8-Amino-adenosine is a potential therapeutic agent for multiple myeloma

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Abstract

Multiple myeloma (MM) is a malignancy of clonal B-cells that accounts for 10% of all hematologic malignancies. We have shown previously that a novel purine analogue, 8-chloro-adenosine, has significant activity for MM in preclinical studies. Objective: Using MM cell lines, we investigated the molecular mechanism of related congener of adenosine, 8-amino-adenosine (8-NH2-Ado). Methods: We employed biological and biochemical assays in MM cell lines to evaluate the clinical potential of 8-NH2-Ado. Results: In MM cell lines both sensitive and resistant to conventional chemotherapies, 8-NH2-Ado is cytotoxic, with IC50 ranging from 300 nmol/L to 3 μmol/L. A mouse leukemic cell line lacking adenosine kinase activity was resistant to 8-NH2-Ado, indicating that phosphorylation of 8-NH2-Ado to its triphosphate form is required for cytotoxicity. A 4-hour incubation of MM cells with 10 μmol/L analogue resulted in an accumulation of >7 mmol/L 8-NH2-ATP with a parallel decline in the endogenous ATP levels. Accumulation of 8-NH2-ATP was dependent on both exogenous concentration of 8-NH2-Ado and incubation time. The accumulation of 8-NH2-ATP was accompanied by a decrease in both RNA and DNA synthesis. The mechanism of 8-NH2-Ado-mediated cytotoxicity was due to apoptosis as measured by an increase in Annexin V binding, a decrease in mitochondrial membrane potential, an increase in caspase activity, cleavage of caspase substrates, and an increase in cells with a sub-G1 DNA content. Conclusion: Based on these results, we conclude that 8-NH2-Ado may hold great potential as a therapeutic agent for the treatment of MM.

Introduction

There are 14,600 cases of multiple myeloma (MM) diagnosed in 2003, with a median survival of only 4 to 5 years (1), which is only modestly improved with high-dose chemotherapy and autologous stem cell transplantation (2). The survival rate for MM has not changed dramatically in the past 30 years. Therefore, new therapies need to be developed. Several nucleoside analogues are currently used for the treatment of acute and chronic hematologic malignancies that include tumors of myeloid and lymphoid lineage. Based on the extensive experience with hematologic tumors, one would expect that nucleoside analogues that have been effective in leukemias and lymphomas should play a role in the treatment of MM (3, 4). The two nucleoside analogues that play a major role in the treatment of B-cell lymphocytic leukemia, hairy cell leukemia, and indolent lymphomas are fludarabine and cladribine (5, 6). Unfortunately, neither of these analogues has exhibited sufficient activity in vitro in myeloma cell lines or in clinical trials to justify continued clinical evaluation in this disease (7). Results of phase II clinical trials in MM with fludarabine (8, 9) and cladribine (3, 4) were completely negative. This may be due to poor phosphorylation of these agents by deoxycytidine kinase or the DNA-directed nature of these arabinoside and deoxyadenosine analogues. However, promising preclinical studies with 8-chloro-adenosine (8-Cl-Ado) indicate that in contrast to the arabinoside (fludarabine) and deoxy-adenosine (cladribine) analogues this adenosine congener may have efficacy in MM. This may be due to an efficient phosphorylation to the triphosphate by adenosine kinase, a decrease in endogenous ATP pools, and a specific inhibition of RNA synthesis (10). These encouraging data provide an impetus to test related adenosine analogues.

The 8-amino derivative of cyclic AMP has been shown previously to be cytotoxic in a rat hepatoma cell line (11) and breast cancer cell lines of human (12, 13) and mouse (14) origin. In these studies, the inclusion of a phosphodiesterase inhibitor to stabilize the cyclic AMP and to enhance its actions resulted in decreased cytotoxicity. Therefore, it was hypothesized that the cytotoxic compound was most likely a metabolite of 8-amino cyclic AMP [i.e., 8-amino adenosine (8-NH2-Ado)]. Saito et al. (14) further investigated the actions of 8-NH2-Ado in an in vivo
breast cancer mouse model and noted a significantly longer survival time for the treated animals indicating efficacy. Based on these observations with 8-aminocyclic AMP and on our promising preclinical studies of 8-Cl-Ado in MM, we investigated the cytotoxicity of 8-NH2-Ado in MM cell lines and further investigated the mechanism of action of 8-NH2-Ado in MM cell lines.

