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Adenosine and deoxyadenosine induces apoptosis in oestrogen receptor-positive and -negative human breast cancer cells via the intrinsic pathway

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Abstract. In this study we have examined the cytotoxic effects of different concentrations of adenosine (Ado) and deoxyadenosine (dAdo) on human breast cancer cell lines. Ado and dAdo alone had little effect on cell cytotoxicity. However, in the presence of adenosine deaminase (ADA) inhibitor, EHNA, adenosine and deoxyadenosine led to significant growth inhibition of cells of the lines tested. Ado/EHNA and dAdo/EHNA-induced cell death was significantly inhibited by NBTI, an inhibitor of nucleoside transport, and 5′-amino-5′-deoxyadenosine, an inhibitor of adenosine kinase, but the effects were not affected by 8-phenyltheophylline, a broad inhibitor of adenosine receptors. The Ado/EHNA combination brought about morphological changes consistent with apoptosis. Caspase-9 activation was observed in MCF-7 and MDA-MB468 human breast cancer cell lines on treatment with Ado/EHNA or dAdo/EHNA, but, as expected, caspase-3 activation was only observed in MDA-MB468 cells. The results of the study, thus, suggest that extracellular adenosine and deoxyadenosine induce apoptosis in both oestrogen receptor-positive (MCF-7) and also oestrogen receptor-negative (MDA-MB468) human breast cancer cells by its uptake into the cells and conversion to AMP (dAMP) followed by activation of nucleoside kinase, and finally by the activation of the mitochondrial/intrinsic apoptotic pathway.

INTRODUCTION

Apoptosis, or programmed cell death, is an orderly and genetically controlled form of cell demise that was first described by Kerr and colleagues (Kerr 1971; Kerr *et al*. 1972). It accounts for the normal physiological death of cells in a multicellular organism and permits the elimination of unwanted or sublethally damaged cells (Los *et al*. 1999; Cassens *et al*. 2003). In contrast,

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necrosis is thought to be a pathological process that results from accidental lethal physical or chemical injuries (Kerr *et al*. 1972; Ellis *et al*. 1991; Kreuter *et al*. 2004). Yet, under certain conditions, intermediate forms of cell death may occur that still follow an enzymatically regulated programme (Los *et al*. 2002; Johar *et al*. 2004).

Among several existing apoptotic signalling pathways, the death receptor-activated pathway (Los *et al*. 1995; Muzio *et al*. 1996) and the apoptosome/mitochondrial pathway (Cecconi *et al*. 1998; Pour-Jafari *et al*. 2005) are probably most frequently activated during various physiological and pathological processes. The key event during the activation of the intrinsic/mitochondrial death pathway is the release of cytochrome c from mitochondria and subsequent formation of the apoptosome (Hill *et al*. 2003). Interestingly, a significant portion of cytochrome c leaves the cell and thus it may serve as an indicator of ongoing apoptotic processes (Renz *et al*. 2001; Barczyk *et al*. 2005). Components of these pathways are frequently mutated during the development of cancer and other diseases (Los *et al*. 2001; Philchenkov *et al*. 2004). The caspase family of proteases plays a key role in execution of the apoptotic programme (Brouckaert *et al*. 2005). Because some of the caspase family members are also involved in the maturation of cytokines, novel anti-cancer and anti-inflammatory drugs frequently target these pathways (Los *et al*. 2003; Chlichlia *et al*. 2005; Hauff *et al*. 2005; Mendoza *et al*. 2005).

The role of adenosine as an endogenous toxic substance received wide attention when Giblett *et al*. (1972) demonstrated that the absence of adenosine deaminase led to a severe combined immunodeficiency syndrome. In adenosine–deaminase-deficient patients, there is an accumulation of adenosine (Ado) and deoxyadenosine (dAdo), which are subsequently phosphorylated. Ado, dAdo, and their nucleotide derivatives have long been recognized to have a multiplicity of effects on cultured cells. Depending upon the experimental conditions, some members of this group may be either toxic and inhibit growth (Henderson & Scott 1980; Henderson *et al*. 1980) or, alternatively, stimulate cell proliferation (Rozengurt 1982; Meininger *et al*. 1988; Kartha & Toback 1992; Rathbone *et al*. 1992a,b; Wang *et al*. 1992; Lemmens *et al*. 1996; Neary *et al*. 1996; Paller *et al*. 1998).

Adenosine analogues can trigger apoptosis or necrosis in various cell types and, in several instances, can inhibit cell growth and interfere with progression of the cell cycle (Ceruti *et al*. 2000). Activation of extracellular adenosine receptors (A1, A2A, A2B and A3), as well as direct intracellular action, have been claimed to play a role, depending upon the cell type and specific pathophysiological conditions prevailing (Abbracchio *et al*. 1997).

The aim of this study is to describe biochemical mechanism(s) by which Ado or dAdo induced apoptosis in MDA-MB468 and MCF-7 cells. These particular breast cancer cell lines were chosen for the study as they are widely accepted in the investigation of compounds and pathways that could be targeted for breast cancer therapy. Here, we show that both Ado and dAdo are toxic to the oestrogen receptor-positive MCF-7 breast cancer cells and also to the oestrogen receptor-negative MDA-MB468 cell line. Furthermore, lack of caspase-3 expression in MCF-7 cells appeared not to affect the toxic potential of either compound. The induced cell death exhibited classical hallmarks of apoptosis, and it was executed in the mitochondrial death pathway.

MATERIALS AND METHODS

Materials and reagents

Adenosine, deoxyadenosine, EHNA, 5′-amino-5′-deoxyadenosine, 8-phenyltheophyline, NBTI, L-homocysteine thiolactone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

(MTT) and all other materials, culture media and related compounds were purchased from Sigma (St Louis, MO, USA). Cell culture plastic ware was from Nunc Co. (Roskilde, Denmark). Caspase-3 colourimetric assay kit (Catalogue no. 101K4019) and DNA ladder marker, 1 kb (61K1778) were obtained from Sigma. The colourimetric caspase-8 substrate IETE-AMC was purchased from Bachem (Heidelberg, Germany). Caspase-9 colourimetric assay kit (Catalogue no. BF10100), and annexin V-FITC apoptosis detection kit (Catalogue no. TA4638) were purchased from R & D Systems (Minneapolis, MN, USA).

