

## **CASPASE DEPENDENT APOPTOSIS INDUCED BY CLADRIBINE IN THE ESTROGEN RECEPTOR NEGATIVE BREAST CANCER CELL LINE, MDA-MB468**

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### **Abstract**

The purpose of the present study is to investigate the cytotoxicity/apoptotic effect of 2-chloro-2'-deoxyadenosine, cladribine, (2-CdA) in the human breast cancer cell line, MDA-MB468 (estrogen receptor negative, ER<sup>-</sup>). MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] assay, annexin V-Fluorescein/PI and Hoechst 33258 staining were used to detect cytotoxicity and cell apoptosis. The activation of caspase-3 and -9 was assayed using caspase activation assay kits. Gel electrophoresis was performed to detect DNA fragmentation. Treatment of MDA-MB468 cells with different concentrations of 2-CdA (50, 100 and 500  $\mu$ M) resulted in a significant increase in the cell death. Annexin V-Fluorescein/PI and Hoechst 33258 staining revealed that the cell death was mainly of apoptotic type. DNA laddering profile was also obtained in the treated MDA-MB468 cells using DNA fragmentation analysis. A significant ( $p < 0.05$ ) increase in the activity of caspase-3 and -9 was observed. Pre-treatment of the cells with kinase inhibitor, 5'-amino-5'-deoxyadenosine inhibited the cytotoxicity effect of 2-CdA. This suggests that intracellular phosphorylation activation reaction plays a key role in the 2-CdA-induced apoptosis. In conclusion, this study showed that high dose of cladribine has an apoptotic effect on ER<sup>-</sup>MDA-MB468 breast cancer cells and its intracellular phosphorylation is necessary for cytotoxicity.

**Keywords:** Cladribine; Apoptosis; MDA-MB468; Caspase-3; Caspase-9

### **Introduction**

Apoptosis is a morphologically and biochemically distinct form of programmed cell death that occurs in

many cell types after exposure to toxic stimuli [1,2]. Studies performed over the past 7 years have demonstrated that aspartate-directed cysteine proteases called caspases play critical roles in the initiation and

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completion of this process [3-8]. In particular, caspase-mediated cleavages destabilize structural components of the cytoskeleton, inactivate key components of DNA repair pathways, and interrupt signal transduction pathways involved in cell survival [8]. At the same time, caspases appear to activate a number of enzymes, including the caspase-activated deoxyribonuclease [9], gelsolin [10,11], and several kinases [8]. Collectively, these cleavages contribute to the stereotypic morphological and biochemical changes [1] that constitute the process of apoptosis.

Due to the importance of caspase role in the apoptotic process, many investigators are currently performing enzymatic assays to assess the activation of these proteases.

2-chloro-2'-deoxyadenosine (cladribine), an analogue of deoxyadenosine is resistant to degradation by adenosine deaminase and is highly toxic to both non-dividing and proliferating lymphocytes [12-15]. It has been effective in the treatment of several hematological malignancies, including hairy cell leukemia and chronic lymphocytic leukemia (CLL) [16]. The cytotoxicity of 2-CdA results from the inhibitory effect of 2-CdATP on various enzymes involved in DNA replication and repair, including ribonucleotide reductase and DNA polymerase [17] and thereby inducing DNA damage and cell death [18]. In view of the potential use of 2-CdA in the treatment of some cancers, the present study was undertaken to examine cladribine for its effect on the induction of apoptosis through caspase activation mechanism in the ER<sup>-</sup> MDA-MB468 cells. The results of this study may prove the efficacy of cladribine in the treatment of breast cancers as well.

## Materials and Methods

### Materials

Cladribine, chemicals, culture media and related compounds were purchased from Sigma Co. (USA). Cell culture plasticware were from Nunc Co. (Denmark). Caspase-3 colorimetric assay kit (Cat. No. 101K4019) and DNA ladder marker, 1 Kb (61K1778) were obtained from Sigma (Germany). Caspase -9 colorimetric assay kit (Cat. No. BF10100) and annexin V-FITC apoptosis detection kit (Cat. No. TA4638) were purchased from R&D systems Co. (USA).