Materials and Methods

Cell Lines

Experiments were conducted using MM cell lines that were developed previously by our group (15, 16). The original cell line (MM.1) was established from the peripheral blood of a MM patient that had been treated with steroid-based therapy. By chronic exposure to glucocorticoid, a steroid-resistant variant was developed. MM.1S cells are sensitive to the killing effects of glucocorticoids, whereas MM.1R cells are resistant to glucocorticoids. RPMI-8226 cells and the multidrug-resistant derivative MDR10V MM cells were obtained from Dr. William S. Dalton (17). All cells were grown in RPMI 1640 (Invitrogen, Baltimore, MD) supplemented with 10% fetal bovine serum, glutamine (2 mmol/L), penicillin (100 units/mL), streptomycin (100 μg/mL), and fungizone (2.5 μg/mL). The L1210 and ED2 cell lines were obtained from Dr. Joseph G. Cory (18). The ED2 variant (lacking Ado kinase) of the mouse L1210 cell line was supplemented with 2,3-dihydro-1H-pyrazole[2,3-c]imidazole (300 μmol/L), Desferal (40 μmol/L), erythro-9-(2-hydroxy-3-nonyl)adenosine (5 μmol/L), and deoxy-adenosine (400 μmol/L). All cells were maintained in a 37°C incubator with 5% CO2.

Drugs and Chemicals

8-NH2-Ado was purchased from Rl Chemical, Inc. (Orange, CA). For high-performance liquid chromatography standards, triphosphates of these analogues were custom synthesized by Biolog (San Diego, CA). N-benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (ZVAD-fmk), Z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethyl ketone (LEHD-fmk), and Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (IETD-fmk) were purchased from Enzyme Systems Products (Livermore, CA). Staurosporine was obtained from Alexis Chemical (San Diego, CA). CMXRos (MitoTracker Red) was from Molecular Probes (Eugene, OR), whereas caspase-8 and caspase-9 fluorometric kits were purchased from R&D Systems (Minneapolis, MN). 2,3-Dihydro-1H-pyrazole[2,3-c]imidazole was obtained from Dr. Robert J. Schultz (Chief, Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, National Cancer Institute, NIH). All other chemicals were obtained from Sigma Chemical (St. Louis, MO). Annexin V-FITC kit was obtained from ImmunoTech, a Beckman Coulter company (Marseille, France).

Cell Proliferation Assay

This assay was done as described previously (19). Briefly, MM cells were cultured into 96-well dishes at a concentration of 25,000 cells/well and incubated with the indicated drugs for 72 hours. Cell proliferation was determined using the MTS Cell Titer aqueous assay (Promega, Madison, WI), which measured the conversion of a tetrazolium compound into formazan by a mitochondrial dehydrogenase enzyme in live cells. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. The data were expressed as the percentage of formazan produced by the cells treated with the control medium in the same assay.

Measurement of Intracellular Nucleoside Triphosphates

Nucleotides were extracted using perchloric acid and the extracts were neutralized with KOH as described previously (20) and stored at −20°C until analyzed. The neutralized extracts were applied to an anion exchange Partisil-10 SAX column and eluted at a flow rate of 1.5 mL/min with a 50-minute concave gradient (curve 7, Waters 600E System Controller, Waters Corp., Milford, MA) from 60% NH4H2PO4 (0.005 mol/L, pH 2.8) and 40% NH4H2PO4 (0.75 mol/L, pH 3.6) to 100% NH4H2PO4 (0.75 mol/L, pH 3.6). The column eluate was monitored by UV absorption at 256 nm and the nucleoside triphosphate was quantitated by electronic integration with reference to external standards (21). The analogue triphosphate was identified by comparing the retention profile and absorption spectrum with that of authentic standard. The intracellular concentration of nucleotides contained in the extract was calculated from a given number of cells of a determined mean volume. The cell number was determined using Coulter counter (Coulter Electronics, Hialeah, FL). This equipment was attached to a channelizer that was used to estimate the mean volume of cells in a given cell population. This volume was used to quantitate the concentration of nucleotides. This calculation assumed that the nucleotides were uniformly distributed in a total cell volume. The lower limit of sensitivity of this assay was 10 pmol in an extract of 5 × 10⁶ cells corresponding to a cellular concentration of 1 μmol/L.

