast). Asterisks are indication of significant difference in comparison with untreated cells (fold difference, 0).

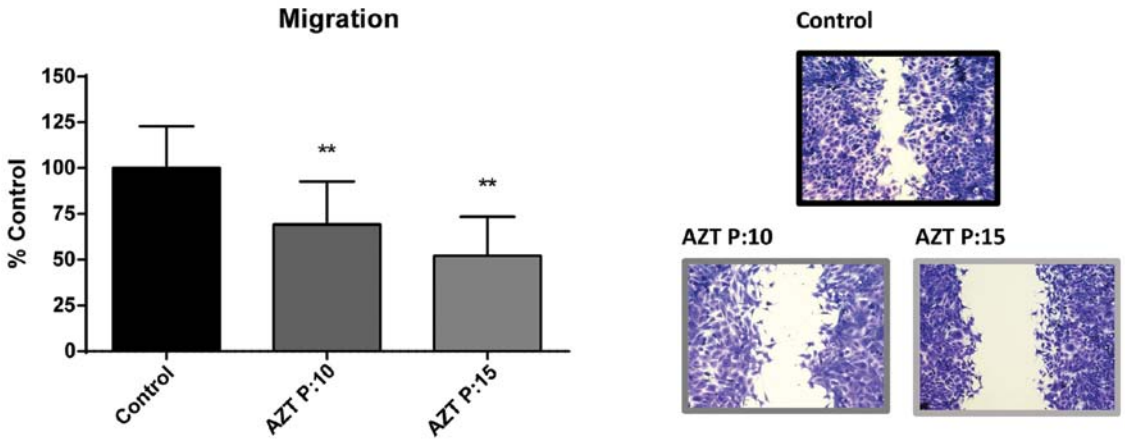

Figure 3. Evaluation of the effect of AZT on cell migration. Evaluated by scratch wound. Crystal violet staining, magnification x200. Quantification was performed using the NIS-Elements 3.0 software. Values represent mean ± SEM (n=6; *p<0.05 and **p<0.01 vs. the control) (ANOVA with Dunnett contrast).

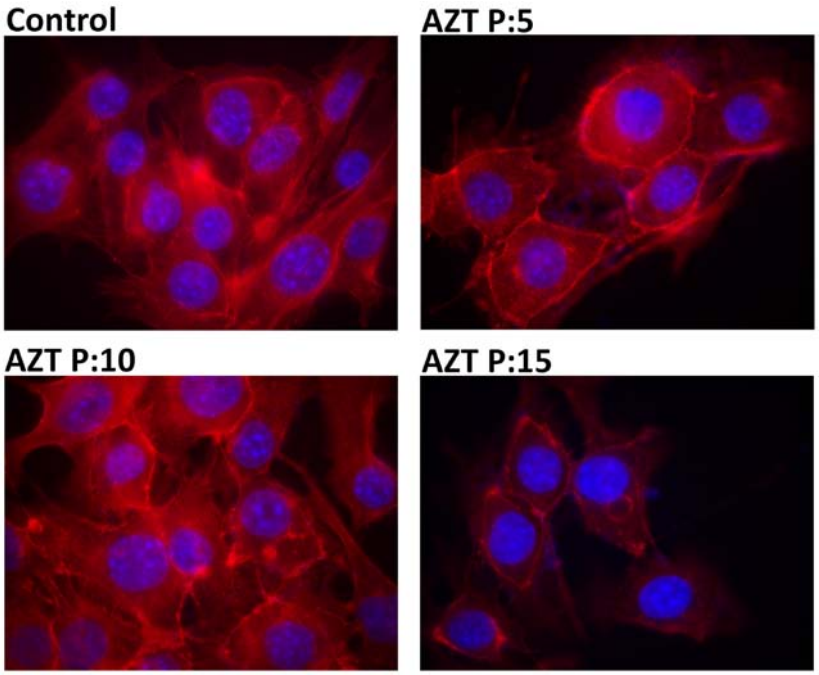

Figure 4. Evaluation of the effect of AZT on the cytoskeleton. Changes in the actin cytoskeleton (phalloidin and DAPI staining) was assessed by fluorescence microscopy. Magnification, x1,000.

