

Original Article

# Effects of azidothymidine on protein kinase C activity and expression in erythroleukemic cell K562 and acute lymphoblastic leukemia cell HSB-2

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## Abstract

Azidothymidine (AZT) is one of the anti-retroviral drugs currently used for the treatment of HIV-infected patients. Several other effects of the drug have been studied *in vitro*, such as the alterations of some enzymes, the inhibition of cell proliferation, and the increase of transferrin receptor expression. In this study, we investigated the alterations of protein kinase C (PKC) activity, PKC $\alpha$  and PKC $\beta$ II expressions and plasmatic membrane fluidity induced by AZT in two cancer cell lines, human chronic myeloid (K562) and human acute lymphoblastic (HSB-2) leukemia cells, respectively. The results showed that both PKC activity and membrane fluidity in HSB-2 cells increased after 24 h of drug incubation. PKC $\alpha$  expression in HSB-2 cells decreased after 48 h of AZT exposure, when the cell growth also decreased. However, in K562 cells, the PKC $\alpha$  and PKC $\beta$ II expressions enhanced in the presence of the drug when the growth was inhibited. The results indicate that AZT is less effective in inhibiting the growth of acute lymphoblastic HSB-2 leukemia cells than inhibiting that of chronic myeloid K562 cells. In fact, after 24 h exposure, the HSB-2 cell growth decreased less than K562 cell growth.

**Key words:** AZT, PKC, K562 cell, HSB-2 cell

## Introduction

The anti-retroviral nucleoside analog zidovudine (3'-azido-3'-deoxythymidine or azidothymidine, AZT) efficiently inhibits human HIV replication. This drug enters the cells very quickly by diffusion and/or active transport. Inside the cell, it can be phosphorylated by thymidine kinase to form 3'-azido-3'-deoxythymidine monophosphate (AZTMP), or phosphorylated by thymidylate kinase to form 3'-azido-3'-deoxythymidine diphosphate. The last derivative 3'-azido-3'-deoxythymidine triphosphate is efficiently and selectively incorporated into the nascent HIV-1 viral DNA via reverse transcriptase and finally blocks the viral DNA synthesis [1]. Besides its anti-retroviral activity, the zidovudine is known for other effects such as the ability to influence some enzymatic activities involved in the glycan processing and the interaction with host cell membranes. AZT causes

down-regulation of erythropoietin receptors [2] and increases transferrin receptor number in K562 cells [3], which might be related to the protein kinase C (PKC) alteration. It has been demonstrated that AZT influences the PKC isoforms, in fact, it causes cardiovascular damage through a mechanism involving the activation of PKC $\delta$  [4]. This old drug may be used as anti-cancer agent. AZT has been shown to act against multiple myeloma [5] and human MOLT-3 lymphoblastoid cells [6], but its clinical use is limited by its side effects.

The PKC family consists more than 12 serine/threonine-specific isoenzymes. Some of these isoforms are characterized by their dependency upon lipids for catalytic activity. The lipids control the amplitude, duration, and subcellular location of signaling by lipid second messenger-responsive kinases [7]. PKCs show tissue and region-specific expression patterns and regulate a wide variety of cellular

processes. They are also involved in both normal and cancer cell proliferation by targeting key proteins of cell cycle regulation such as the cyclin D3 [8]. The role of PKCs in cell proliferation is dependent on the cell line, timing of activation, and intracellular localization of the isoforms. In some types of melanoma, for example, PKC $\alpha$  activation is associated with increased cell proliferation and decreased differentiation; consequently the PKC $\alpha$  inhibitors may be used as anti-cancer drugs [9]. PKCs are involved in both normal and cancer hematopoietic cell proliferation and differentiation. AZT has various biological effects and it may affect the cell proliferation via PKC. Furthermore, we have reported that AZT inhibits the PKC activity when treating human chronic myeloid (K562) cells up to 3 h; AZTMP appears to be responsible for PKC inhibition [10]. In the present study, we aimed to evaluate the effect of AZT on cell growth in a longer exposure time and the possible correlation with the PKCs. To better elucidate the AZT/PKC interaction in two cancer cell lines, we treated the human erythroleukemic (K562) and the acute lymphoblastic (HSB-2) leukemia cells at different concentrations of AZT up to 72 h and checked the growth and viability of the cells, the expressions of PKC $\alpha$  and PKC $\beta$ II isoforms involved in cell growth and differentiation. Furthermore, we also used phorbol-12-myristate-13-acetate (PMA), an agent already known to cause the differentiation of HSB-2 and K562 cells [11,12] as well as the alterations of PKC $\alpha$  level and PKC $\beta$ II level. Finally, we measured the activity of PKC as well as the plasmatic membrane fluidity related to lipid peroxidation that influenced the membrane-bound enzyme function.