Inhibition of Nucleic Acid Synthesis by Nucleoside Analogues

Exponentially growing cells were incubated with the indicated concentrations of nucleoside analogues for 4 hours. After 3 hours, [3H]thymidine or [3H]uridine (2 μCi/mL) was added to these cultures and incubation was continued for an additional 1 hour in a multiscreen assay system (Millipore Corp., Bedford, MA). The cells were then collected on multiscreen GV filters under vacuum and washed four times each with ice-cold 8% trichloroacetic acid, water, and 100% ethanol. The radioactivity in the acid-insoluble material retained on the filters was measured by scintillation counting and expressed as the percentage of control (untreated) value of cells.

Flow Cytometry

To determine the distribution of cells within the cell cycle, aliquots of cells (1 × 10⁶ each) were pelleted (500 × g for 5 minutes at 4°C) and washed twice in ice-cold PBS (8.1 g NaCl, 1.14 g Na2HPO4, 0.22 g KCl, 0.25 g/L KH2PO4), fixed in ice-cold 70% ethanol, and stored at 4°C until analyzed. Before analysis by flow cytometry, the fixed cells...
were pelleted, washed in PBS, and resuspended in ice-cold flow buffer (PBS containing 0.5% Tween 20, 15 g/mL propidium iodide, 5 g/mL DNase-free RNase). The stained cells were analyzed using an EPICS Profile II flow cytometer (Coulter Electronics).

**Immunoblotting Analysis**

MM1.S cells were cultured and grown at a concentration of $10 \times 10^6$ in tissue culture medium (30 mL) in 75 cm² flasks. Cells were treated with 8-NH$_2$-Ado (10 μmol/L) for the indicated times and harvested. Staurosporine (100 nmol/L) was used as a positive control for apoptosis. Cell pellets were homogenized in lysis buffer [10 mmol/L KPO$_4$ (pH 7.0), 1 mmol/L EDTA, 5 mmol/L EGTA, 10 mmol/L MgCl$_2$, 50 mmol/L β-glycerol phosphate, 1 mmol/L sodium orthovanadate, 2 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5% NP40, 0.1% deoxycholate]. Homogenates were centrifuged at 4°C for 10 minutes at 16,100 x g and the supernatants were collected and stored at −20°C until time of use. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Protein at a concentration of 40 μg was mixed with sample buffer [125 mmol/L Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mmol/L DTT, 0.05% bromophenol blue] and fractionated on a precast 8% to 16% Tris-glycine gel (Invitrogen/Novex, Carlsbad, CA). The following day, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Following protein transfer, membranes were blocked with 5% nonfat milk in PBS and subsequently incubated with the primary antibody at 1:1,000 dilution overnight at 4°C. To detect for poly(ADP-ribose) polymerase (PARP), a mouse monoclonal antibody was used (PharMingen, San Diego, CA). After washing with PBS-T, the blots were developed using horseradish peroxidase–linked secondary antibody (Amersham, Arlington Heights, IL). After incubation with the primary antibody, blots were visualized with a X-ray film (Hyperfilm, Amersham).

**Annexin V-FITC Assay**

As cells undergo apoptosis, the integrity of the cell membrane is disrupted and phosphatidylserine is exposed. Using a fluorescent conjugate of Annexin V, the presence of phosphatidylserine is detected by Annexin V binding, which has a high selective affinity for phosphatidylserine. MM1.S cells were grown in 25 cm² flasks at a concentration of 2.5 × 10^6 cells in 5 mL medium. Cells were treated with 8-NH$_2$-Ado (10 μmol/L), and in some conditions, the cells received ZVAD-fmk (40 μmol/L) 1 hour prior to the addition of drug. Cells were then harvested and washed twice with cold PBS and collected by centrifugation. Samples were resuspended in diluted binding buffer provided with the kit and cells were stained following manufacturer’s directions. Samples were analyzed by flow cytometry using Coulter EPICS XL instrument and data were analyzed using System II software package.

**Caspase-8 and Caspase-9 Fluorometric Assay**

Cells were cultured in 25 cm² flasks with 5 × 10^6 cells in 5 mL medium. Cells were treated with 8-NH$_2$-Ado (10 μmol/L) or 8-Cl-Ado (10 μmol/L) for the indicated times. After collection, the cells were washed twice with cold PBS and resuspended with cell lysis buffer following the manufacturer’s protocol. Protein concentrations in all cell lysates were determined using Bio-Rad protein assay (Bio-Rad Laboratories) and total protein (100 μg) was used for each examined sample. Samples were added to the reaction buffer with the appropriate caspase fluorogenic substrate. Reactions were incubated for 2 hours at 37°C and fluorescence was determined using Packard FluoroCount fluorescent microplate reader that allowed for light excitation at 400 nm wavelength and collected emitted light at a wavelength of 505 nm.