Cell culture

MDA-MB-468 and MCF-7 breast cancer cell lines, obtained from the National Cell Bank of Iran (NCBI), were grown in RPMI-1640 supplemented with 10% foetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. They were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air. Cultures were regularly examined using an inverted microscope (Micros, Vienna, Austria).

MTT assay

To evaluate the cytotoxicity effect of adenosine or deoxyadenosine on the MCF-7 and MDA-MB468 breast cancer cell lines, the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] colourimetric assay was applied (Ghavami *et al*. 2005). Briefly, asynchronously growing cells (3×10^4 cells/ml) were transferred into 48-well culture plates containing 500 μl of medium and incubated for 24 h. The cells were then treated with stimulants and/or inhibitors as described in RESULTS. After that the MTT assay was performed and the percentage cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100.

Analysis of nuclear morphology

Cells were plated in eight-well chamber slides and were allowed to adhere. Treated and untreated (control) cells were fixed with methanol-acetic acid 3 : 1 (v/v) for 10 min, after which staining was performed with Hoechst 33258 (200 μg/ml). Slides were then washed with phosphate-buffered saline (PBS; pH 7.4) and were examined using an epifluorescence microscope (Micros). Apoptotic cells were recognized on the basis of nuclear morphological changes they had undergone, such as chromatin condensation and fragmentation (Ghavami *et al*. 2004).

Pretreatment with adenosine deaminase inhibitor, receptor antagonist, kinase inhibitor and nucleoside transporter inhibitor

To examine the role of extracellular adenosine receptors in Ado and dAdo-induced cell death, cells were pretreated with 10 μM of 8-phenyltheophylline, a receptor antagonist, for 30 min prior to treatment with various concentrations of Ado and dAdo. In order to provide evidence for intracellular phosphorylation of Ado and dAdo, a 30-min pre-exposure to the kinase inhibitor, 5′-amino-5′-deoxyadenosine (20 μM) was performed. To test whether Ado and dAdo were required for cells to exert their cytotoxicity, a 30-min pre-exposure to the nucleoside transport inhibitor, NBTI (20 μM) was performed.

Caspase-3, -8 and -9 activation assay

Caspase-3, -8 and -9 colourimetric assays were used to investigate caspase-3 and caspase-9 activation in the treated MCF-7 and MDA-MB468 cells. The caspase-8 assay also was performed as described previously (Ghavami *et al*. 2005). Briefly, to estimate caspase-3 activity, cells were lysed by incubation with cell lysis buffer on ice for 15 min and were then centrifuged at 20 000 *g* for 10 min (at 4 °C). For the 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, 5 μl of the relevant fluorescent caspase substrate 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 had been included. Protein content was estimated by the Bradford method (Bradford 1976). Activities were expressed as nmol/min/mg protein.

DNA electrophoresis

Treated and untreated cells were trypsinized, washed twice with ice-cold PBS and then centrifuged. Pellets were lysed using lysis buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 10 mM NaCl, and 1% (w/v) sodium dodecyl sulphate (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 was precipitated with ethanol overnight at −20 °C. Precipitated DNA was washed once in 70% ethanol, re-suspended in tris-acetate (TAE) buffer and then was applied on a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide; electrophoresis was performed using TAE running buffer. A ladder of 1 kb was used as a marker.

Flow cytometry

Flow cytometric estimation of apoptosis was performed using annexin-V and propidium iodide (PI) staining kits according to the manufacturer's instructions. Briefly, cells plated to a density of $0.2–0.3 \times 10^6$ cells per well in 6-well plates were incubated for 24 h. Then treatment was carried out and the plates were once more incubated for 48 h. Cells were collected, washed and stained for 15 min at room temperature with annexin-V-fluorescein and PI, and then were examined using a flow cytometer (Coulter EPICS-XLCoulter, Miami, FL, USA). Cells that were negative for both annexin-V and PI were considered live; those positive for annexin-V but negative for PI were considered early apoptotic; and those positive for both annexin-V and PI were considered to be in late apoptosis or necrosis.

Statistical analysis

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

RESULTS

Adenosine and deoxyadenosine are cytotoxic for both oestrogen receptor-positive and oestrogen receptor-negative breast cancer cells, but only in the presence of adenosine deaminase inhibitor.

We examined the growth inhibitory effect of adenosine, and deoxyadenosine in the presence and in the absence of adenosine deaminase inhibitor (EHNA) on MDA-MB-468 (oestrogen receptor-negative) and MCF-7 (oestrogen receptor-positive) human breast cancer cell lines. As shown in Fig. 1, adenosine and deoxyadenosine alone tested at concentrations of up to 500 μM did not significantly inhibit the cell population growth of either cell line. Next we tested the toxicity of Ado and dAdo in the presence of 40 μM EHNA. As shown in Fig. 2, pretreatment of cells with EHNA and then subsequent treatment of cells with Ado and dAdo resulted in significant growth inhibition at concentrations ≥ 100 μM Ado and dAdo ($P < 0.05$).

