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Report

Chronic *in vitro* exposure to 3′-azido-2′, 3′-dideoxythymidine induces senescence and apoptosis and reduces tumorigenicity of metastatic mouse mammary tumor cells

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Summary

Normal cells in culture divide a certain amount of times and undergo a process termed replicative senescence. Telomere loss is thought to control entry into senescence. Activation of telomerase in tumors bypasses cellular senescence and is thus a requirement for tumor progression. We reported previously the preferential incorporation of 3′-azido-2′, 3′-dideoxythymidine (AZT) in telomeric sequences of immortalized cells in culture. In this work, we have investigated the effects of chronic *in vitro* AZT exposure on F3II mouse mammary carcinoma cells. We demonstrate, for the first time, that AZT-treated tumor cells have a reduced tumorigenicity in syngeneic BALB/c mice. Tumor incidence was reduced and survival was prolonged in animals inoculated with AZT-treated cells when comparing with control counterparts. The number and size of spontaneous metastases were also decreased in animals inoculated with AZT-treated cells. In addition, we present evidence of morphological and biochemical signs of senescence, as shown by the staining for senescence associated ß-galactosidase activity, and induction of programmed cell death, as demonstrated by an increase of caspase-3 activity, in tumor cells exposed to AZT. These data indicate that chronic exposure of mammary carcinoma cells to AZT may be sufficient to induce a senescent phenotype and to reduce tumorigenicity.

Introduction

Normal diploid cells in culture divide a certain amount of times and enter a non-dividing state termed senescence. Telomere loss is thought to control entry into senescence. Telomeres consist of specific repeats at chromosome ends, which are synthesized by a reverse transcriptase called telomerase [1]. Telomerase is not expressed in most somatic tissues, however in most tumors telomerase activity is detectable [2]. Activation of telomerase in tumors bypasses cellular senescence and is thus a requirement for tumor progression [1].

Telomere maintenance is a prerequisite for continued tumor growth, therefore telomere shortening is a tumor suppressing mechanism [3]. In a previous report, we demonstrated the preferential incorporation of 3′-azido-2, 3′-dideoxythymidine (AZT) in telomeric sequences of CHO cells, indicating that such incorporation could be telomerase-mediated [4]. Later, we demonstrated that HeLa cells subjected to long-term exposure with AZT exhibited an irreversible telomere shortening without detectable signs of senescence [5]. Like many antitumor drugs, AZT appears to have, under certain circumstances, a potential tumorigenic effect [6, 7]. However, AZT has been used in phase I and II clinical trials alone or in combination with other drugs in gastrointestinal cancers, and some tumor regression has been reported [8, 9].

In this report, we subjected telomerase-expressing F3II mouse mammary carcinoma cells to chronic *in vitro* AZT exposure, and explored the ability of the compound to induce senescence and apoptosis and to decrease tumorigenesis *in vivo.*

Material and methods

Compound

AZT was purchased from Sigma (St. Louis, MO). For *in vitro* treatment, the compound was dissolved in deionized water and then diluted into culture medium.

Tumor cells and culture conditions

The sarcomatoid mammary carcinoma cell line F3II is a highly invasive and metastatic variant established from a clone of a spontaneous BALB/c mouse mammary tumor. Upon subcutaneous injection in the flank, F3II cells grow as spindle-cell carcinoma tumors with a high local invasiveness and a 90–100% incidence of lung metastases [10]. Stock F3II cells were maintained in minimal essential medium (MEM 41500, Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 80 μg/ml gentamycin, and 20 μg/ml tetracyclin in monolayer culture. For harvesting, cells were trypsinized using standard procedures and incubated in serum-free MEM for 30–60 min at 37°C for recovery.

Chronic in vitro *AZT treatment*

F3II tumor cell cultures were exposed to long-term AZT treatment at a concentration of 800 μM [5]. This concentration had relevant cytotoxic effects neither on log phase growing cells nor on semiconfluent, slowly-growing monolayers in the present cellular system. Control F3II cells were maintained in the same culture conditions without AZT, and subcultured twice a week. To assess *in vitro* growth of tumor cells, quantification of cell number was made by hemocytometer counting and doubling times were calculated in the logarithmic growth phase.