## Materials and Methods

### Materials

RPMI-1640 medium and fetal calf serum were from Labtek Eurobio (Les Ulis Cedex, France). PMA, AZT, L- $\alpha$ -phosphatidyl-L-serine, 1,2-dioctanoyl-sn-glycerol (C8:0), 5-doxyl-stearic acid (5-DSA), 16-doxyl-stearic acid (16-DSA), Trypan blue, and the other chemical compounds were obtained from Sigma (St Louis, USA); PepTag Assay Kit used to determine enzyme activity was purchased from Promega (Madison, USA); the monoclonal anti-actin, anti-PKC $\alpha$ , and anti-PKC $\beta$ II antibodies were from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Santa Cruz, USA); polyvinylidene fluoride (PVDF) membranes were from Bio-Rad (Richmond, USA); the reagents for enhanced chemiluminescence detection were obtained from Amersham Life Science (Pittsburgh, USA).

### Cell cultures

Human K562 cells and HSB-2 cells were purchased from American Type Culture Collection (Rockville, USA). The cells were grown in suspension at a density of  $1.0 \times 10^5$  and  $2.0 \times 10^5$  cells/ml, respectively, in RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 0.1 mg/ml penicillin, and streptomycin, in humidified air with 5% CO $_2$  at 37°C. Cells in logarithmic phase of growth were used in all experiments. Cell viability was checked by 0.1% Trypan blue exclusion assay.

### Detection of PKC activity

K562 cells were treated with 40  $\mu$ M AZT for 48 and 72 h. HSB-2 cells were treated for a time span ranging from 5 min to 72 h. Both cell lines were homogenized in 20 mM Tris-HCl buffer (pH 7.5), containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol tetraacetic acid, 10 mM 2-mercaptoethanol, 10  $\mu$ g/ml leupeptin

and aprotinin, 1 mM NaF, 1 mM Na $_3$ VO $_4$ , 2 mM phenylmethylsulfonyl fluoride, and 0.5% (v/v) Nonidet P-40. The homogenates were incubated in an ice bath for 30 min and centrifuged at 6,000 *g* for 5 min. The protein concentration of supernatant was determined as previously reported [13]. PKC activity was measured using the PepTag non-radioactive assay [12]. K562 and HSB-2 cell lysates were incubated for 30 min at 30°C with reaction mixture according to the manufacturer's protocol. The reactions were stopped by placing the tubes in a boiling water bath and the samples were loaded onto 0.8% agarose gel and run at 100 V for 15 min. After excision and solubilization of the phosphorylated bands, the absorbance was read at 570 nm and the PKC activity was expressed as nmol/(min  $\times$  mg) of total proteins.

### PKC $\alpha$ and PKC $\beta$ II expression

K562 and HSB-2 cells ( $5 \times 10^6$ ) were treated with 40  $\mu$ M AZT, 10 nM PMA, or 40  $\mu$ M AZT plus 10 nM PMA for 8, 24, 48, and 72 h. At the end of each treatment, the cells were washed with 10 mM phosphate-buffered saline (PBS) and lysed for 30 min at 4°C by addition of the buffer containing 10 mM Tris, pH 7.2, 5 mM MgCl $_2$ , 1 mM EDTA, 0.2% (v/v) Nonidet P-40, and 10  $\mu$ g/ml aprotinin and leupeptin. The proteins (60–80  $\mu$ g/lane) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The expressions of PKC isotypes were assessed by isotype-specific mouse monoclonal antibodies followed by peroxidase-conjugated anti-mouse secondary IgG antibodies. Actin was detected with a monoclonal anti-actin antibody (1:200 dilution). Immune complexes were detected by a chemiluminescent detection system, quantified by ImageJ software and normalized to  $\beta$ -actin levels.