**Mitochondrial Membrane Potential Assay**

Cells were cultured in 25 cm² flasks with 5 × 10^6 cells in 5 mL medium. A concentration of ZVAD-fmk (40 μmol/L) was added to the appropriate flasks 1 hour prior to drug therapy. Cells were treated with 8-NH$_2$-Ado (10 μmol/L) or 8-Cl-Ado (10 μmol/L) for 24 hours. Cells were washed with cold PBS and samples were transferred into two tubes with 1 mL PBS per tube. Samples were incubated with CMXRos fluorescent dye (10 nmol/L) for 15 minutes at 37°C and analyzed by flow cytometry using Coulter EPICS XL instrument with the data analyzed using the System II software package. A decrease in fluorescence indicates a decrease in the mitochondrial membrane potential ($\Delta \psi_{m}$).

**Results**

**Cytotoxicity of 8-NH$_2$-Ado**

The structure of 8-NH$_2$-Ado is shown in Fig. 1. To determine the growth inhibitory effect of 8-NH$_2$-Ado, we examined MM cell lines that are sensitive (MM.1S) and resistant (MM.1R and RPMI-8226; MDR10V) to traditional chemotherapies for this malignancy. The MM.1R and RPMI-8226 cell lines are resistant to glucocorticoids, whereas the MDR10V cell line is a multidrug-resistant MM cell line (17). Cells were incubated for 72 hours with the concentrations of 8-NH$_2$-Ado indicated in Fig. 2A. The results indicate that 8-NH$_2$-Ado causes a decrease in cell number with an IC$_{50}$ ranging from 300 nmol/L to 3 μmol/L. Normal human lymphocytes isolated from the peripheral blood mononuclear cell fraction had a...
decrease in cell number, with an IC₅₀ of 3 μmol/L in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, inner salt (MTS) assay (data not shown). This is a range of concentrations that has been achieved in plasma with another 8-substituted purine analogue (ref. 22; i.e., 8-Cl-Ado). Similar cytotoxicity has been measured in cells from epithelial cancers (prostate, breast, and cervix), indicating 8-NH₂-Ado may also be beneficial in some solid tumors (data not shown).

Cytotoxicity Requires Phosphorylation of 8-NH₂-Ado

To explore whether phosphorylation of 8-NH₂-Ado is required for cytotoxicity in MM cell lines, we examined the effect of this compound in the ED2 mouse leukemia cell line that is deficient in adenosine kinase activity (18). The cytotoxicity of 8-NH₂-Ado in ED2 cells was compared with the L1210 parental mouse leukemia cell line that retains adenosine kinase activity and the results are shown in Fig. 2B. The ED2 (AK⁻) and L1210 (AK⁺) cells were treated with the indicated concentrations of 8-NH₂-Ado for 72 hours and the formazan absorbance was determined in the MTS assay. The L1210 cells are sensitive to 8-NH₂-Ado with an IC₅₀ in the range of 1 to 3 μmol/L, whereas the ED2 cells are not affected even at 10 μmol/L analogue. These data indicate that adenosine kinase activity is required for 8-NH₂-Ado cytotoxicity.

Concentration-Dependent Metabolism of 8-NH₂-Ado in MM Cells

To determine the metabolism of 8-NH₂-Ado to its triphosphate, exponentially growing MM.1S cells were incubated with 8-NH₂-Ado (1, 3, 10, or 30 μmol/L) for 4 hours. As shown in Fig. 3A, there was a concentration-dependent increase in 8-NH₂-ATP with a maximal accumulation of 5,800 μmol/L. At 30 μmol/L exogenous drug concomitant with analogue triphosphate accumulation, there was a dose-dependent decrease in the endogenous level of ATP. At 4 hours with 8-NH₂-Ado (30 μmol/L), the cellular concentration of ATP was decreased to 25% of the control value. Using a 10 μmol/L concentration of 8-NH₂-Ado, the accumulation of 8-NH₂-ATP was measured from 0 to 12 hours. As shown in Fig. 3B, an increase in 8-NH₂-ATP concentration was noticed up to 6 hours where the level of triphosphate reached >8,000 μmol/L. After 6 hours, the levels of 8-NH₂-ATP decreased and returned to ~2,500 μmol/L by 12 hours. The decrease is most likely attributable to cell death caused by 8-NH₂-Ado. With the measured decrease in cell number, with an IC₅₀ of 3 μmol/L in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, inner salt (MTS) assay (data not shown). This is a range of concentrations that has been achieved in plasma with another 8-substituted purine analogue (ref. 22; i.e., 8-Cl-Ado). Similar cytotoxicity has been measured in cells from epithelial cancers (prostate, breast, and cervix), indicating 8-NH₂-Ado may also be beneficial in some solid tumors (data not shown).