Telomerase activity determination

Telomerase activity of F3II mouse mammary carcinoma was determined in cultured cells and tumors with a photometric enzyme immunoassay by a modification of the telomere repeat-amplification protocol (TRAP) described by Kim et al. [2], using a telomerase PCR ELISA Kit (Roche, Indianapolis, IN). Samples are regarded as telomerase-positive if the difference in absorbance is >0.2 units, according to the instructions given by the manufacturer.

Telomere length determination

Telomere length was examined in DNA from F3II cells exposed to 800 μM AZT for 20, 34 and 51 *in vitro* passages, as described previously [5]. Telomere length was compared to control F3II cells before AZT exposure, as well as cells cultured for 51 passages without the compound.

Senescence associated beta-galactosidase (SA-ß-gal) assay

F3II cells cultured in the presence or absence of AZT were tested for SA-ß-gal activity as described by Dimri et al. [11]. Briefly, semiconfluent monolayers were washed with PBS, fixed for 5 min with 0.5% glutaraldehyde in PBS, washed in PBS with 1 mM $MgCl_2$, and incubated for 24 h at 37°C in fresh staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (Promega, Madison, WI), 0.5 mM $K_3Fe[CN]_6$, 0.5 mM $K_4Fe[CN]_6.3H_20$, 1 mM $MgCl_2$ in PBS, pH 6.0.

Determination of caspase-3 activity

Semiconfluent monolayers from F3II cells cultured with or without AZT were washed with PBS to eliminate serum traces and incubated in serum-free MEM for 16 h, in the presence or absence of the caspase inhibitor Z-VAD-FMK. Caspase-3 activity was determined by using the colorimetric CaspACE Assay System (Promega), following the instructions given by the manufacturer. Cell extracts from 1×10^6 cells were used as enzyme source.

In vivo *studies*

Female BALB/c inbred mice from the Animal Care Division of the Institute of Oncology Angel H. Roffo (Buenos Aires, Argentina), with an age of 12–14 weeks and a weight of 20–25 g, were used. They were housed in plastic cages under standard conditions and had access to rodent chow and water *ad libitum* [12]. Group of five animals received 2×10^5 or 5×10^4 F3II cells, exposed or not to AZT for 38 *in vitro* passages. Tumor cells were injected in the subcutis of the right flank. The time of appearance of local tumors was monitored by palpation and further confirmed by histopathology. In all cases, tumors were diagnosed as spindle-cell carcinomas. Tumor size was measured with a caliper twice a week and tumor volume was