### Cell labeling and electron paramagnetic resonance measurements

The electron paramagnetic resonance (EPR) study was carried out to detect the plasmatic membrane fluidity alterations induced by AZT. The spin labels 5-DSA or 16-DSA which were incorporated into the cell membranes were used. The nitroxide group moves quickly around the point of attachment and its position, C5 or C16, allows different observations: the spin label C5 (5-DSA) monitors the variation in the region near the water-lipid interface of the membranes while the C16 (16-DSA) reveals the state of the membrane internal 'core' [14]. K562 cells were treated with 40  $\mu$ M AZT for 24, 48, and 72 h; HSB-2 cells were treated with 40  $\mu$ M AZT for 3, 24, 48, and 72 h. After being washed, K562 cells were incubated with  $2.5 \times 10^{-5}$  M 5-DSA for 5 min or  $2.5 \times 10^{-5}$  M 16-DSA for 15 min; HSB-2 cells were incubated with  $1.2 \times 10^{-5}$  M 5-DSA or 16-DSA for 5 min. The free probes were removed by further washing with 10 mM PBS, pH 7.4. And the pellets were finally re-suspended in 200  $\mu$ l of the same buffer and used for EPR measurements. The X-band spectrometer (9.5 GHz) was composed of various Bruker units: a magnet B-M8, the resonant cavity 4108 TMH/9101, and a microwave bridge ER040XR, equipped with the field controller BH15. The EPR signal was collected by means of a lock-in amplifier EG&G, model 5210. The spectrometer operates at central magnetic field near 3480 G, 100 G span. Instrument settings were points 1024, sweep time 300 s, microwave power 280 mW, and attenuation 10 dB. The measurements were carried out at a constant temperature of 20°C. The membrane fluidity was measured by calculating the order parameter *S* of EPR spectra of 5-DSA and the ratio of amplitudes of central peak and third peak of the signal ( $a_2/a_3$ ) of 16-DSA [15,16]. The greater values correspond to a membrane fluidity decrease.

## Statistical analysis

Data were presented as the mean  $\pm$  standard error (SE) from three or five separate experiments. The comparisons of the data were statistically analyzed by Student's *t*-test. *P* < 0.05 were considered significant.

## Results

### Effect of AZT on cell growth

After treatment with 40  $\mu$ M AZT, 10 nM PMA, and AZT plus PMA for the indicated time, the viability of K562 erythroleukemic cells was evaluated. The concentration of 40  $\mu$ M AZT was chosen, because it had been used in the previous experiments which had already been published [10]. It was found that AZT decreased K562 cell growth by 35% after 24 h exposure, with a further reduction at 72 h. As expected, 10 nM PMA alone or in combination with 40  $\mu$ M AZT, caused a total block of the growth (Fig. 1A). In HSB-2 cells, the AZT effect on cell growth and viability was evaluated after 24, 48, and 72 h treatment under the concentrations of 10, 40, and 100  $\mu$ M, respectively. As shown in Fig. 1B, 10  $\mu$ M AZT was almost ineffective, while 40  $\mu$ M AZT led a 21% inhibition at 72 h, which was similar to 100  $\mu$ M AZT treatment. But because of the high lethality (15%) under 100  $\mu$ M AZT, 40  $\mu$ M AZT was chosen as the best concentration for further experiments. Figure 1C shows the effect on HSB-2 cell proliferation after treatments up to 72 h with 40  $\mu$ M AZT, 10 nM PMA, or AZT plus PMA. After 24 h exposure to AZT, the cell viability decreased by 9% and this effect was more pronounced (24%) at longer exposure times. PMA alone or combined with AZT almost totally inhibited HSB-2 cell growth.