### Cell Culture

MDA-MB468 breast cancer cell line obtained from National Cell Bank of Iran (NCBI) were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml

penicillin and 100 µg/ml streptomycin. They were incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub> and 95% air. Cultures were regularly examined using inverted microscope (Micros, Austria).

### MTT Assay

To evaluate the cytotoxicity effect of 2-CdA on the MDA-MB468 breast cancer cell lines, MTT colorimetric assay was applied [19]. Briefly, asynchronously growing cells ( $3 \times 10^4$  cells/ml) were transferred into 48-well culture plates containing 500 µl of medium and incubated for 24 h. 2CdA, at 25, 50, 100 and 500 µM concentrations were added to each plate and incubated for 24 and 48 h after which MTT assay was performed and frequency of cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100.

### Pretreatment with Receptor Antagonist and Kinase Inhibitor

To examine the role of extracellular adenosine receptors in the 2-CdA-induced cell death, cells were pretreated with 10 µM of 8-phenyltheophylline, a receptor antagonist, for 30 min prior to the treatment with 2-CdA.

In order to provide evidence for the intracellular phosphorylation of 2-CdA, a 30 min pre-exposure to the kinase inhibitor, 5'-amino-5'-deoxyadenosine (20 µM) was performed.

### Analysis of Nuclear Morphology

MDA-MB468 cells were grown in 8 well chamber slides consisting of microscope slide base, to which cells adhere, covered by a detachable plastic cover. Cells were fixed with methanol-acetic acid 3:1 (v/v) for 10 min after which staining was carried out with Hoechst 33258 (10 µg/ml) at 37°C in dark (10 min). Slides were then washed with PBS (pH 7.4) and examined by fluorescence microscope (Micros, Austria). Annexin V/PI staining was done according to the kit manual. Early apoptotic cells show green fluorescence.

### Caspase-3 and -9 Activation Assay

A caspase-3 and -9 colorimetric assay kits were used to investigate caspase-3 and caspase-9 activation in the treated MDA-MB468. Briefly, to estimate caspase-3 activity, cells were lysed by incubation with cell lysis buffer on ice for 15 min and then centrifuged at 20,000 g for 10 min (at 4°C). For caspase-9 activation assay,

cells were lysed by incubation with cell lysis buffer on ice for 10 min and then centrifuged at 10,000 g for 1 min (at 4°C). Enzymatic reactions were carried out in a 96 well flat bottom microplate. To each reaction samples 5 and 50 µl of cell lysate (100-200 µg total protein) were added for caspase-3 and -9, respectively. Additional controls, one free from cell lysate and the other lacking substrate as well as caspase-3 positive control has been included. Protein was estimated by Bradford method [20]. The activities were expressed as nmole/min/mg protein.

### DNA Laddering

Cells, treated with different concentrations of 2-CdA for different times, were trypsinized, washed twice with ice-cold PBS and centrifuged. The pellets were lysed using lysis buffer containing 50 mM Tris-HCL (pH 8.0), 20 mM EDTA, 10 mM NaCl, 1% (w/v) sodium dodecylsulfate (SDS). The lysate was incubated sequentially with 20 µg/ml RNase (at 37°C for 60 min) and 100 µg/ml proteinase K (at 37°C for 3-5 h). DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and precipitated with 0.1 vol sodium acetate (pH=5.2) and 2.5 vol of iced cold ethanol and incubated overnight at -20°C. The precipitated DNA was washed once in 70% ethanol, resuspended in TAE buffer and then was applied on 1.8% agarose gel containing 0.5 µg/ml ethidium bromide and electrophoresis was performed for 2 h at 100 V using TAE running buffer. A ladder of 1 Kb was used as a marker.