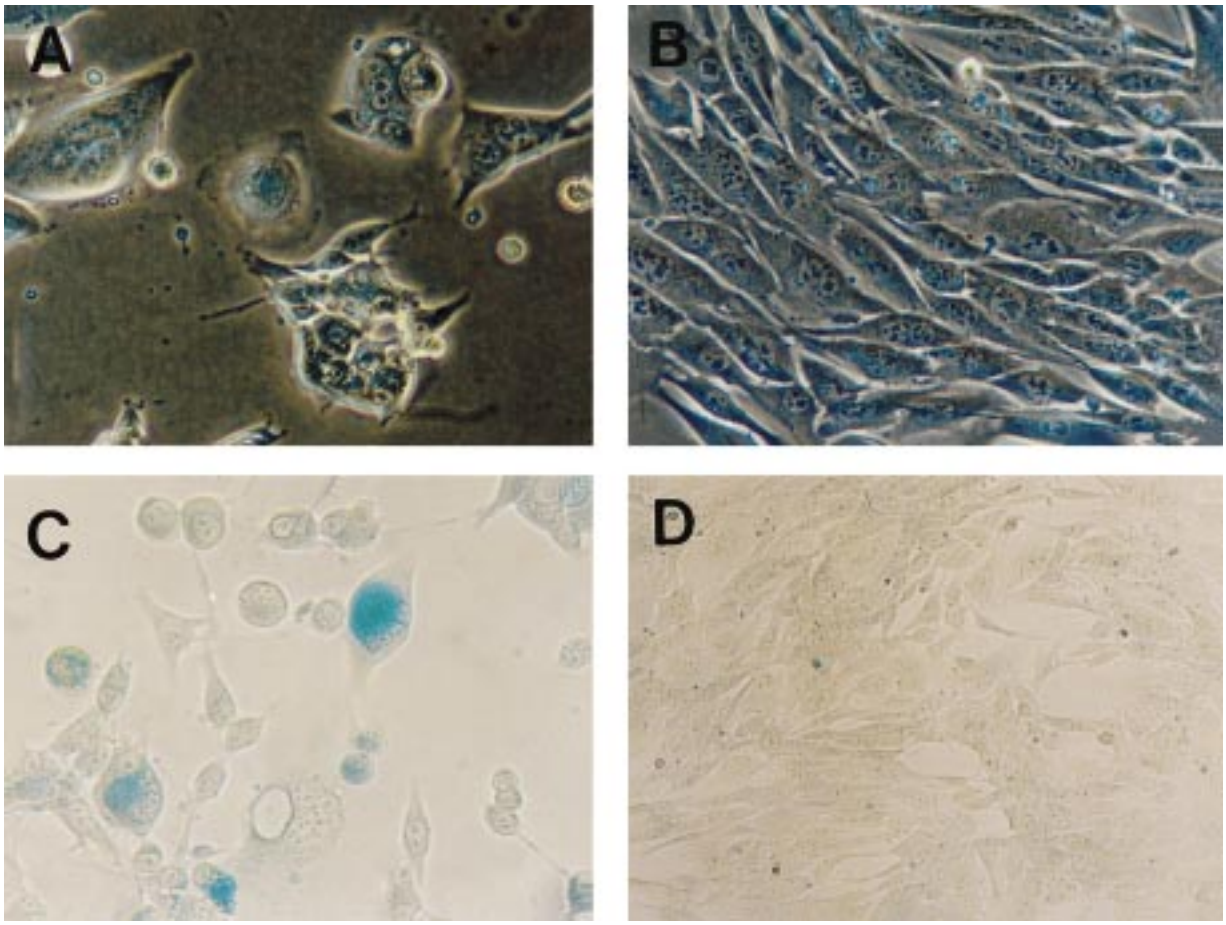

Figure 1. Morphological changes and SA-ß-gal activity in chronically *in vitro* AZT-treated F3II cells. (A) phase-contrast micrographs of F3II cultures demonstrated characteristic morphology of senescent cells after 34 passages *in vitro* in the presence of AZT. (B) untreated control cells. (C) histochemical staining for SA-ß-gal activity was positive in AZT-treated cells. (D) untreated control cells were negative for SA-ß-gal activity staining. The photographs were taken at × 200 (A, B and C) and × 100 (D).

calculated by the formula: $\pi/6 \times \text{width}^2 \times \text{length}$. Animals were sacrificed and necropsied when it became moribund or when the tumor exceeded 15,000 mm^3. Tumors were removed and fixed in 10% formalin and paraffin-embedded sections were stained with hematoxylin/eosin. Mitotic index was measured and expressed as mitoses per 10 high-power fields (HPF). The proliferating cell nuclear antigen (PCNA) was detected by immunohistochemistry. Apoptosis was determined in tumor sections by the TdT 'TUNEL'-like *in situ* non-isotopic end labeling kit (Oncogene Research Products, Boston, MA), following the instructions given by the manufacturer, and apoptotic index was also measured. To investigate the presence of spontaneous metastases a group of animals were sacrificed and necropsied on day 60; lungs were removed, fixed in Bouin's solution and the number of surface lung nodules were determined by two independent observers under a dissecting microscope, as described in detail [12].

Results

AZT inhibits telomerase activity in F3II cells

Telomerase expression on F3II cells by telomerase PCR-ELISA assay was assessed in two independent experiments. Treatment with 800 µM AZT for at least 30 passages completely inhibited telomerase activity of F3II mammary carcinoma cells. Telomere length was determined by Southern blot analysis with a telomeric probe. Telomere length determination did not show a significant decrease in telomeric fragment size in AZT-treated cells (data not shown).

Chronic AZT treatment reduces in vitro *growth rate of F3II cells*

The effect of chronic AZT treatment on the *in vitro* growth rate of F3II cells was evaluated in the logarithmic growth phase. After 42 passages, AZT-treated tumor cell cultures showed a significant increase in the population doubling time (Control: 16.9 ± 2.3 h; AZT: 41.1 ± 5.3 h; $p < 0.05$, Student's t-test).