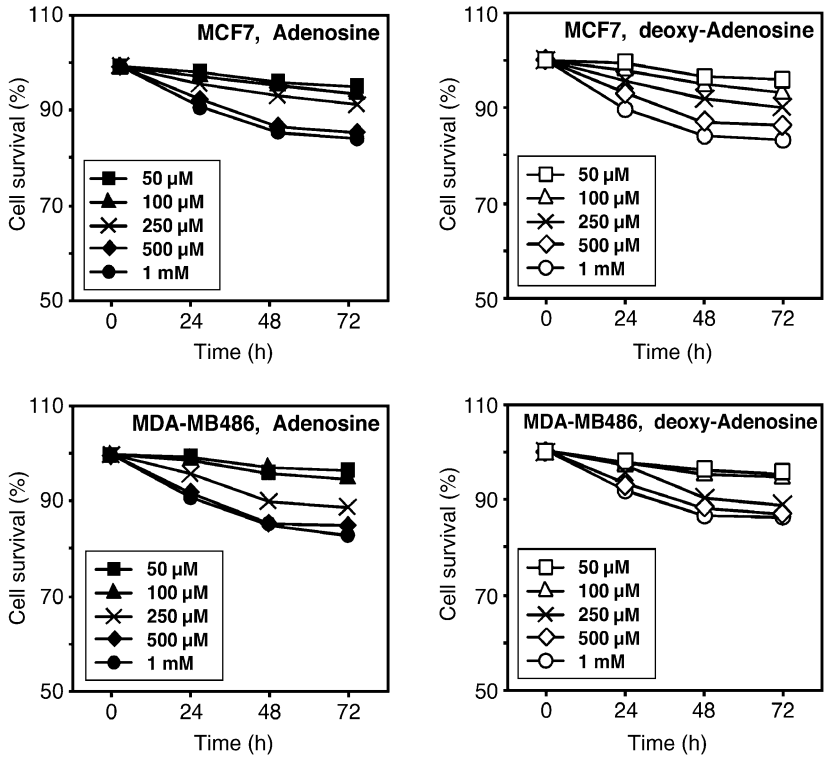

Figure 1. Effect of adenosine and deoxyadenosine on cell growth in breast cancer cell lines, MCF-7 and MDA-MB468. Cells were treated with various concentrations of adenosine and deoxyadenosine for 24, 48 and 72 h, and viability was assessed by MTT assay. Results are expressed as percentage of the corresponding control and represent the mean ± SD of six repeats. The standard deviation bars were omitted in order to preserve the clarity of the figure, however, they never exceeded 9%.

Adenosine and deoxyadenosine operate intracellularly and require the action of adenosine kinase for their toxicity

To determine whether the site of action of the Ado/EHNA or dAdo/EHNA was extra or intracellular, cells were grown in Ado/EHNA or dAdo/EHNA in the presence of the non-specific Ado receptor antagonist, 8-phenyltheophyline. As shown in Fig. 3, pretreatment of cells with 8-phenyltheophyline (10 µM) had no effect on Ado/EHNA or dAdo/EHNA-mediated toxicity. Next, we blocked cellular import of both Ado and dAdo by a specific nucleoside transport inhibitor, NBTI. As shown in Fig. 4, cytotoxicity of Ado/EHNA or dAdo/EHNA was significantly abrogated by NBTI, suggesting that the Ado/EHNA or dAdo/EHNA combination acts intracellularly and not via cell surface receptors.

In ADA deficiency, Ado and dAdo accumulate and exert lymphotoxic effects either directly or after conversion to phosphorylated derivatives such as AMP and dATP (Green & Chan 1973; Mitchell *et al*. 1978; Hershfield & Mitchell 1995; Yang & Cortopassi 1998). Thus, we tested the effects of adenosine kinase inhibition on the toxicity of both compounds. Treatment with 5′-amino-5′-deoxy-Ado (20 µM), that inhibits adenosine kinase, reduced the growth inhibition induced by combination of adenosine and EHNA or deoxyadenosine and EHNA (Fig. 5) significantly ($P < 0.05$). These findings indicate that Ado-mediated cell killing proceeds via an intracellular route that requires the action of adenosine kinase.

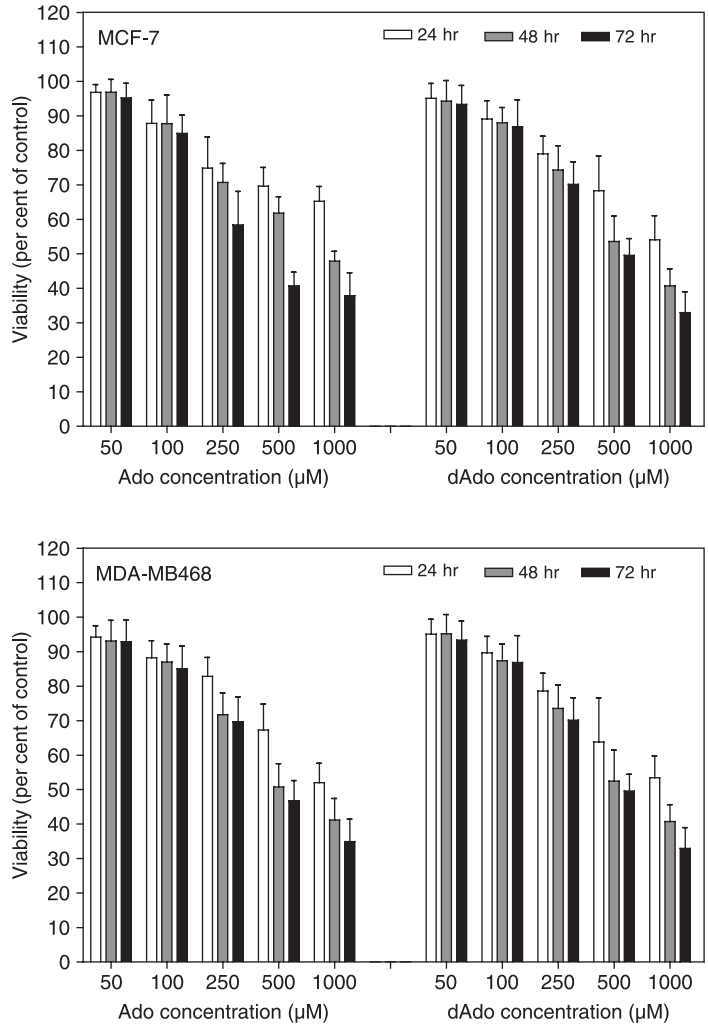

Figure 2. Adenosine deaminase inhibitor (EHNA) strongly potentiates the toxicity of adenosine and deoxyadenosine. MCF-7 and MDA-MB468 cells were pretreated with 40 μM EHNA, various concentrations of adenosine and deoxyadenosine for 24, 48 and 72 h; viability was assessed by MTT assay. Results are expressed as percentage of the corresponding control and represent the mean ± SD of six repeats.