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increase in 8-NH₂-ATP, there is a decrease in the endogenous ATP, ultimately approaching 0% of control by 12 hours. In the glucocorticoid-resistant MM.1R cells treated with 8-NH₂-Ado, there is a comparable level of 8-NH₂-ATP accumulation, indicating a similar mechanism of cytotoxicity (data not shown). In normal lymphocytes, which are less sensitive to the toxic effects of 8-NH₂-Ado, there is a much lower accumulation of 8-NH₂-ATP (13% of the accumulation in MM.1S cells) and a modest decrease in the endogenous ATP pools (data not shown). This indicates that sensitivity to the drug may be linked to the accumulation of analogue triphosphate.

Effect of 8-NH₂-ATP on Macromolecular Synthesis

Because 8-NH₂-ATP is an analogue of ATP, we investigated whether it has an effect on nucleic acid synthesis. Both MM.1S and MM.1R cells were incubated with 8-NH₂-Ado (10 μmol/L) for the indicated times; however, during the last hour of incubation, the DNA and RNA precursors, [³H]thymidine and [³H]uridine, respectively, were added to the medium. In both glucocorticoid-sensitive and glucocorticoid-resistant MM cell lines, 8-NH₂-Ado treatment results in a profound inhibition of both RNA and DNA synthesis (Fig. 4). This is in contrast to either deoxyadenosine analogues currently in clinical use, which cause an inhibition of DNA synthesis, or the investigational adenosine analogue (8-Cl-Ado), which inhibits only RNA synthesis without an effect on DNA synthesis (10). Therefore, 8-NH₂-Ado seems to be unique in its ability to profoundly inhibit both DNA and RNA synthesis.

8-NH₂-Ado Induces Apoptosis in MM Cells

The observed 8-NH₂-Ado-induced cytotoxicity could be the result of apoptosis or decreased proliferation. Cells undergoing apoptosis have a reduced DNA content caused by cleavage and loss of small DNA fragments. Using cell cycle analysis, apoptotic cells are identified as those cells in the sub-G₁ fraction of the cell cycle. As shown in Fig. 5, in a 24-hour treatment, 8-NH₂-Ado (10 μmol/L) caused a significant decrease of cells in the G₀/G₁ and G₂-M phases of the cell cycle and an increase to 66.1% in the number of cells in the sub-G₁ fraction. 8-Cl-Ado at an equimolar concentration has a less marked effect, with only 19.4% of cells in sub-G₁. Similar to the decreased cytotoxicity of 8-NH₂-Ado in 8226-S myeloma cells, we measured a decreased percentage of these cells (18.1%) undergoing apoptosis (data not shown). Accumulation of the sub-G₁ population of cells at the 10 μmol/L concentration of 8-NH₂-Ado was blocked by the pan-specific caspase inhibitor ZVAD-fmk, indicating that caspase activity is required. These results indicate that 8-NH₂-Ado stimulates a more robust response than 8-Cl-Ado causing cytolysis by the induction of apoptosis, an event that requires caspase activity.

MM.1S cells were treated with 8-NH₂-Ado for 24 hours and Annexin V binding and PI staining were measured by

![Figure 4](image_url)

**Figure 4.** Effect of 8-NH₂-Ado on DNA and RNA synthesis in MM.1S (A) and MM.1R (B) cells (n = 3). Cells were incubated with 8-NH₂-Ado (10 μmol/L) for the indicated times. One hour prior to harvesting the cells, either [³H]thymidine or [³H]uridine was added to the medium as described in Materials and Methods. Incorporation of [³H] into either DNA or RNA. Points, percentage of incorporation in untreated control cells; bars, SE. Average of three determinations.