Chronic AZT treatment induces senescence in F3II cells

As shown in Figure 1, long-term *in vitro* treatment with AZT induced morphological and biochemical signs of senescence. At passage 34, F3II cells acquired the rounded and enlarged morphology characteristic of senescent cells (Figure 1A and B). Histochemical staining with the biomarker for senescence SA-ß-gal, was positive in AZT-treated cells (Figure 1C). Control cells show no staining (Figure 1D).

Chronic treatment with AZT increases the activity of caspase-3

To evaluate whether replicative senescence produced by long-term AZT treatment leads to apoptosis, caspase-3 activity was measured. Caspase-3 is a member of the caspase family of aspartate-specific cysteine proteases that play a central role in the execution of programmed cell death [13]. Caspase-3 activity was significantly increased in AZT-treated tumor cells, with values 2.6-fold higher than control cells after more than 50 *in vitro* passages in the presence of the compound (Figure 2).

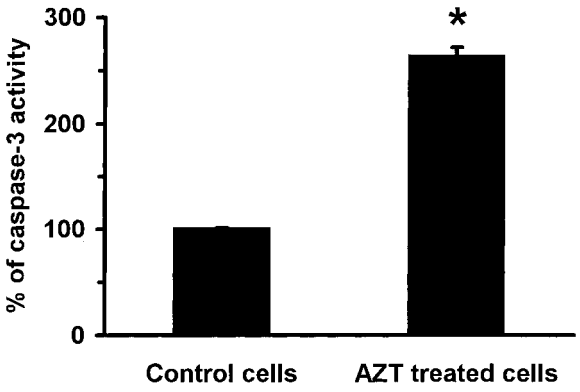

Figure 2. Increasing of caspase-3 activity in AZT-treated tumor cells. F3II cells were cultured for 59 *in vitro* passages in the presence of AZT. Caspase-3 activity was determined by colorimetric CaspACE Assay System. Results are expressed as the percent of untreated control and represent the average of two independent experiments, each one performed in duplicate. $^{*}p < 0.005$, Student's t-test.

Tumor cells chronically treated with AZT are less tumorigenic in vivo

AZT-treated cells were tested for tumorigenicity *in vivo* in syngeneic female BALB/c mice by subcutaneous injection. Animals receiving 2×10^5 AZT-

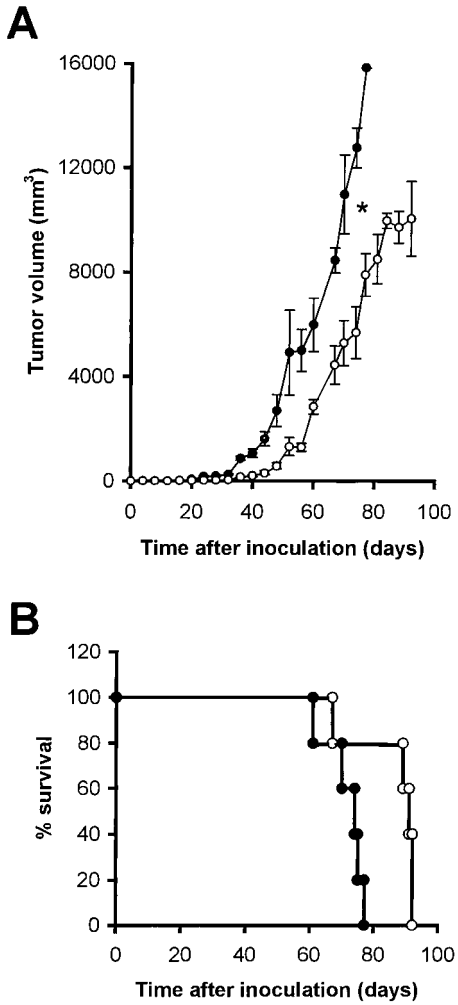

Figure 3. Effect of chronic *in vitro* AZT exposure on the growth of murine mammary tumors (A) and survival of mice (B) after inoculation with F3II cells. Animals received 2×10^5 AZT-treated F3II cells (open circles) or untreated control cells (filled circles). Data points are representative of two independent experiments with at least five animals per group. See 'Materials and methods' for further experimental details. $^{*}p < 0.001$ from day 40 to the end of the experiment, Student's t-test. Survival of tumor-bearing mice was analyzed by the Kaplan-Meier method (Log-Rank test, $p = 0.0274$; Wilcoxon test, $p = 0.0374$).