Cell death induced by adenosine or deoxyadenosine exhibits typical morphological hallmarks of apoptosis

In order to examine the type of cell death induced by Ado or dAdo, we measured the degree of phosphatidylserine externalization and the total cell-death burden, by flow cytometry. MDA-MB468 and MCF-7 cells were incubated with different concentrations of adenosine and deoxyadenosine in the presence of adenosine deaminase inhibitor (EHNA), and the percentages of early apoptotic and late apoptotic cells were assessed by flow cytometric analysis of annexin-V binding and PI permeability. It has been reported earlier that loss of plasma membrane asymmetry is an early cell type-independent apoptotic event, resulting in exposure of phosphatidylserine at the cell surface (Vermes *et al.* 1995). Annexin-V binds with high affinity to phosphatidylserine,

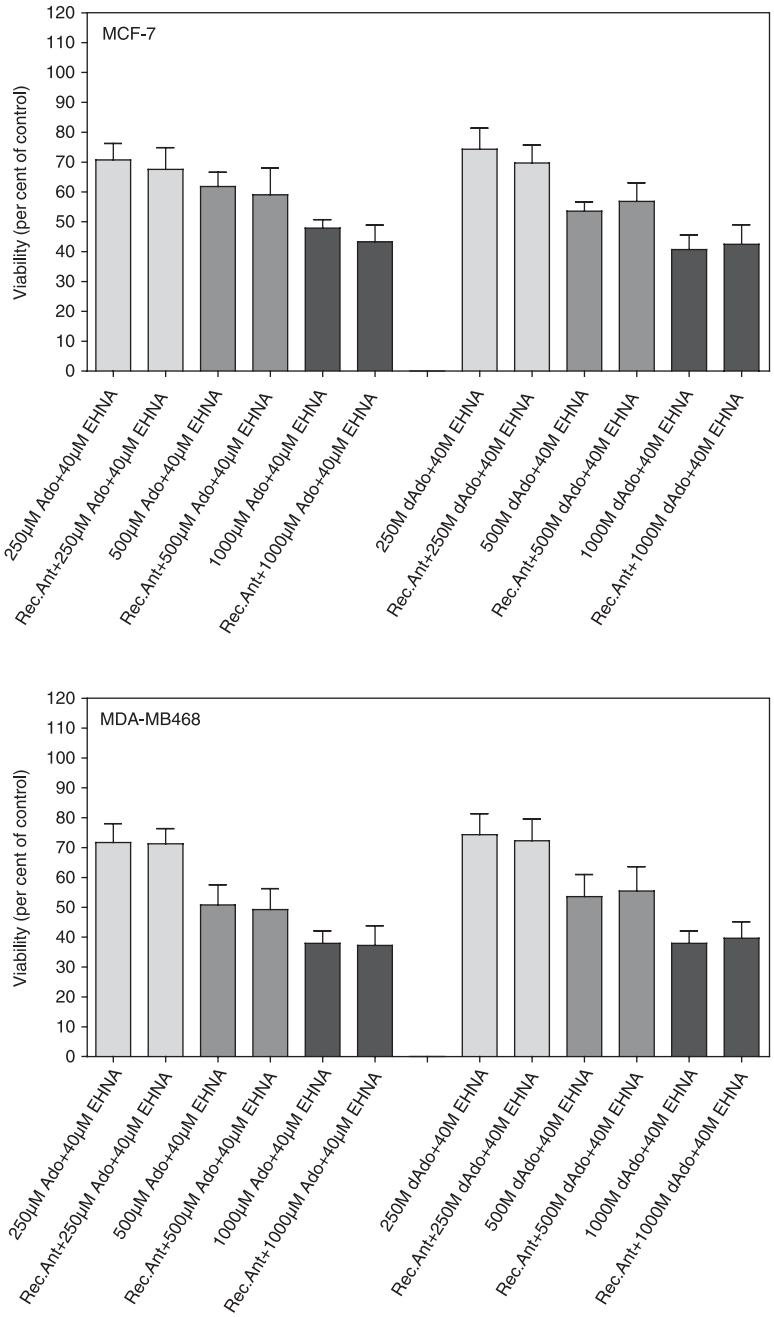

Figure 3. The effect of non-specific Ado receptor antagonist 8-phenyltheophylline on adenosine and deoxyadenosine on the growth of the breast cancer cell populations. We have tested the effect of non-specific Ado receptor antagonist 8-phenyltheophylline (10 μM) on adenosine and deoxyadenosine toxicity (in the presence of 40 μM EHNA) in breast cancer cell lines, MCF-7 and MDA-MB468. Pretreatment of cells with 10 μM 8-phenyltheophylline had no effect upon the Ado/EHNA or dAdo/EHNA-mediated toxicity for 48 h. Results are expressed as percentage of the corresponding control and represent the mean ± SD of six repeats.