### Statistical Analysis

The results were expressed as the mean±SD. and statistical differences were evaluated by one way ANOVA.  $P < 0.05$  was considered significant.

## Results

### Cytotoxicity Assay

To evaluate cytotoxicity of cladribine, viability tests were applied using MTT assay. As it is shown in Figure 1, treatment of the ER<sup>-</sup>MDA-MB468 cells with 50, 100, 500 µM concentrations of cladribine resulted in a significant ( $p < 0.05$ ) increase in the cell death (Fig. 1A). 2-CdA at concentrations below 50 µM had no significant effect. Pretreatment of the cells with kinase inhibitor, 5'-amino-5'-deoxyadenosine inhibited the induction of apoptosis (Fig. 1B). Pretreatment with 8-phenyltheophylline, a receptor antagonist however, did

not affect 2-CdA-induced effect (data not shown).

### Detection of Apoptosis by Annexin V/PI Staining and Hoechst 33258

Morphological changes observed in the treated (in comparison to the untreated) cells are shown in Figure 2A. Fluorescein-conjugated annexin V/PI staining (detected by epifluorescence microscope) were used to distinguish apoptotic cells. As shown in Figure 2B, cells at early apoptotic stage show a green (annexin V positive/PI negative) and those at late apoptotic or necrotic stage illustrate a green and red (annexin V positive/PI positive) fluorescence which indicates that the cell death induced by 2-CdA is mainly of apoptotic type. Non-apoptotic (untreated) cells were annexin V negative/PI negative. Furthermore, the apoptotic changes in the nuclear morphology, using Hoechst 33258 staining, are also shown in Fig. 2C.

### Caspase-3 and -9 Activation

To explore the possible biochemical mechanisms underlying 2-CdA-induced apoptosis, activities of caspase-3 and caspase-9 were assayed. The results demonstrated that the activity of caspase-3 and -9 was significantly elevated in treated ER<sup>-</sup>MDA-MB468 breast cancer cell lines (Figs. 3A, B).

### DNA Laddering

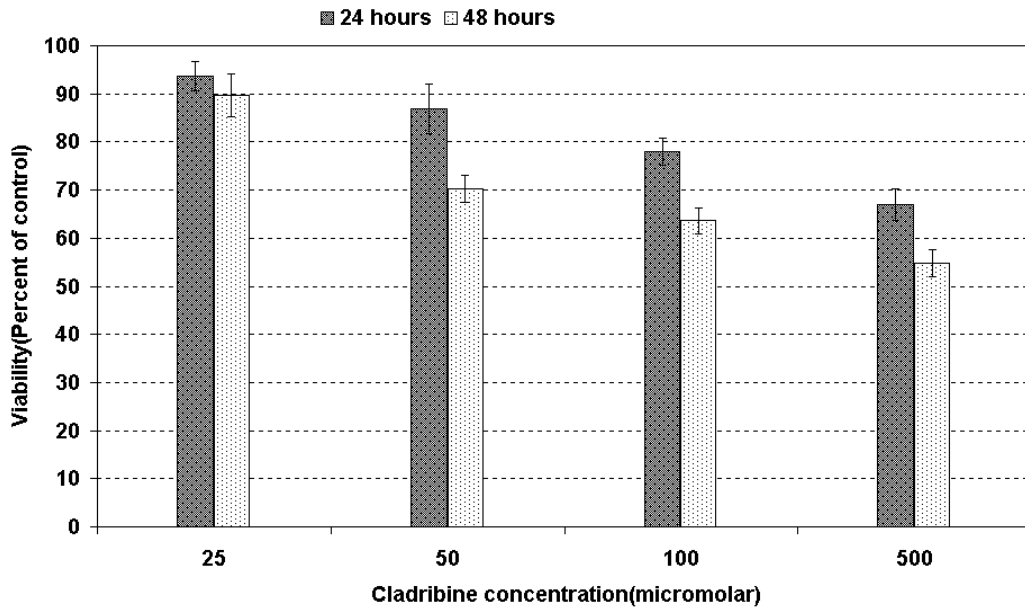
Electrophoretic analyses of DNA extracted from untreated and treated cells are shown in Figure 4. The DNA from untreated, control, cells were unfragmented. Addition of 500 µM 2-CdA to the ER<sup>-</sup>MDA-MB468 resulted in a DNA-ladder like fragmentation.