### Influence of AZT on PKC expression

In this set of experiments, the influence of AZT on the PKC expressions of two conventional isoforms PKC $\alpha$  and PKC $\beta$ II was assessed in K562 cells. After 72 h of AZT treatment at different concentrations, the drug induced an increase of PKC $\beta$ II with a maximum effect at 40  $\mu$ M AZT (Fig. 2A). The effect was time-dependent. After 8 h treatment, the PKC $\beta$ II slightly increased, and this modulation was more pronounced showing the maximal response at 48 h. On the contrary, the incubation with 10 nM PMA induced a decrease of PKC $\beta$ II at all monitored time points. When both AZT and PMA were added, AZT-induced PKC $\beta$ II enhancement disappeared (Fig. 2B).

Regarding the PKC $\alpha$ , as shown in Fig. 3A, our experiments demonstrated an increase of its expression at 24 h, which is more evident at 72 h. The treatment of K562 cells with PMA caused an increase of the PKC $\alpha$  expression with a time course similar to that observed with AZT. When PMA and AZT were added together to K562 cells, the

PKC $\alpha$  expression remained unchanged when compared with that obtained by AZT alone. In HSB2 cells (Fig. 3B), only PKC $\alpha$  expression was affected by AZT with a significant increase at 24 h of incubation, although the effect disappeared after longer exposure time (48 h).

### Influence of AZT on PKC activity

The basal activity measured in K562 cells was  $0.793 \pm 0.041$  nmol/min  $\times$  mg of total proteins. An exposure to 40  $\mu$ M AZT for 48 and 72 h slightly increased the PKC activity. Figure 4 shows the effect of AZT on PKC activity in HSB-2 cells at different incubation time. It is evident that, at short incubation time (<3 h), the enzyme activity was not modulated, whereas it increased to a maximum level at 24 h, which appeared to be 30% more than that of the control. Longer time of treatment, up to 72 h, did not cause further increment of PKC activity.

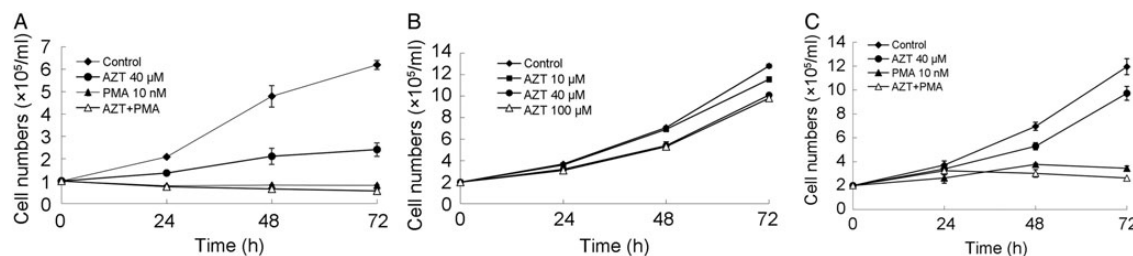
### AZT treatment modified the membrane fluidity

The possible alteration of cell membrane fluidity upon AZT treatment was evaluated by EPR spectroscopy. The EPR spectra of spin labels embedded in the plasmatic membrane were used to detect the dynamic physical state of lipids. The erythroleukemic K562 cells were treated with 40  $\mu$ M AZT at 24, 48, and 72 h and then incubated with the spin labels 16-DSA and 5-DSA. The data reported in Table 1 outlined that there are no significant differences in the EPR parameters after drug treatment.