![Figure 5](image_url)

**Figure 5.** 8-NH₂-Ado induces apoptosis in MM.1S cells. Cells were incubated with the indicated treatments for 24 hours. Cells were harvested and fixed overnight with ethanol and DNA content was stained with propidium iodide and analyzed for cell cycle by flow cytometry. Control (light gray columns); 10 μmol/L 8-NH₂-Ado (black columns); 10 μmol/L 8-NH₂-Ado + 40 μmol/L ZVAD-fmk (white columns); 10 μmol/L 8-Cl-Ado (hatched columns). Representative experiment of three individual experiments. Bars, SE.
flow cytometry with the results shown in Fig. 6A. Increased Annexin V binding correlates with the movement of phosphatidylserine to the outer leaflet of the plasma membrane during early events in apoptosis. As a positive control for Annexin V binding, MM.1S cells were treated with staurosporine (100 μmol/L) for 24 hours with 50.9% of the cells binding Annexin V. 8-NH2-Ado treatment causes a loss of Δψm with 95.4% of the cells expressing low fluorescence, whereas 8-Cl-Ado treatment is less effective with 37.8% of the cells expressing low fluorescence. The loss of Δψm is unchanged by the addition of ZVAD-fmk, indicating that caspase activity is not required for the loss of Δψm. Taken together, the data in Fig. 6 indicate that 8-NH2-Ado is causing apoptosis in MM.1S cells and these early apoptotic events do not require the activation of caspases.

8-NH2-Ado Promotes Activation of Caspases

We have shown that 8-NH2-Ado-induced cell death is associated with caspase activation (Fig. 5). Here, we show cleavage of a caspase substrate and further characterize which initiator caspases are activated. PARP is a substrate of caspase-3 that is cleaved during apoptosis. MM.1S cells were treated with 8-NH2-Ado (10 μmol/L) for times indicated in Fig. 7A and whole cell lysates were examined by immunoblotting with an antibody against PARP. There is significant cleavage of PARP by 6 hours of drug incubation, which is complete by 12 hours. The addition of ZVAD-fmk (40 μmol/L) blocks PARP cleavage, completely indicating that caspase activity is required for this action. These results indicate that caspases are rapidly

Another hallmark of apoptosis is the decrease in Δψm (23) as measured by decreased binding of the mitochondrial cationic fluorescent dye CMXRos. Using this approach, we have examined the Δψm in MM.1S cells following 24 hours of treatment with 8-NH2-Ado (10 μmol/L; Fig. 6B). In staurosporine-treated cells, a positive control for apoptosis, 95.4% of the cells are in the low fluorescence population, indicating a loss of Δψm. Similarly, the 8-NH2-Ado treatment causes a loss of Δψm with 95.4% of the cells expressing low fluorescence, whereas 8-Cl-Ado treatment is less effective with 37.8% of the cells expressing low fluorescence. The loss of Δψm is unchanged by the addition of ZVAD-fmk, indicating that caspase activity is not required for the loss of Δψm. Taken together, the data in Fig. 6 indicate that 8-NH2-Ado is causing apoptosis in MM.1S cells and these early apoptotic events do not require the activation of caspases.

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activated by 8-NH2-Ado. Caspase-3 is an effector caspase and can be activated by the initiator caspase-8 or caspase-9. We examined which initiator caspses are activated in response to 8-NH2-Ado to gain insights into the apoptotic pathways used by 8-NH2-Ado.

In apoptosis, there are two classic pathways that lead to activation of initiator caspases. Activation of the extrinsic death receptor pathway leads to caspase-8 activation and the intrinsic mitochondrial injury pathway leads to activation of caspase-9; however, there may be cross-talk between these two pathways (24). MM.1S cells were treated with 8-NH2-Ado (10 μmol/L) for the times indicated in Fig. 7B and caspase-8 and caspase-9 activity was measured in an enzymatic assay. Both caspase-8 and caspase-9 are activated by as early as 6 hours and activation increases up to 24 hours. The activation of both caspase-8 and caspase-9 is completely blocked by the addition of ZVAD-fmk (40 μmol/L), the pan-specific caspase inhibitor. 8-Cl-Ado also increases activation of both caspase-8 and caspase-9 albeit to a lesser extent.