## Discussion

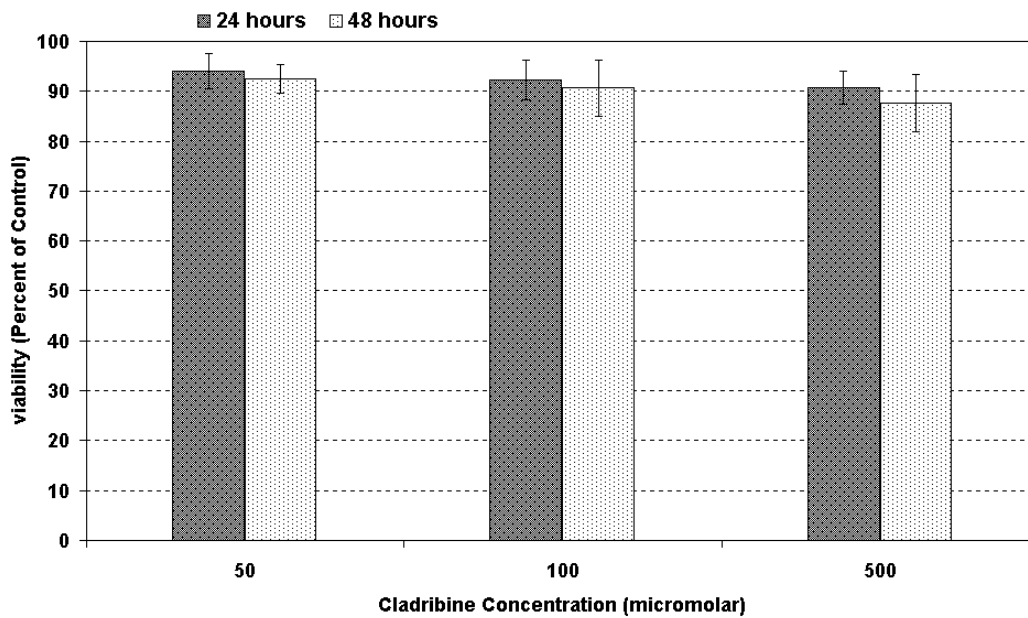
The cytotoxic effect of 2-CdA on the different cancer cell lines, other than breast cancer, has been reported. Therefore, in the present study we examined the apoptotic effect of this agent on the ER<sup>-</sup>MDA-MB468 breast cancer cell line.

Cell cytotoxicity evaluated by MTT method showed a significant increase in the death of cells treated by 2-CdA. Apoptosis was detected by morphological analysis of treated cells using annexin V/PI and Hoechst 33258 staining. The concentrations at which significant apoptosis was observed in the MDA-MB468 cells was equal or greater than 50 µM. This finding is different from that obtained for other cancer cell lines including

A)