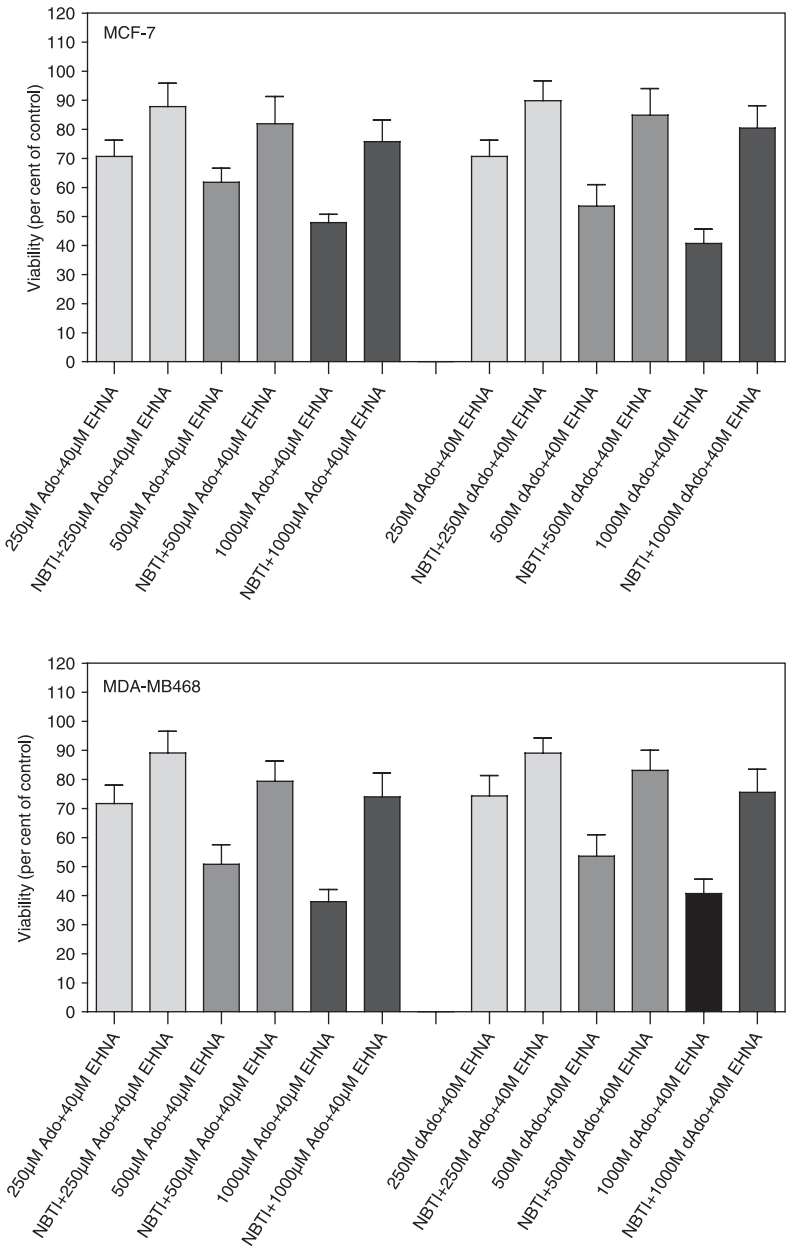

Figure 4. Intracellular but not extracellular adenosine or deoxyadenosine is toxic to these cells. Pretreatment with 20 μM nucleoside transport inhibitor, NBTI, significantly abrogated cytotoxicity of Ado/EHNA or dAdo/EHNA within 48 h. Results are expressed as percentage of the corresponding control and represent the mean ± SD of six repeats.

enabling detection of early apoptosis. Thus, Annexin-V is frequently applied to detect apoptotic cells *in vitro* and even *in vivo* (Lahorte *et al*. 2004). Cells at an early stage of apoptosis appeared green (annexin-V positive/PI negative) and those at a late apoptotic or necrotic stage appear green and red (annexin-V positive/PI positive). Early and late stages of apoptosis induced by Ado/EHNA or dAdo/EHNA in both MCF-7 and MDA-MB468 breast cancer cell line are shown in Fig. 6.

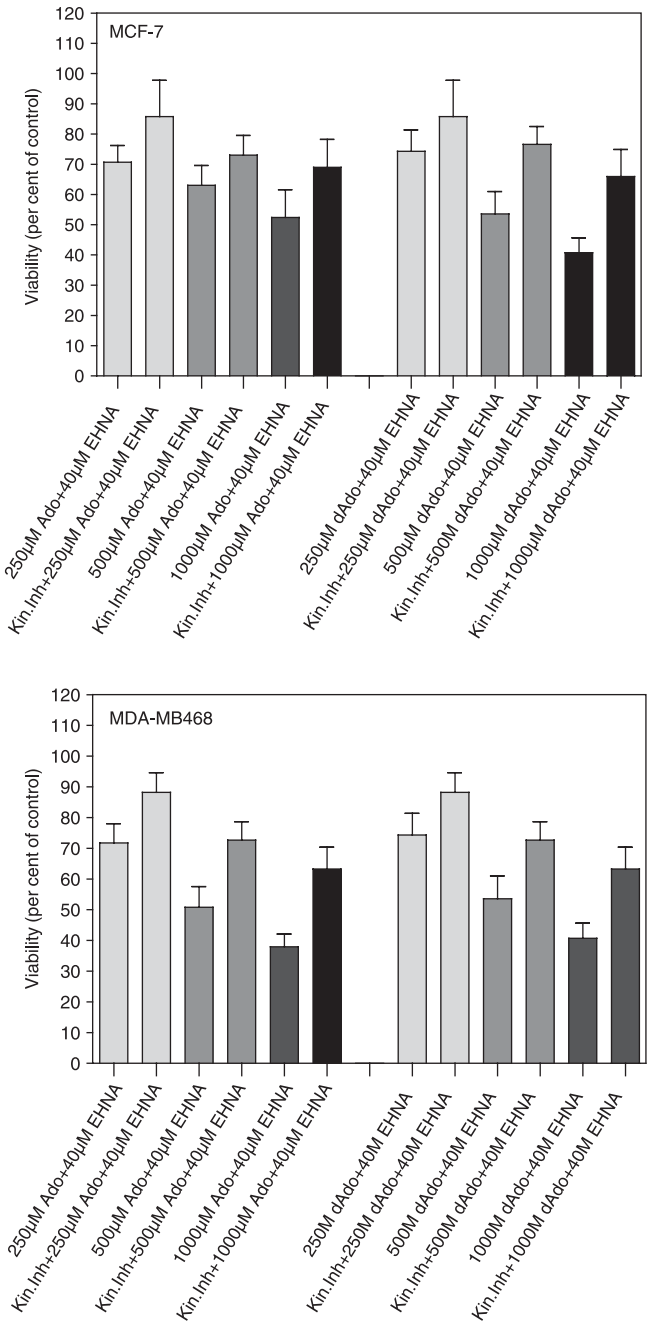

Figure 5. Adenosine kinase is required for adenosine or deoxyadenosine toxicity. Pretreatment with 5′-amino-5′-deoxyadenosine (20 μM), an inhibitor of adenosine kinase, significantly reduced the growth inhibition induced by the combination of adenosine and EHNA or deoxyadenosine and EHNA within 48 h. Results are expressed as percentage of the corresponding control and represent the mean ± SD of six repeats.