Table 1. Effect of chronic *in vitro* AZT exposure on F3II mammary tumor formation

Cells[a]	Tumor incidence at day 10 (positive per total (%))	Tumor incidence at day 25[c] (positive per total (%))	Tumor latency[d] (days)
Control	2/5 (40)	5/5 (100)	13.4 ± 6.1
AZT-treated[b]	0/5 (0)	1/5 (20)*	20

[a] 5×10^4 F3II cells were injected s.c. in the right flank of 5 female mice on day 0.
[b] F3II cells were treated *in vitro* with AZT for 38 passages.
[c] Similar results were obtained at day 120.
[d] Tumor latency represents the time between the injection of F3II cells and the appearance of detectable tumors. Results are presented as mean ± SD, considering positive animals at the end of the experiment.
* $p < 0.05$, Fisher's exact test.

Table 2. Effect of chronic *in vitro* AZT exposure on F3II mammary tumor growth and spontaneous lung metastases

Cells[a]	Tumor growth rate[c] (mm^3/day)	Incidence of lung metastases (positive per total (%))	Number of lung metastases[d] (median (range))
Control	141 ± 39	7/8 (88)	16 (0–74)
AZT-treated[b]	38 ± 36*	5/12 (42)	0 (0–3)**

[a] On day 0, 2×10^5 F3II cells were injected s.c. in the right flank of at least eight female mice.
[b] F3II cells were treated *in vitro* with AZT for 46 passages.
[c] The average tumor volume was recorded twice a week and tumor growth rate was calculated during the exponential growth phase. Values represent mean ± SEM.
[d] The number of spontaneous lung metastases was determined 60 days after s.c. tumor cell inoculation.
* $p < 0.001$, Student's t-test.
** $p < 0.01$, Mann-Whitney U test.

treated cells showed a significant reduction in primary tumor volumes (Figure 3A), and survived longer than control mice (Figure 3B). Furthermore, when mice received a minor tumor burden (5×10^4 cells), mammary tumor formation was dramatically reduced in animals inoculated with AZT-treated cells when compared with animals inoculated with untreated tumor cells (Table 1).

In a different set of experiments, animals receiving 2×10^5 tumor cells were sacrificed on day 60, and s.c. tumors and lungs were evaluated. In tumors, mitotic index was similar in both control and treated groups (Control: 4.2±1.6 mitoses per 10 HPF; AZT: 4.0±1.3 mitoses per 10 HPF; non-significant, Student's t-test). Accordingly, there was no difference in PCNA expression. Although telomerase activity was positive in tumors from both control and treated groups, apoptotic index was dramatically increased in tumors derived from AZT-treated cells (Control: 9.5 ± 4.2 apoptosis per 10 HPF; AZT: 90.0 ± 10.3 apoptosis per 10 HPF; $p < 0.001$, Welch's t-test for samples with different variances). Tumor growth rate was significantly diminished and the number of spontaneous metastases was decreased in animals inoculated with AZT-treated cells with respect to the control group (Table 2). The size distribution of lung nodules in control animals was 56% for small (< 1 mm in diameter), 31% for medium (1–2 mm) and 13% for large (> 2 mm) nodules. In contrast, 100% of lung nodules in animals receiving AZT-treated cells was very small (< 0.5 mm in diameter).

Discussion

In this paper we demonstrate for the first time that AZT-treated mouse mammary carcinoma cells have a reduced tumorigenicity and metastatic potential *in vivo*. Additionally, we present evidence of morphological and biochemical signs of senescence as well as induction of programmed cell death upon long-term *in vitro* AZT treatment of mammary carcinoma cells.

In a previous report we demonstrated the preferential incorporation of AZT in telomeric sequences of CHO cells, indicating that such incorporation could be telomerase-mediated [4]. Later, we demonstrated that HeLa cells subject to long-term exposure with AZT suffered an irreversible telomere shortening [5].

Rhyu affirmed that drugs designed for telomerase inhibition could provide a therapy with relatively limited side effects. But these hopes, she concluded, rest on a couple of unresolved issues, being the central one: Do shortening of telomeres cause senescence? [14]. In the present work, telomerase activity of F3II cells was completely abolished at passage 30. After 34 passages in the presence of 800 μM AZT, noticeable signs of senescence were documented in F3II cells. AZT-treated mammary tumor cells modified their morphology, with an enlarged, flattened shape and increased granularity. Accordingly, SA-β-gal activity, a biomarker for senescence, was positive in AZT-treated tumor cell cultures, in contrast with its absence in their control counterparts.