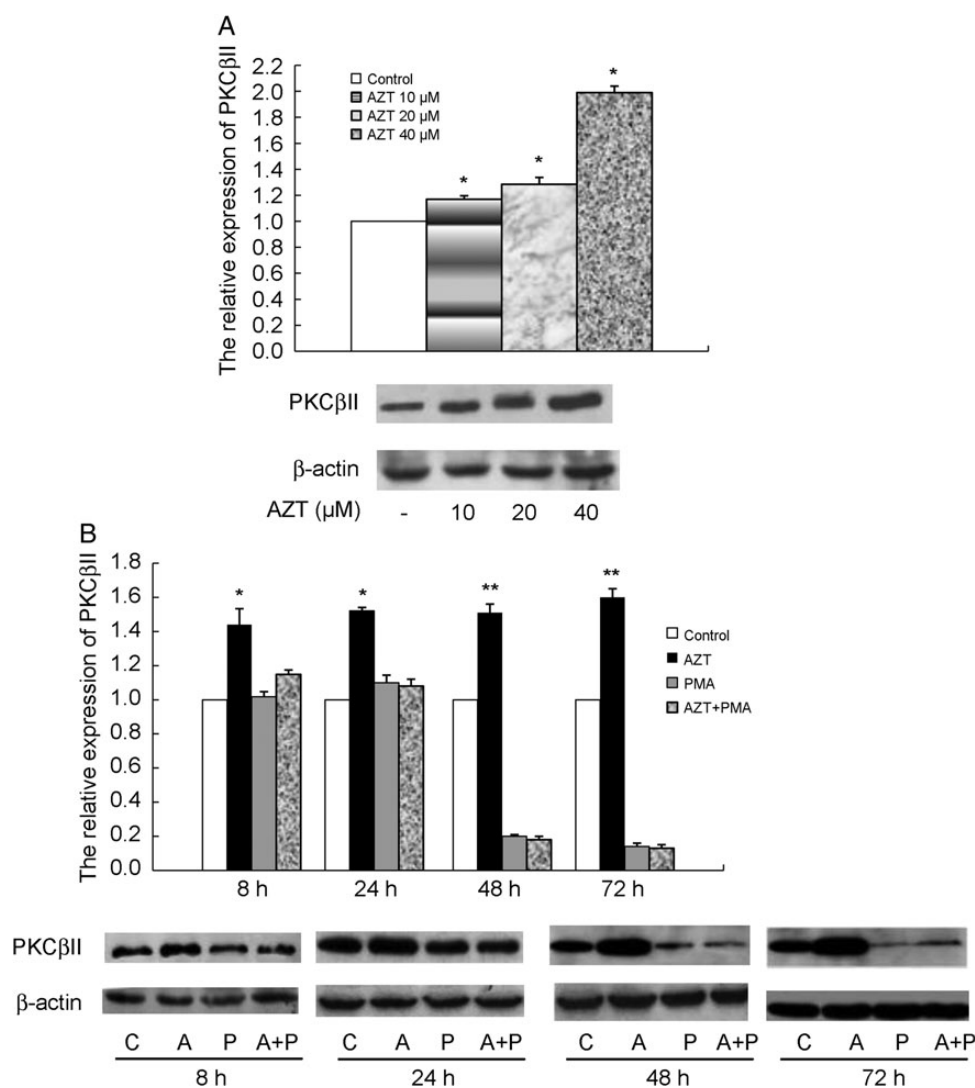
When the lymphoblastoid HSB-2 cells were exposed to AZT, no change was observed by using the 5-DSA spin label (Fig. 5A). On the contrary, at 24, 48, and 72 h, a membrane fluidity increase was found using the 16-DSA spin label, which indicates a major disorder in the deep zone of the lipid core, as demonstrated by the significant reduction (20%) of the EPR parameter (Fig. 5B).

## Discussion

The essential role played by PKC in the growth of both normal and tumor cells [8], as well as the action of AZT as anti-neoplastic drug, is well known [17]. In this study, we focused on the effect of this drug on PKC in two cell lines namely human chronic myeloid (K562) and human acute lymphoblastic (HSB-2) leukemia cells. The effect of AZT on the cell growth is less remarkable in HSB-2 than in K562 cells. The sensitivities of the cells to AZT depend on several factors such as the delivery of the drug into cells [18] and the intracellular thymidine kinase activity that converts the drug to AZTMP according to a previously published study [19]. Therefore, it was suggested that K562 and HSB-2 cells had different levels of AZTMP. Comparing the effects of



**Figure 1. Effect of AZT and PMA on cell growth** (A) Effect of 40  $\mu$ M AZT, 10 nM PMA or 40  $\mu$ M AZT plus 10 nM PMA treatments on K562 cell growth. (B) Effect of different AZT concentration on HSB-2 cell growth. (C) Effect of 40  $\mu$ M AZT, 10 nM PMA or 40  $\mu$ M AZT plus 10 nM PMA treatments on HSB-2 cell proliferation. Data are the mean  $\pm$  SE from four to six independent experiments.