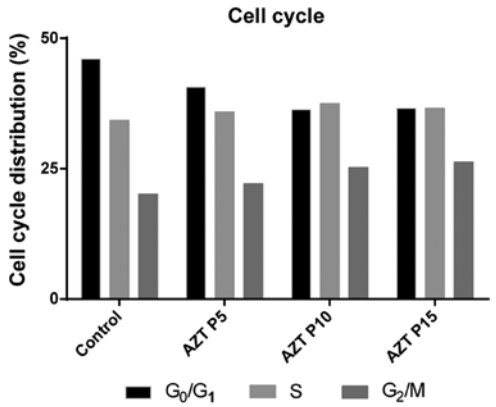

Figure 5. Effect of AZT on the cell cycle. The assay was performed by flow cytometry with propidium iodide staining (BD FACSCalibur). Events (10,000)/sample were acquired.

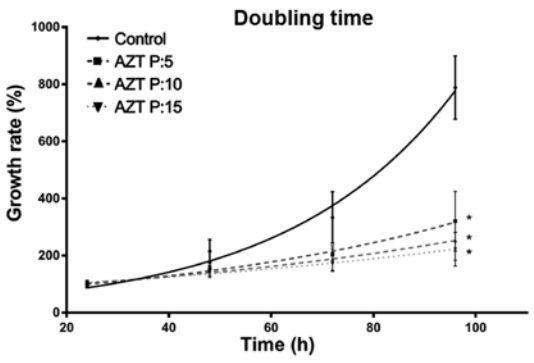

Figure 6. Analysis of AZT treatment on cell doubling time. Cells (15,000/well) were seeded in 12-wells. Plates with crystal violet and reading at 570 nm revealed every 24 for 96 h. A growth curve given as 100% measured at 24 h for each treatment was built. Statistical analysis was performed by comparing the non-linear adjustment curves through its parameters.

Discussion

3-Azido-2,3-dideoxythymidine [azidothymidine (AZT) or zidovudine] was the first reported telomerase inhibitor. The mechanism of AZT antitumor action still remains elusive. AZT is able to transform an immortal cell in a mortal one. It has been said that this was due to its action as a chain terminator. Although the experimental results support this view, AZT by itself could produce a specific antitumor action by other means, independently of its action as a telomerase inhibitor. Lately, a number of non-telomeric telomerase functions have been described. For instance, TERT, the main component of telomerase, allows the regulation of target genes of the signaling pathway Wnt/β-catenin (15), highlighting Cyc-D1 and C-myc. In the present study, we intend to demonstrate that canonical and non-canonical functions of telomerase are affected by AZT in the same cell line and with the same dose integrating the different mechanisms of action underlying the role of AZT in tumor biology (16).

Chronic treatment with a dose of 600 μM of AZT progressively decreased the telomerase activity along 15 passages in F3II cell line without effects on cell viability. Our group was the first to find this effect using 800 μM (6). Later, many other authors confirmed this finding. In the present study we found at 600 μM, that expression levels of TERT were significantly reduced through the passages evaluated. Given that, it has been reported that TERT acts as a modulator of β-catenin/BRG1 transcriptional complex, which acts on target genes of the signaling pathway Wnt/β-catenin, it is evident to expect that a decrease in the expression levels of TERT negatively impacts on expression of C-Myc and Cyc-D1 genes. Our results revealed a continuing decline, corresponding with the amount of passages, in the expression of both genes, reaching expression levels up to two-fold reduction for C-Myc and one-fold reduction to Cyc-D1. This result allows us to infer that the decline in TERT expression due to treatment with AZT, could be affecting its role as a positive regulator of these genes, coinciding with studies in the literature who analyze knockdown models of TERT (17). Ji *et al* demonstrated in 2005 that intermittent treatment with AZT initially suppressed hTERT and then c-Myc. Similar results were found by Jin *et al* in 2012 (18).