We further investigated the possible mechanism for 8-NH2-Ado activation of caspase-8 by examining whether the Fas pathway is used. Using ZB4, an antibody that blocks Fas, we examined whether the ability of 8-NH2-Ado to induce apoptosis in MM.1S cells was impaired. ZB4 treatment did not block the ability of 8-NH2-Ado to induce apoptosis in MM.1S cells (data not shown), indicating that the 8-NH2-Ado-induced activation of caspase-8 does not require Fas activity but rather occurs by another mechanism. Caspase-8 may also be activated secondary to stimulation of the intrinsic pathway. In this scenario, activation of caspase-9 would ultimately lead to activation of caspase-8. To examine this possibility, we used specific inhibitors for both caspase-9 (LEHD-fmk) and caspase-8 (IETD-fmk) to determine whether subsequent activation of caspase activity is impaired. When caspase-9 activation is blocked by LEHD-fmk, there was a concomitant decrease in the activation of caspase-8. This result would indicate that perhaps caspase-8 activation is subsequent to caspase-9 activation. However, when caspase-8 activation was blocked with IETD-fmk, there was no measurable activation of caspase-9, indicating that the activation of caspase-9 and caspase-8 by 8-NH2-Ado is complex and that there is a likely a cross-talk between the activation pathways.

**Discussion**

We have reported previously promising preclinical studies in MM for the RNA-directed nucleoside analogue 8-Cl-Ado (10) and this drug is progressing to phase I clinical trials. Based on our earlier observations on the activity of 8-Cl-Ado (10, 25) in MM cell lines and on the literature hypothesizing the activity of 8-NH2-Ado activity in cell lines derived from solid tumors (11–14), we have tested the activity of 8-NH2-Ado in MM cell lines and determined the mechanism of action of this compound. We report here that 8-NH2-Ado causes cell death at a lower concentration and at a faster time course and induces a more robust apoptotic response than 8-Cl-Ado. The mechanism of cytotoxicity requires phosphorylation in the cells by adenosine kinase, ultimately resulting in accumulation of 8-NH2-ATP to levels of 7 mmol/L, whereas endogenous ATP levels are dramatically decreased. The cellular consequences of the accumulation of amino-modified ATP include the inhibition of both RNA and DNA synthesis. Cell death occurs by apoptosis as measured by increased Annexin V binding, decreased Δψm, increased activity of both caspase-8 and caspase-9, and cleavage of cellular substrates including PARP and DNA.

We have found that 8-NH2-Ado has characteristics that are similar to 8-Cl-Ado; however, the amino compound is more effective in causing cell death and also shows unique qualities. The range of concentrations required for cytolsis is 10-fold lower for 8-NH2-Ado in comparison with that achieved with 8-Cl-Ado (10), indicating that 8-NH2-Ado may be more effective in causing cell death. The glucocorticoid-resistant MM.1R cells require a slightly higher concentration of 8-NH2-Ado to achieve cytotoxicity than the glucocorticoid-sensitive MM.1S cells, perhaps indicating some amount of cross-resistance to 8-NH2-Ado. Although the primary mechanism of cell death for both compounds is via apoptosis, the apoptosis achieved with the amino compound is more rapid with significant PARP cleavage by 6 hours of incubation as opposed to 24 hours for 8-Cl-Ado (data not shown). In addition, the amplitude of the apoptotic response is more robust for the amino compound. Following 24 hours of incubation with a maximal concentration of the analogues, for the amino compound, 66.1% of the cells are in the sub-G1 fraction of the cell cycle and 95% of the cells exhibit a decreased Δψm as compared with 8-Cl-Ado where 19.4% of the cells are in sub-G1 and 37% of the cells have a decreased Δψm.