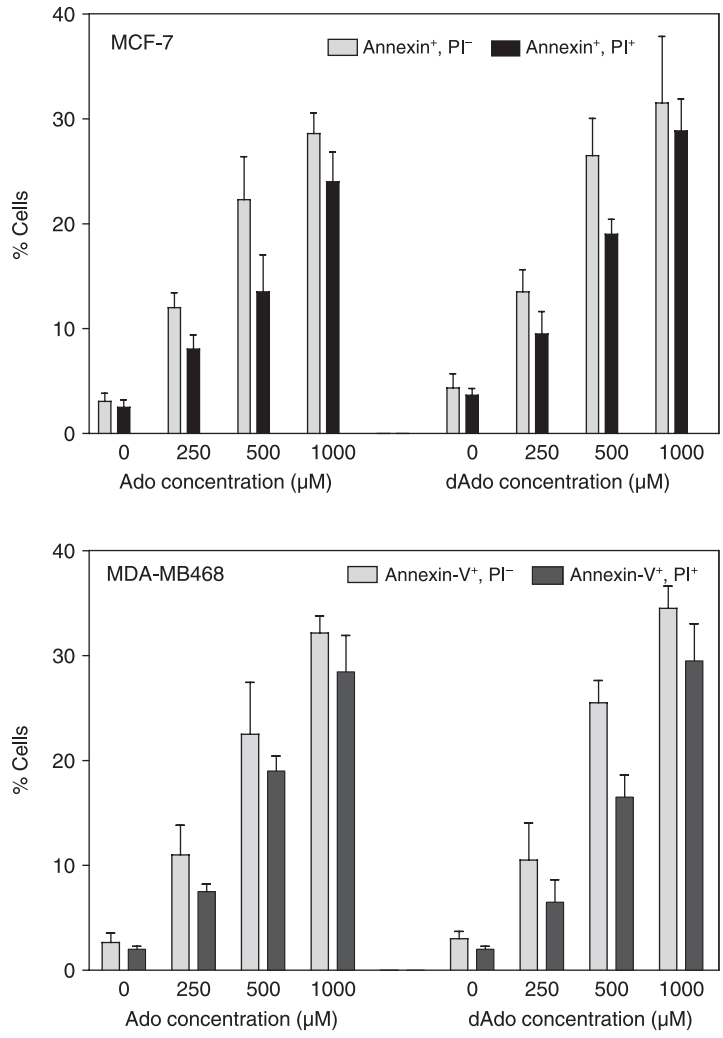

Figure 6. Treatment with adenosine or deoxyadenosine induces typical apoptotic loss of cell membrane symmetry. Cells were treated with various concentrations of adenosine and deoxyadenosine in the presence of adenosine deaminase inhibitor (EHNA) for 48 h, and percentages of early and late apoptotic cells were assessed by flow cytometric analysis of annexin-V and PI binding. Results are represent the mean ± SD of four repeats.

To confirm the data obtained by flow cytometry, we examined the morphology of dying cells upon treatment with Ado/EHNA or dAdo/EHNA; cell morphology was first examined by Nomarski-contract microscopy. To confirm apoptotic cell death, cell nuclei were stained with the DNA marker Hoechst 33258. As shown in Fig. 7 (right), both Ado/EHNA and dAdo/EHNA caused typical apoptotic changes in nuclear morphology, with pronounced condensation of nuclei and nuclear fragmentation. These experiments confirm that both Ado and dAdo induce predominantly apoptotic death in target cells.

Internucleosomal DNA fragmentation is one of the hallmarks of apoptosis. Such DNA, when resolved on an agarose gel, appears as a series of bands with molecular weight in multiples of ~180–200 bp (the length of DNA wrapped around a single nucleosome), and referred to as a

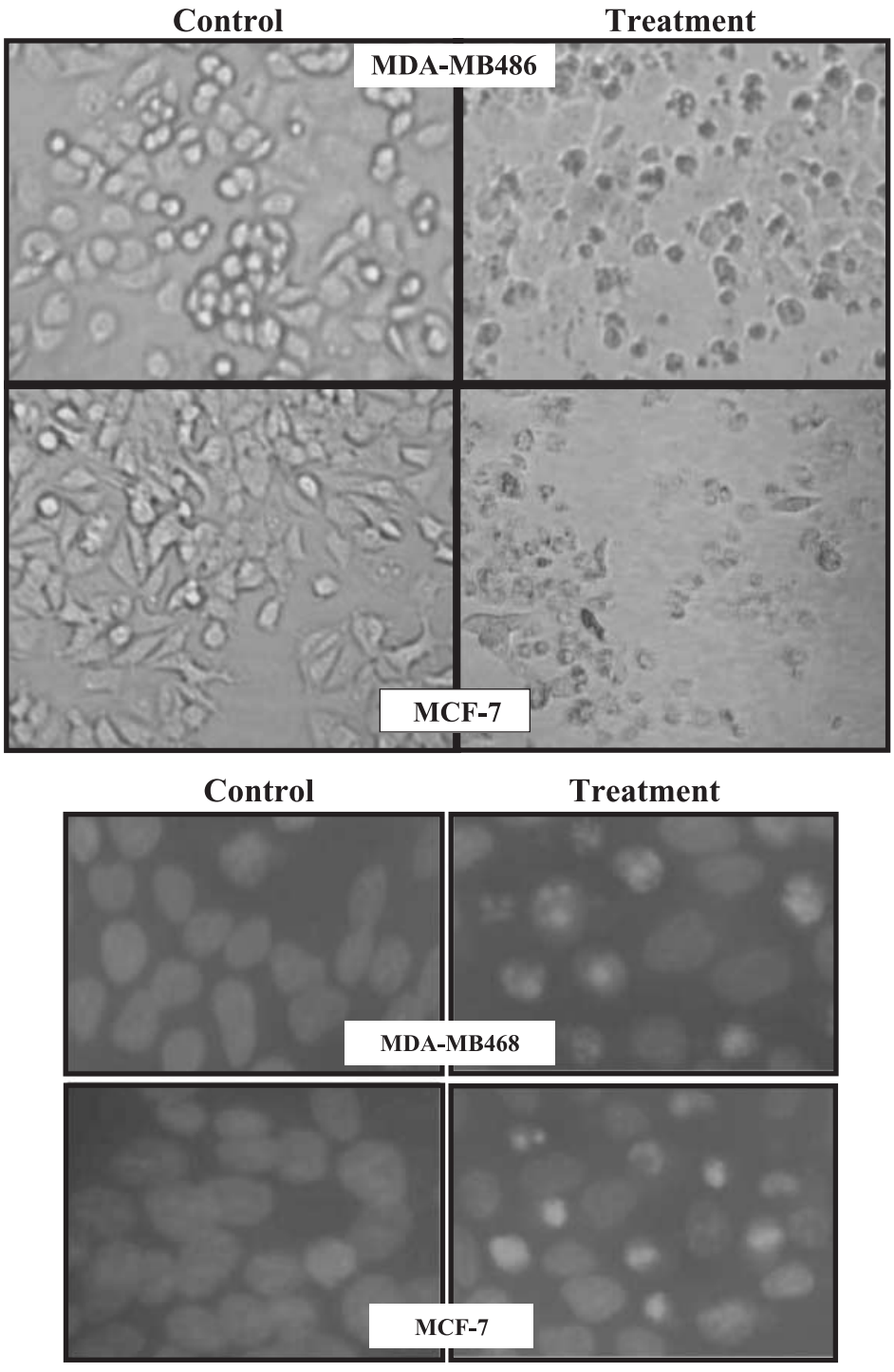

Figure 7. Cells treated with adenosine or deoxyadenosine have typical apoptotic morphology. The morphology of both the types of breast cancer cells, treated with either adenosine or deoxyadenosine, was examined by microscopy after Hochest 33258 staining.