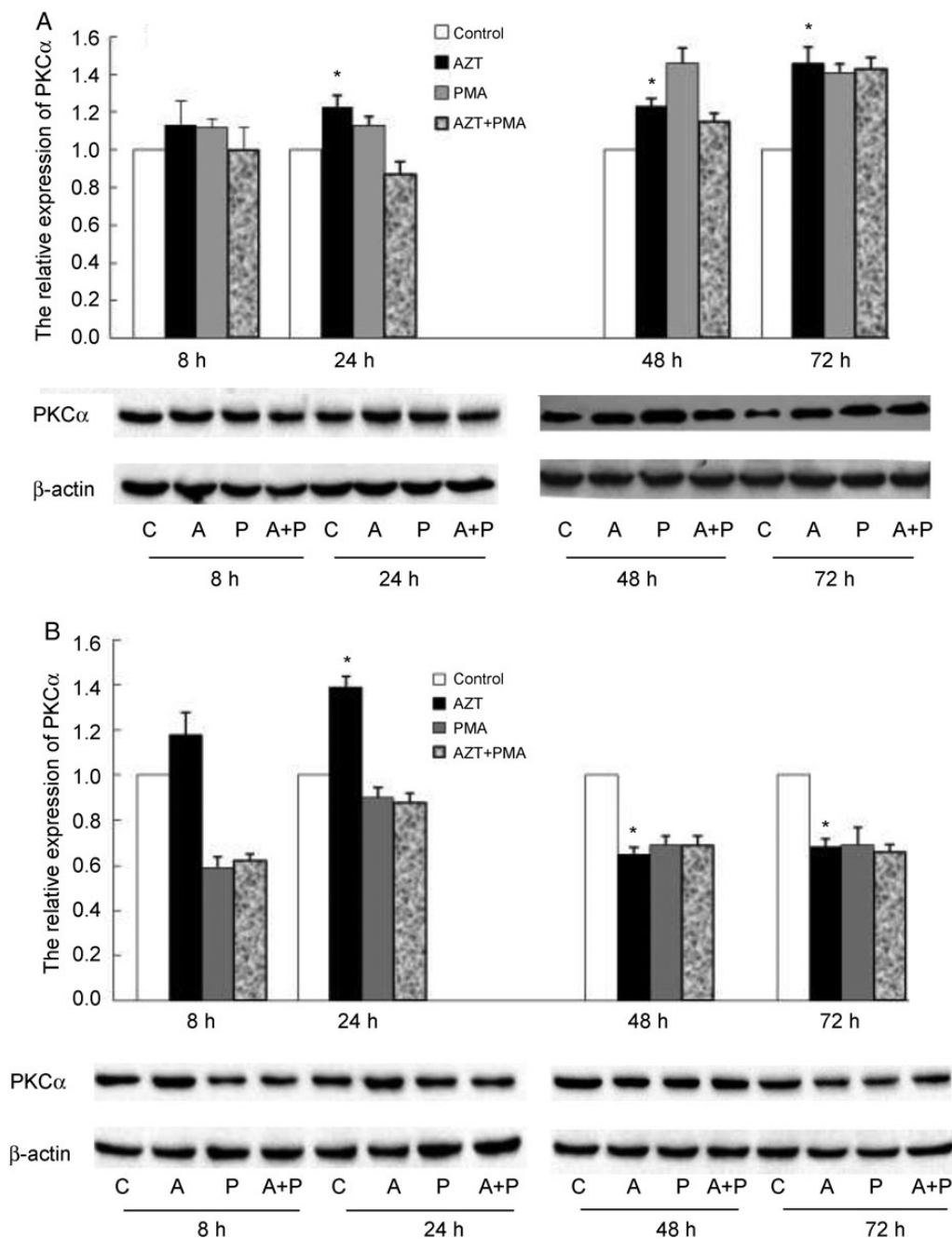
**Figure 2. Effect of AZT and PMA on PKCβII expression** (A) PKCβII expression in K562 cells treated with 10, 20, and 40 μM AZT for 72 h. (B) PKCβII expression in K562 cells. C, untreated cells; A, 40 μM AZT; P, 10 nM PMA; A + P: 40 μM AZT plus 10 nM PMA. Representative immunoblot of three independent experiments with similar results. Data have been obtained assigning the value 1 to the control. \* $P < 0.05$  vs. control. \*\* $P < 0.01$  vs. control.