Regarding variations in Cyc-D1 after AZT treatment, we found a passage-dependent decrease reaching 38.24% at 15 passages. Downregulating expression of Cyc-D1 is a clear marker of changes or modulation in the cell cycle, being the protein which is required for G_1/S cell cycle progress. The decrease of this factor represents a cycle arrest in phase G_0/G_1 cell cycle. Due to the evidence collected regarding the expression of Cyc-D1, cell cycle analysis was performed by flow cytometry. As a result of this assay, we determined there was no such arrest. Similarly, Datta *et al* showed that long-term AZT-treated MT-2 cells arrest in all phases of the cell cycle (19). Fang *et al* found that cyclin-A was increased at early times after AZT treatment cyclin-dependent kinase 1 was decreased at later time points, while it was found that AZT-treated compared to untreated mice downregulated Cyc-D2 (p=0.0003) (20). However, some authors have found different results in other models. Olivero *et al* found that AZT induced an upregulation of Cyc-D1 accompanied by a downregulation of the Cyc-D1-associated inhibitors P18 and P57 (21). Regarding the decline found in a Cyc-D1 expression after treatment, it is important to highlight the key role played by this protein in cell cycle entry. The first approximation of this effect was based on the impact it would have on this process expecting an increase in the proportion of cells in state G_0/G_1 (22). The results obtained on the cell cycle assay did not allow us to observe this phenomenon, showing a decrease in the proportion of cells in G_1 phase; a constant proportion of cells in S phase and an increased G_2/M phase at the long-term AZT-treated cells. One possible interpretation for this observed phenomenon could be based on the fact that treatment with AZT increased levels of p21 mRNA (23). This protein is known for its role in controlling the cell cycle through inhibition of complex E/cdk2 and A/cdk2, being able to stop the progression of the cell cycle. In addition to this, during S phase, it is also able to inhibit CDK1-cyclin-A and CDK2-cyclin-A, which are required for progression through S and G_2, respectively (24). Another possible alternative to this alteration of the cell cycle, which also involves p21, is the relationship between this protein and c-Myc, being the last one presented as a repressor of p21 expression. At lower levels of expression of c-Myc, p21 levels increase, leading the regulation of different cell cycle cyclins. These facts could explain the differences between the cell amount in each phases of the cell cycle observed after treatment (25).

In addition, has been reported that c-Myc has an important role in what concerns mitosis and DNA replication, being a regulator of the target genes Cdc6, Cdt1, MCM3, 4 and 5 and other genes associated with this processes (22). This explanation coincides with was observed in our results of doubling time performed on cells at different passages (0, 5, 10 and 15), where the average doubling time is increased significantly along passages, suggesting that the AZT could impact in mitosis and duplication of the treated cells.

Chronic treatment with AZT decreases cell migration, and substantially modifies the reorganization of actin cytoskeleton, finding passage-dependent response in a high aggressiveness model as F3II. The literature suggests that there is a link between the expression of c-Myc and invasive phenotype of the cell that correlates high expression of this gene with increased migratory ability (26). Our results agree with this evidence, observing that along the treatment the expression of this gene is diminished as well as migration and actin filament formation, indicating that the treated cells have an epithelial phenotype which it was associated with a less aggressive profile. This link between c-Myc, cytoskeleton and migration is described in literature and it was related to the effect of c-Myc on signaling via RhoA/ROCK, which is highly related to the regulation of epithelial-mesenchymal transition, motility cell signaling and cytoskeletal organization (27). Focussing on the results obtained, we can postulate that AZT treatment has an effect on cell migration, probably through the regulation of c-Myc and TERT by AZT, leading tumor cells to a less aggressive phenotype, being a remarkable event in the field of extratelomerics activities. To the best of our knowledge no previous study has focused on cell migration after AZT treatment.

In conclusion, the results and information presented here, are intended to clarify and contribute to the study of how

AZT exerts its antitumoral effect. Telomerase stands at the crossroads of multiple signaling pathways and its upregulation/reactivation leads to the modulation of critical cellular processes, including gene expression and metabolism. Understanding AZT antitumoral action is of utmost importance, even more since the recent report of Bhushan and Kush on the pharmocophoric studies of anti-telomerase drugs. They found that AZT and few non-nucleoside HIV-RTIS have exquisite selectivity, not requiring bioactivation by kinases to triphosphate and not attaching into growing DNA chain. Instead, binding to allosteric site of the reverse transcriptase that is distinct from the substrate binding site. Therefore, they bind reverse transcriptase near catalytic site and instantly denature it by the non-competitive mechanism (28).

Although most of the described effects were known, this is the first time that the mentioned variables were analyzed after treatment with AZT using the same treatment, in the same cell line and at the same time, providing evidence that they are not interfering among them, and that AZT inhibitory action may be due to a mix of canonical and non-canonical effects.

Even knowing that more experiments are needed, we started a differential display study with cells treated or not with AZT. Preliminary results point toward the described dual effect.

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