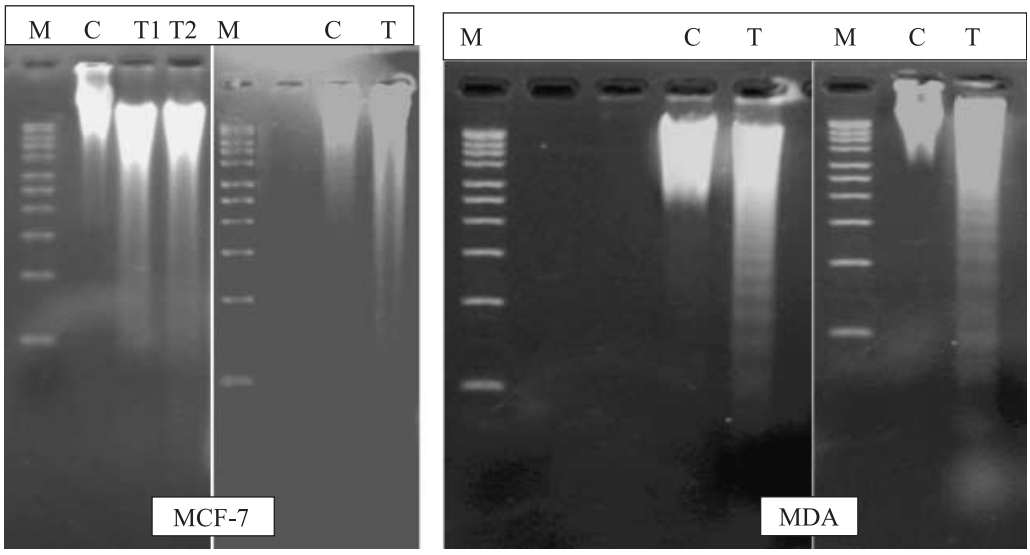

Figure 8. Adenosine and deoxyadenosine induce DNA-fragmentation in target cells. Agarose gel electrophoresis of DNA extracted from untreated (C), treated (T) MCF-7 and MDA-MB468 cells plus 1 kb DNA marker (M). Cells were treated with 500 μM adenosine or deoxyadenosine in the presence of 40 μM EHNA for 48 h. DNA was then extracted and electrophoresis was performed.

DNA ladder. Thus, we have tested whether Ado or dAdo induces DNA laddering in the target cell lines. Addition of 500 μM adenosine or deoxyadenosine in the presence of 40 μM EHNA to the MDA-MB468 cells resulted in DNA ladder-like fragmentation (Fig. 8). As expected, in MCF-7 cells, a DNA laddering profile was not obtained because of the cells' lack of caspase-3; they are unable to activate CAD-DNase, the main molecule responsible for DNA ladder formation in many cell types. Hence, these results confirm that Ado or dAdo induces apoptotic cell death in our experimental system.

Caspase-9 and caspase-3 become activated upon adenosine or deoxyadenosine treatment

To explore the possible biochemical mechanisms underlying Ado/EHNA or dAdo/EHNA-induced apoptosis, the activation of caspases-3, -8 and -9 were assayed using DEVDase-, IETDase-, and LEHDase-enzymatic assays, respectively. As shown in Fig. 9, the activity of caspase-9 was significantly ($P < 0.05$) elevated in both treated breast cancer cell lines. No activation of caspase-8 was observed in either cell line (data not shown). As expected, the activity of caspase-3, was significantly ($P < 0.05$) elevated only in MDA-MB468 cells, but was not observed in the caspase-3-negative MCF-7 cells on treatment with Ado/EHNA or dAdo/EHNA. Nevertheless, our earlier results indicate that both cell lines were sensitive to the combination of stimuli mentioned above. Thus, caspase-3 appears to be dispensable for cell death induction by either Ado or dAdo or their metabolites.

DISCUSSION

Adenosine deaminase (ADA) catalyses the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. Mutations in the ADA gene, that