AZT under our experimental conditions with PMA, which is a differentiating agent for HSB-2 cells [11] recently used in multidrug resistance studies [20], we found that both drugs could inhibit cell growth in K562 and HSB-2 cells, but the PMA exhibited a more powerful effect which was not observed with AZT. When AZT and PMA were used simultaneously, the growth was blocked after 24 h treatment, which showed that the differentiating action of PMA was prevalent on AZT effect.

Several studies have reported the relationships between cell growth and PKC isoforms expression [21]. According to our data, in K562 erythroleukemic cells, AZT induced an increase in both PKCβII and PKCα expressions. The effect on the PKCα is weaker than that on PKCβII enzyme, and only can be detected at longer exposure time. PKC isozymes are involved in the action of anti-cancer drugs by different mechanisms, promoting or inhibiting the growth, depending on the cell type. Some researchers demonstrated that PKCβII is necessary in the antitumor action of gnidimacrin [22], while another antitumor drug called enzastaurin is a PKCβ inhibitor. This drug reduces colon cancer by inhibiting the pro-proliferative genes [23]. With regard to

other cell line, in acute lymphoblastic (HSB-2) leukemia cells, AZT induced an alternating effect on PKCα expression. In fact, this protein increased at 24 h and then diminished after 48 h of AZT exposure. This trend was similar to the HSB-2 cell growth inhibition that occurred only after 48 h of treatment. As already known, PKCα depletion reduces the proliferation of cancer cells [24]. In the light of the above reported observations, we also compared the effects of AZT with PMA, which differentiated the K562 cells [25] and regulated the PKCα and PKCβII expressions [26]. In our experiments, PMA removed the effects induced by AZT on PKCα and PKCβII expressions when the cells were treated simultaneously with the two drugs. This was more evident on PKCβII, since the increase of this isoform caused by AZT is almost abolished by PMA. The effects induced by AZT on the PKC are opposite to those induced by PMA [10,26], thus supporting a different action pathway of this drug.

However, with regard to the time course of the PKC activity, the drug induced an increase in the HSB-2 cells which was not observed in the other cell type. AZT acts as an oxidant agent in many types of cells such as macrophages [27], and it induces an increase of oxidized

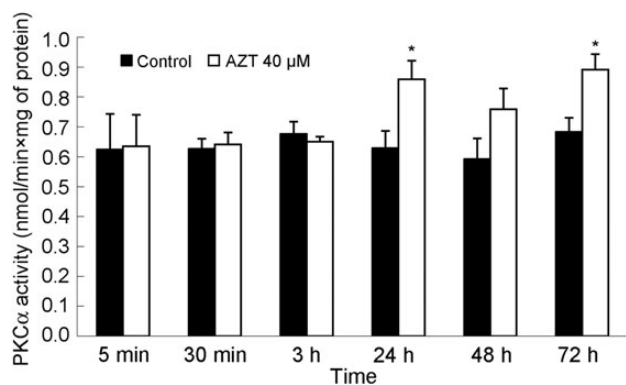


**Figure 3. Effect of AZT and PMA on PKC $\alpha$  expression** (A) PKC $\alpha$  expression in K562 cells. (B) PKC $\alpha$  expression in HSB-2 cells. C, untreated cells; A, 40  $\mu$ M AZT; P, 10 nM PMA; A + P, 40  $\mu$ M AZT plus 10 nM PMA. Representative immunoblot of four independent experiments with similar results. Data have been obtained assigning the value 1 to the control. \* $P < 0.05$  vs. control.