8-NH2-Ado requires phosphorylation of the compound to its triphosphate form for the toxicity observed. The initial reaction is the conversion to the monophosphate form by adenosine kinase and ultimately to the triphosphate. Those cells with higher adenosine kinase activity will most likely be more susceptible to the cytotoxic actions of 8-NH2-Ado. In addition, the high specific activity of adenosine kinase in lymphocytes may limit toxicity in other tissues (26). We plan to examine the effects of 8-NH2-Ado in normal bone marrow progenitor cells to assess potential toxicity issues. A comparison of the reported literature for triphosphate accumulation with other adenosine-based nucleoside analogues is shown in Table 1. There is a 40- to 400-fold higher accumulation of 8-NH2-ATP when compared with the triphosphate accumulation for 8-Cl-Ado, cladribine, fludarabine, and clofarabine. The formation of 8-NH2-ATP seems to be the favored reaction over the production of ATP because the concentration of endogenous ATP is significantly reduced in the face of elevated 8-NH2-ATP formation. These changes in cellular ATP levels not only contribute to apoptosis by affecting nucleic acid synthesis but also may affect the bioenergetics of the cell, as the endogenous ATP levels are depleted by 12 hours of incubation with 8-NH2-Ado.
The extensive cellular accumulation of 8-NH₂-ATP has an inhibitory effect on both RNA and DNA synthesis in both glucocorticoid-sensitive and glucocorticoid-resistant myeloma cell lines. This is in contrast to the deoxyadenosine analogues (cladribine and pentostatin), which inhibit DNA synthesis without effect on RNA synthesis. Fludarabine is an arabinoside-adenosine analogue that inhibits DNA synthesis in proliferating cells; however, in indolent cells such as chronic lymphocytic leukemia cells, fludarabine has an inhibitory effect on RNA transcription (27). The experimental adenosine analogue 8-Cl-Ado inhibits RNA synthesis without effect on DNA synthesis in MM cell lines. Because MM cell proliferate very slowly, it is plausible that inhibition of RNA synthesis may be an important growth inhibitory effect in indolent cells, thus contributing to the 8-NH₂-Ado-induced cytotoxicity in MM cell lines. We hypothesize that the 8-NH₂-Ado-induced decrease in DNA synthesis is secondary to the inhibition of RNA synthesis and the ensuing cell death. Future studies are planned to examine this issue in detail.

Based on the dramatic reduction in the ΔVm and depleted endogenous ATP levels, we speculate that 8-NH₂-Ado may have a direct effect on the mitochondria to promote apoptosis as has been reported for other chemotherapeutics (28). To examine this hypothesis, we investigated the activation of initiator caspases. As anticipated, we measured 8-NH₂-Ado-induced activation of caspase-9 indicating the involvement of mitochondrial damage. However, we did not anticipate the activation of caspase-8. To understand more about the mechanism of caspase-8 activation, we investigated whether Fas activation was involved and determined that Fas activation was not required for caspase-8 activation. Fas-independent activation of caspase-8 by chemotherapeutic compounds has been reported in cell lines from breast cancer (29), hepatomas (30), and Jurkat cells (31). In addition, activation of caspase-8 independent of Fas has been reported in B-lymphoid cells and found to occur downstream of caspase-3 activation (32). Studies using caspase-8 and caspase-9 specific inhibitors were inconclusive; therefore, further studies are needed to determine the mechanism of caspase-8 activation by 8-NH₂-Ado in myeloma cells.

MM is an incurable hematologic malignancy, which would benefit from the development of new therapeutic approaches. Myeloma cells are slowly replicating, making them candidates for agents that affect transcription and cellular bioenergetics. DNA-directed purine nucleoside therapy has been effective in several lymphoid malignancies, the mechanism of action of which occurs through metabolic conversion to the triphosphate form (nucleotide) causing an inhibition of DNA synthesis by direct incorporation and chain termination or by interference with DNA polymerase and nucleotide reductase. Nucleoside analogues with closely related structures and that share metabolic pathways and inhibit similar target enzymes can still exhibit a diverse spectrum of anticancer activity in human tumor types in the clinic. Although the current DNA-directed purine nucleoside analogues in clinical use (cladribine, pentostatin, and fludarabine) have not been effective in MM patients, novel analogues may still be effective in MM and should be tested for clinical efficacy. Based on our favorable data presented here, 8-NH₂-Ado seems to have a unique mechanism of action and holds great promise as a potential therapeutic agent for the treatment of MM.

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References

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Table 1. Comparison of analogue triphosphate in hematologic cell lines

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Chemical name</th>
<th>Cell line</th>
<th>Triphosphate accumulation (µmol/L)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>8-Amino-adenosine</td>
<td>8-NH₂-Ado</td>
<td>MM.1S</td>
<td>5,800</td>
<td>Fig. 3A</td>
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<td>8-Chloro-adenosine</td>
<td>8-Cl-Ado</td>
<td>MM.1S</td>
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<td>Cladribine</td>
<td>2CdA</td>
<td>Raji</td>
<td>15</td>
<td>(33)</td>
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<tr>
<td>Fludarabine</td>
<td>F-ara-9-β-d-arabinofuranosyladenine</td>
<td>CCRF-CEM</td>
<td>30</td>
<td>(33)</td>
</tr>
<tr>
<td>Clofarabine</td>
<td>Cl-F-ara-9-β-d-arabinofuranosyladenine</td>
<td>K562</td>
<td>52</td>
<td>(34)</td>
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</tbody>
</table>


8-Amino-adenosine is a potential therapeutic agent for multiple myeloma

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