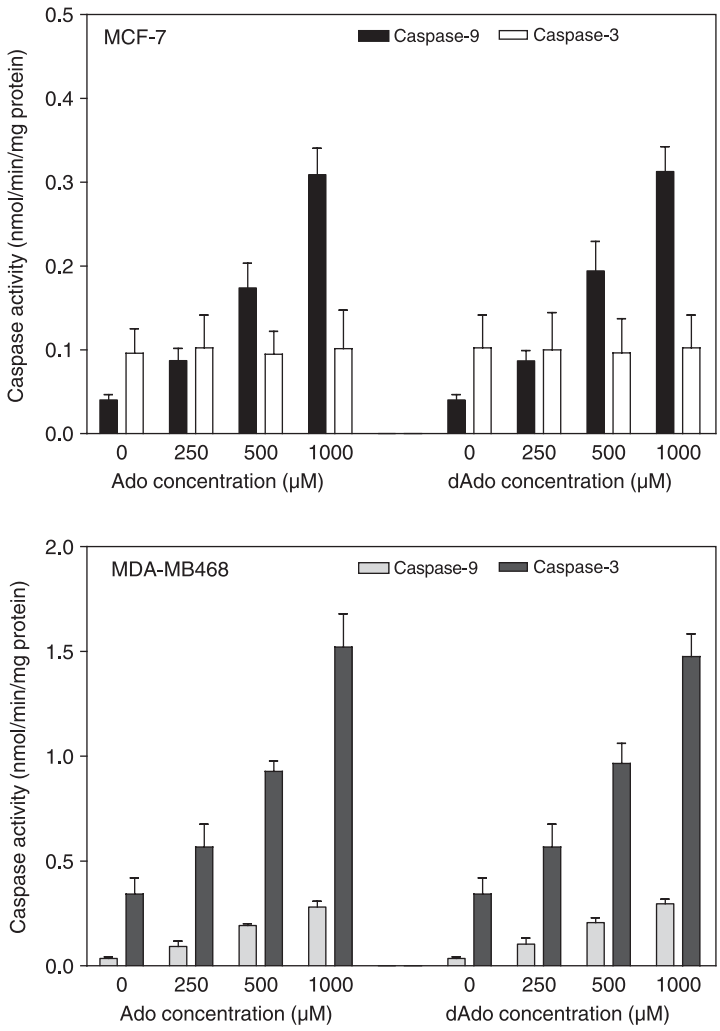

Figure 9. Activation of caspases after adenosine and deoxyadenosine treatment. The enzymatic activity of caspases-3 and -9 was measured in MCF-7 and MDA-MB468 cells treated with various concentrations of adenosine and deoxyadenosine in the presence of the adenosine deaminase inhibitor (EHNA) for 48 h. Results represent the mean ± SD of four repeats.

result in loss of enzyme activity, cause severe combined immunodeficiency (Giblett *et al*. 1972), sometimes called ADA-deficiency. In ADA deficiency, Ado and dAdo accumulate and exert lymphotoxic effects either directly or after conversion to phosphorylated derivatives such as AMP and dATP (Green & Chan 1973; Mitchell *et al*. 1978; Hershfield & Mitchell 1995; Yang & Cortopassi 1998). Because the Ado kinase inhibitor 5′-amino-5′-deoxyadenosine has protected the cells, it seems also likely that, in our experimental system, the phosphorylation of Ado with a resultant elevation in AMP, ADP, and/or ATP is required for growth inhibition. It has been described previously that Ado and dAdo are toxic to a variety of cell types (Henderson & Scott 1980; Henderson *et al*. 1980; Kizaki *et al*. 1988; Szondy 1994; Wakade *et al*. 1995; Kohno *et al*. 1996; Dawicki *et al*. 1997; Dubey *et al*. 1997; Yao *et al*. 1997; Barry

& Lind 2000; Saitoh *et al*. 2004). Recent studies have shown that adenosine induces apoptosis *via* both receptor-mediated and non-receptor-mediated pathways (Barry & Lind 2000; Peyot *et al*. 2000; Appel *et al*. 2001; Schrier *et al*. 2001; Zhao *et al*. 2002). For example, A2b and A3 adenosine receptors appear to induce apoptosis in arterial smooth muscle cells, glial cells and glomerular mesangial cells (Peyot *et al*. 2000; Appel *et al*. 2001; Zhao *et al*. 2002). The intrinsic pathway becomes activated in epithelial cancer cells originating from breast, colon, gastric and ovary plus from neuroblastoma cells. This occurs when extracellular adenosine enters the cell and becomes converted to AMP (Barry & Lind 2000; Schrier *et al*. 2001; Saitoh *et al*. 2004).

In our study, adenosine and deoxyadenosine alone did not significantly inhibit the growth cell populations of either cell line tested, indicating that adenosine and deoxyadenosine are metabolized by adenosine deaminase (ADA) and converted to inosine and deoxyinosine, respectively, which are not toxic to these cells. Both adenosine and deoxyadenosine become toxic, however, if the deamination process is blocked. The cytotoxicity effect of Ado/EHNA or dAdo/EHNA on our breast cancer cells was not affected by the receptor antagonist, 8-phenyltheophyline. Thus, presence of adenosine receptors did not trigger apoptosis in our experimental system. In contrast, it was significantly inhibited by the nucleoside transport inhibitor, NBTI, or by the kinase inhibitor, 5′-amino-5′-deoxyadenosine. Because the nucleoside kinase inhibitor, 5′-amino-5′-deoxyadenosine, protected the cells, it seems likely that phosphorylation of Ado and dAdo with resultant elevation in AMP, ADP, ATP and dAMP, dADP, dATP is required for growth inhibition. The inhibitory effect of the nucleoside transport blocker on apoptosis has been demonstrated in several cell types (Tanaka *et al*. 1994; Wakade *et al*. 1995; Lewis *et al*. 1997).

In conclusion, the results of the present study indicate that extracellular adenosine and deoxyadenosine induces apoptosis in both the oestrogen receptor-positive MCF-7 cells, and in the oestrogen receptor-negative MDA-MB468 breast cancer cells via the mitochondrial/intrinsic cell death pathway. Inhibition of deamination is critical for the toxicity of both compounds. Prior to triggering of apoptosome formation, both adenosine and deoxyadenosine have to be transported through the cell membrane. Furthermore, they become phosphorylated by adenosine kinase. The phosphorylation step appears to be important, but not critical for the toxicity induced by both compounds, as 5′-amino-5′-deoxyadenosine, a specific inhibitor of adenosine kinase, was not able to fully protect cells from adenosine- or deoxyadenosine-induced toxicity. The inhibitor study may indicate that multiple pathways become triggered by Ado and dAdo and their metabolites, to kill cancer cells. It is worth noticing that some phospho-metabolites, like ATP or dATP, directly participate in activation of the intrinsic, apoptosome-dependent cell death pathway (Cecconi *et al*. 1998). Moreover, as both the caspase-3-positive MDA-MB468 cells and the caspase-3-negative MCF-7 cells were killed by adenosine or by deoxyadenosine, caspase-3 may be dispensable for the propagation of cell death induced by both compounds. This is not surprising as other caspases can take over most of the proteolytic functions of caspase-3. It has been reported previously that lack of expression of a single caspase is frequently compensated for by up-regulation of expression of other family members (Zheng & Flavell 2000; Zheng *et al*. 2000). While it is unlikely that direct phospho-metabolites of Ado or dAdo will ever be considered as candidates for the development of new inhibitors of proliferation, as anti-cancer chemotherapeutics they may become attractive leads that, upon further modification, may show a sufficiently large therapeutic window. Besides differential activities of cellular pathways in normal or in cancer cells, the efficacy of transmembrane transport of Ado and dAdo, may potentially be explored in this context.

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