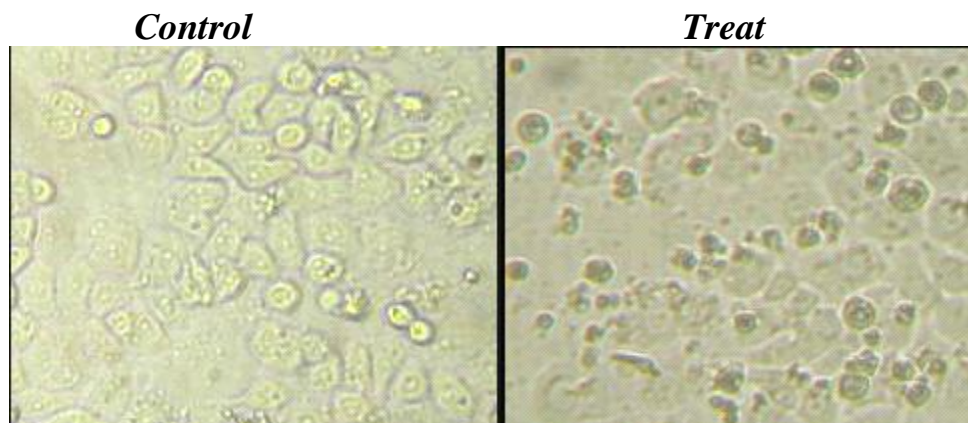


B)

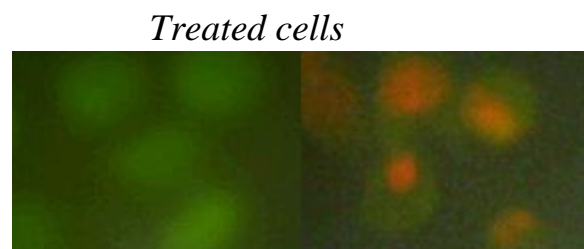


**Figure 1.** Effect of 2-CdA on the growth of breast cancer cell line, MDA-MB468. Cells were treated with different concentrations of 2-CdA for 24 and 48 h, and the viability was assessed by MTT assay (1A). Pretreatment with 5'-amino-5'-deoxyadenosine (kinase inhibitor) inhibited the cytotoxicity effect of 2-CdA in the MDA-MB468 cells (1B). Results are expressed as percent of corresponding control and represent the mean  $\pm$  SD of 6 repeats.

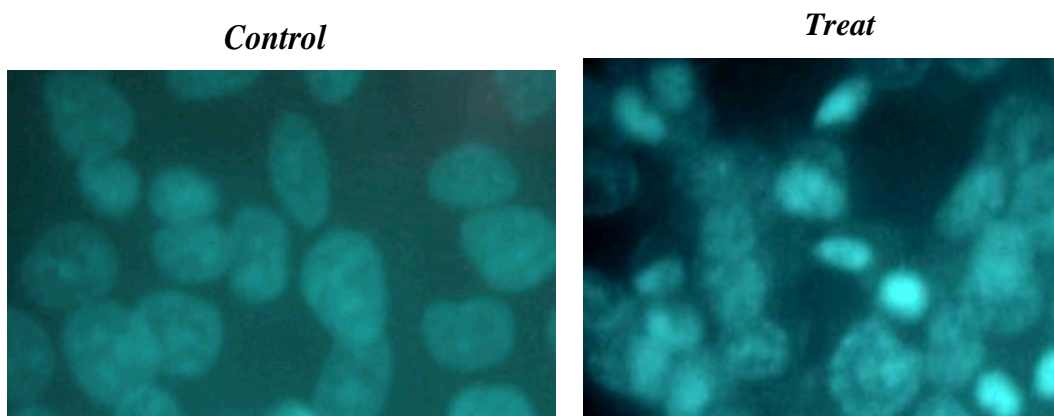
A)



B)