glutathione (GSSG) levels in tumor cells [28]. It was previously reported that reactive oxygen species (ROS) are able to directly interact with PKC which can be oxidized and activated [29]. As is known, the basal content of glutathione (GSH) in K562 cells is almost 10-fold higher than that in HSB-2 cells [30]. Although we have no experimental evidence to prove it, our results seem to suggest that K562 cells should be able to counteract the damage of ROS better than HSB-2 cells. The increasing effect on PKC activity in HSB-2 cells could be related, at least in part, to the oxidative action of AZT. The activity of some membrane-bound proteins is influenced by oxidation processes and PKC activity increases in parallel with the lipid peroxidation

[31,32]. ROS produced by AZT, change the lipid peroxidation and increase the membrane fluidity [33,34]. According to our data, the plasma membrane fluidity of K562 cells did not change after drug treatment, while it resulted in modification of HSB2 cells. Therefore, this cell line is more sensitive than K562 cells to the AZT action on the plasma membrane. The results on PKC activity and membrane fluidity could be related to the oxidative damage induced by AZT, which could be greater in HSB-2 cells than in K562 cells.

Potential limitations of this study include lack of data to clarify the mechanisms that may explain the different responses of the two cell types. Our results indicated that AZT inhibited cell growth in both



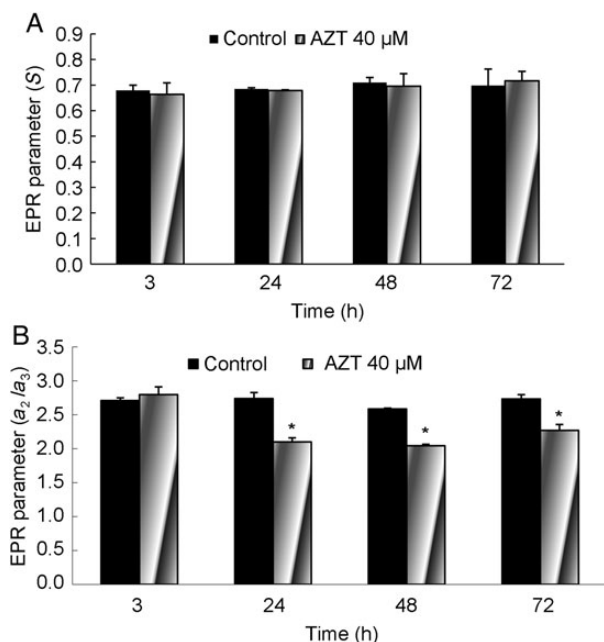
**Figure 4.** PKC activity in HSB-2 cells treated with 40  $\mu$ M AZT. Control (solid columns); AZT (dashed columns). Data are the mean  $\pm$  SE from four independent experiments. \* $P < 0.05$  vs. control.

**Table 1.** Effect of 40  $\mu$ M AZT on K562 cells membrane fluidity

	16-DSA	5-DSA
Control 24 h	3.265 $\pm$ 0.033	<sup>a</sup>
AZT 24 h	3.358 $\pm$ 0.132	<sup>a</sup>
Control 48 h	3.323 $\pm$ 0.101	0.680 $\pm$ 0.006
AZT 48 h	3.169 $\pm$ 0.190	0.598 $\pm$ 0.046
Control 72 h	3.280 $\pm$ 0.073	0.660 $\pm$ 0.015
AZT 72 h	3.248 $\pm$ 0.045	0.654 $\pm$ 0.029

The values are the  $a_2/a_3$  parameters for 16-DSA and  $S$  parameters for 5-DSA spin label.

<sup>a</sup>These data were published in a previous paper. Data are the mean  $\pm$  SE from three to five independent experiments.



**Figure 5.** Effect of 40  $\mu$ M AZT on HSB-2 cells membrane fluidity. (A) The values are the parameters  $S$  of 5-DSA spectra. (B) The values are the  $a_2/a_3$  parameters of 16-DSA spectra. Data are the mean  $\pm$  SE from four independent experiments. \* $P < 0.05$  vs. control.

cancer cell lines, modulated the PKC activity, and also regulated the PKC $\alpha$  and PKC $\beta$ II expressions. These findings can contribute to identify a new use of AZT as a drug able to control cell growth in some types of cancer. In conclusion, our study may be useful to improve the knowledge on the effect induced by AZT on human chronic myeloid (K562) and human acute lymphoblastic (HSB-2) leukemia cells.

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