Some reports have demonstrated that AZT shortens the telomeres of *Tetrahymena* [15], decreases the telomere length in two immortalized human cell lines by telomerase inhibition [16], and inhibits cell growth and telomerase activity of breast cancer cells *in vitro* [17]. Yegorov et al. described, based on morphological observations, the induction of senescence-like processes in AZT cultured mouse fibroblasts [18].

As telomeric repeats are non-palindromic and lack restriction sites, they remain as relatively long terminal restriction fragments, which can be identified by probing with labeled telomeric oligonucleotides. We measured telomeric length and did not observe a significant telomeric shortening. In mice, very long repeated sequences have been described and shortening has not been observed upon aging or oncogenesis. These observations in the mouse have challenged the concept of telomere shortening in relation to aging and immortalization. However, Mark et al. [19] using quantitative fluorescence *in situ* hybridization demonstrated that telomere length in mice was very heterogeneous, but specific chromosomes had similar telomere length. They described that telomere length reduction was observed not only for overall telomeres but also for the shortest. Therefore, it can be hypothesized that the shortest telomeres in a cell rather than its overall length, as evaluated by Southern blot, may be rate-limiting its proliferative capacity.

Having found morphological and biochemical signs of senescence we analyzed if AZT-treated cells expressed markers of apoptosis. We found an increase of almost 300% in the activity of caspase-3, an early regulatory event in the programmed cell death process, in tumor cells treated with AZT. Lee et al. have demonstrated in telomerase deficient mice, that telomerase deficiency induces, in late generation animals, defective spermatogenesis with increased apoptosis and decreased proliferation in the testis [20].

In our experimental model, analysis of the tumorigenicity of F3II carcinoma cells revealed that mammary tumor formation was reduced and survival was prolonged in animals inoculated with AZT-treated cells when compared with control non-treated tumor cells. Furthermore, the number and size of spontaneous metastases were significantly decreased. Taking together these data indicate a reduced tumorigenicity and a delay in the disease. To further understand what biological phenomenon is governing this effect, we analyzed sections from tumors and explore their mitotic and apoptotic compartments as well as telomerase expression. Mitotic index and PCNA expression did not show any variation between tumors originated from AZT-treated or control cells. Similarly, telomerase expression was found equally positive in both groups of tumors, which is consistent with the fact that telomerase resumes its activity when treatment with AZT is discontinued [5]. However, apoptotic index was dramatically increased in tumors originated from AZT-treated cells. This confirms our hypothesis that tumor cells chronically exposed to AZT are able to enter in a program of replicative senescence ending in apoptosis.

Along similar lines, Kondo et al. transfected an antisense vector against the human telomerase RNA into human malignant glioma cells exhibiting telomerase activity. After 30 doublings, some subpopulations of transfectants expressed high levels of interleukin-1ß-converting enzyme, underwent apoptosis and exhibited reduced tumorigenicity in nude mice [21]. Although in general, the phenotypic characteristics of human tumors growing in nude mice reflects that of the original tumor, their evolution and metastatic capabilities are not as well represented as in syngeneic models [22].

Immortality may be thought of as an abnormal escape from cellular senescence. AZT, by its action of inducing senescence and apoptosis in tumor cells, appears as a candidate drug in the treatment of cancer, since all evidence points towards its specific action in telomerase positive cells. Two main considerations should be taken into account regarding the antitumorigenic potential of this drug. First, as other chemotherapeutic agents, AZT appears to have, under certain circumstances, a potential tumorigenic effect [6, 7]. Second, being the shortening of telomeres a slow process, the dynamics of the disease could put in risk the life of the patient before the action of AZT becomes noticeable. But, it could constitute an excellent adjuvant therapy in cases where conventional drugs reduce the bulk of tumor mass giving time to AZT to act in the remnant surviving tumor cells.

Cancer cells acquire an indefinite growth capacity, escaping from cell mortality ultimately through activation of telomerase and maintenance of telomeres. Our observations provide direct evidence that chronic AZT treatment may be sufficient to reverse tumor cell immortality and cause a senescent phenotype.

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