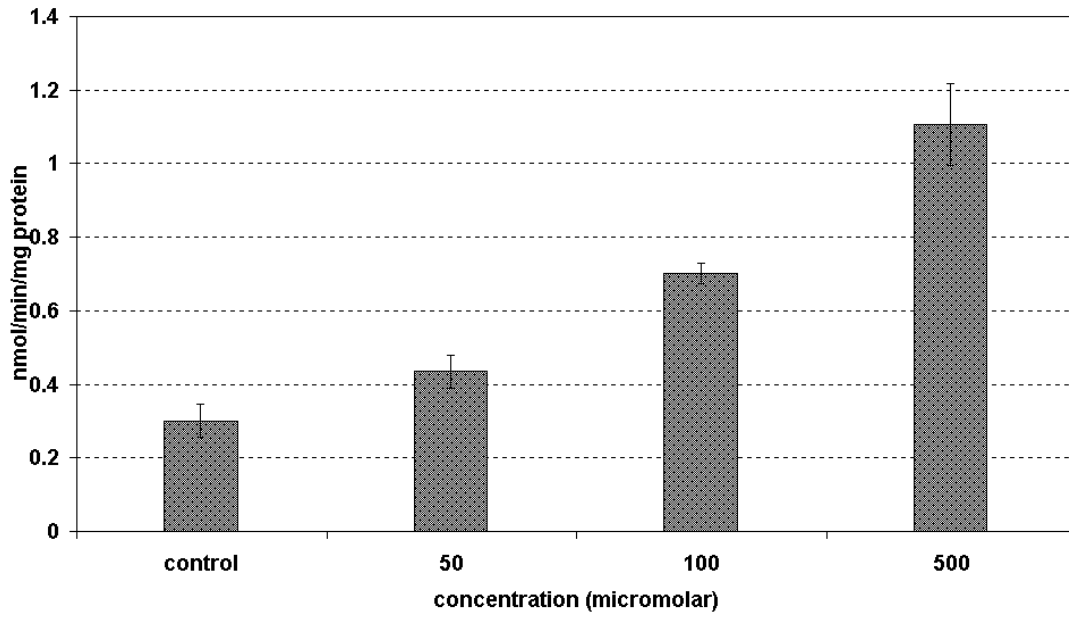


C)



**Figure 2.** Cell morphology of both treated and untreated cells detected by inverted microscope (2A). Morphological apoptotic changes using annexin V/PI staining (2B) which shows that non-apoptotic cells are annexin V<sup>-</sup>/PI<sup>-</sup> whereas early apoptotic cells are annexin V<sup>+</sup>/PI<sup>-</sup> and late apoptotic cells are annexin V<sup>+</sup>/PI<sup>+</sup>. Hoechst 33258 staining (2C) shows nuclear condensation and fragmentation characteristic of apoptotic cells.

A)



B)

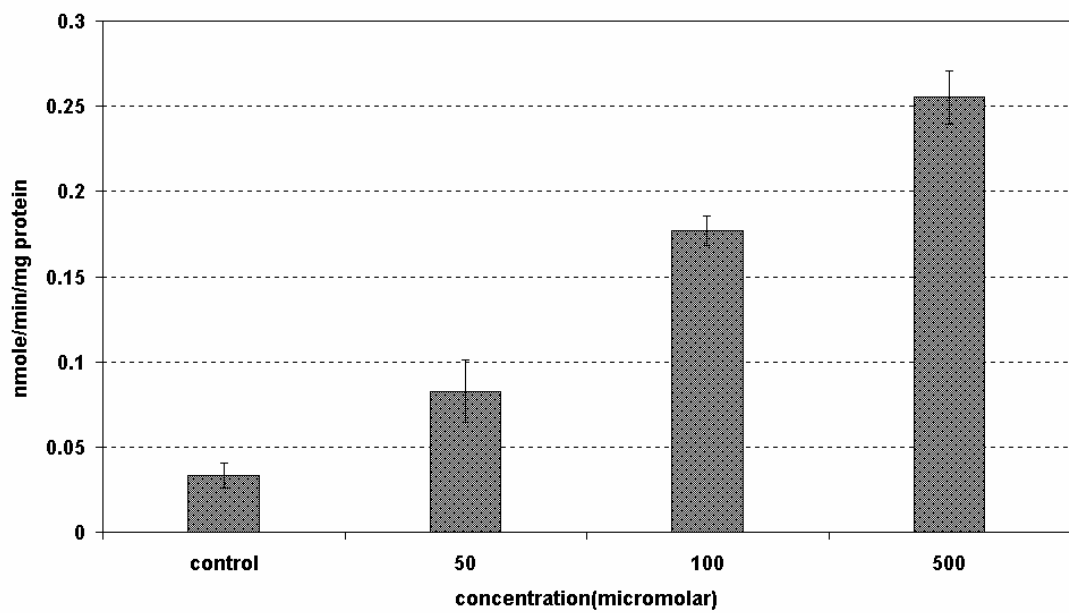
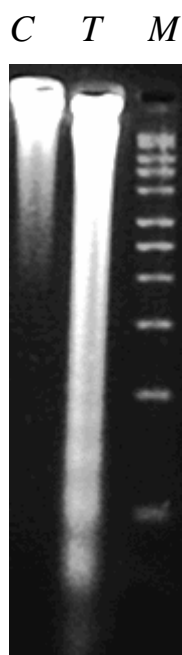


Figure 3. Activity of caspase-3 (2A) and -9 (2B) in the MDA-MB468 breast cancer cells after treatment with 2-CdA for 48 h. Results are expressed as activity of the enzyme and represent the mean±SD of 6 repeats.



**Figure 4.** Agarose gel electrophoresis of DNA from untreated (C), treated (T) MDA-MB468 as well as a 1 Kb DNA marker (M).

human astrocytoma cell line, colorectal carcinoma cell line HCT116, K562 cells, human leukemia cell lines HSB2 and Jurkat [18,21-23] indicating that these cell lines are more sensitive to the cytotoxicity effect of 2-CdA. Enzymatic factors important in 2-CdA biotransformation include deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK) and 5'nucleotidase (5'NT) [23]. Therefore, the lack or low expression of these enzymes which are responsible for the intracellular activation of this adenosine analog in MDA-MB468 breast cancer cells, may be responsible for the differential effect exerted by 2-CdA. To examine this possibility, the cells were pretreated with 5'-amino-5'-deoxyadenosine, a kinase inhibitor. The results revealed that this inhibitor is able to prevent the induction of apoptosis by 2-CdA and thus indicating the involvement of phosphorylation reaction in the observed cytotoxicity effect of 2-CdA. Furthermore, high-dose 2-CdA therapy has been used for chronic myelogenous leukemia [24]. These investigators treated patients with doses (21.5 mg/m<sup>2</sup> daily) that were higher than the usual use, and our results are highly relevant to this cladribine therapy trial. In addition, other investigators using 2-chloroadenosine-treated rheumatoid fibroblasts found a dose response of equal or greater than 50  $\mu$ M [25]. Activation of extracellular adenosine receptors did not play any significant role in the apoptotic effect

induced by 2-CdA, since pretreatment of the cells with 8-phenyltheophylline, a receptor antagonist, did not change the 2-CdA-induced effect. Therefore, 2-CdA needs to be phosphorylated inside the cells to induce cell death.

Activation of caspases is generally considered to be a requisite event during apoptosis. Our results also demonstrated that apoptosis induced by 2-CdA was dependent on the activation of caspase-3 and -9, suggesting a possible mitochondrial apoptotic pathway, e.g. through calcium-dependent cytochrome c release or a change in  $Bax/Bcl-2$  ratio. These findings are in agreement with those obtained in the 2-CdA-treated CCRF-CEM cell line [23].

Concerning caspase-3 activation, we have also previously shown that caspase-3 activation occurs upon treatment with tamoxifen [26] suggesting a caspase-3 dependent mechanism for the induction of apoptosis in the ER<sup>-</sup>MDA-MB468 human breast cancer cells.

In conclusion, to our knowledge, this is the first study demonstrating the efficacy of 2-CdA in the induction of apoptosis in ER<sup>-</sup>MDA-MB468 breast cancer cell line via caspase-3 dependent pathway.

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