8-Amino-adenosine induces loss of phosphorylation of p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2, and Akt kinase: Role in induction of apoptosis in multiple myeloma

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Abstract
Multiple myeloma is a slowly proliferating B-cell malignancy that accumulates apoptosis-resistant and replication-quiescent cell populations, posing a challenge for current chemotherapeutics that target rapidly replicating cells. Multiple myeloma remains an incurable disease in need of new therapeutic approaches. The purine nucleoside analogue, 8-amino-adenosine (8-NH2-Ado), exhibits potent activity in preclinical studies, inducing apoptosis in several multiple myeloma cell lines. This cytotoxic effect requires phosphorylation of 8-NH2-Ado to its triphosphate form, 8-amino-ATP, and results in a concomitant loss of endogenous ATP levels. Here, we show the novel effect of 8-NH2-Ado on the phosphorylation status of key cellular signaling molecules. Multiple myeloma cells treated with 8-NH2-Ado exhibit a dramatic loss of phosphorylation of several important signaling proteins, including extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, and Akt kinase. Cells depleted of ATP independent of 8-NH2-Ado do not exhibit the same decrease in phosphorylation of vital cellular proteins. Therefore, the significant shifts in endogenous ATP pools caused by 8-NH2-Ado treatment cannot account for the changes in phosphorylation levels. Instead, 8-NH2-Ado may influence the activity of select regulatory protein kinases and/or phosphatases, with preliminary data suggesting that protein phosphatase 2A activity is affected by 8-NH2-Ado. The distinctive effect of 8-NH2-Ado on the phosphorylation status of cellular proteins is a novel phenomenon for a nucleoside analogue drug and is unique to 8-NH2-Ado among this class of drugs. The kinetics of 8-NH2-Ado-mediated changes in phosphorylation levels of critical prosurvival and apoptosis-regulating proteins suggests that the modulation of these proteins by dephosphorylation at early time points may be an important mechanistic step in 8-NH2-Ado-induced apoptosis. [Mol Cancer Ther 2005;4(4):569–77]

Introduction
Multiple myeloma is a plasma cell malignancy resulting in significant morbidity and mortality. About 2% of all cancer deaths are attributed to multiple myeloma. There is no cure for this hematologic cancer and median survival from diagnosis is 3 to 4 years with conventional therapy. Although high-dose chemotherapy and stem cell transplantation are successful in inducing remission, patients eventually develop drug-resistant disease and relapse (1, 2). Thus, there is a great need for alternate effective anti-myeloma therapies and treatment options.

Among the earliest chemotherapeutic agents successfully introduced for antitumor therapy, cytotoxic purine and pyrimidine nucleoside derivatives belong to a pharmacologically diverse family containing cytotoxic, antiviral, and immunosuppressive agents. Although several nucleoside analogues are currently used for the treatment of acute and chronic hematologic malignancies (3–5), these analogues do not exhibit sufficient activity in vitro or in clinical trials to justify continued clinical evaluation in multiple myeloma (3, 4). However, promising in vitro data with the purine nucleoside analogues 8-chloro-adenosine (8-Cl-Ado) and 8-amino-adenosine (8-NH2-Ado) indicates that, in contrast to the arabinosino-adenosine and deoxyadenosine analogues, these adenosine congeners may have efficacy in multiple myeloma (6). 8-NH2-Ado was originally shown to be cytotoxic in a rat hepatoma cell line (7) and in human and mouse breast cancer cell lines (8–10). We have shown that 8-NH2-Ado is also cytotoxic to several multiple myeloma cell lines. 8-NH2-Ado profoundly inhibits both DNA and RNA synthesis and induces caspase activation and subsequent apoptotic cell death in multiple myeloma cell lines that are either sensitive or resistant to traditional therapies (11).

Here, we report a novel effect of a nucleoside analogue drug on key cellular signaling pathways. Multiple myeloma cells treated with 8-NH2-Ado exhibit a rapid and dramatic loss of phosphorylation of several important...
signaling proteins, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), and Akt kinase, whereas other pyrimidine and purine analogues do not alter phosphorylation levels. The kinetics of 8-NH₄-Ado-mediated changes in phosphorylation levels of these critical prosurvival and antiapoptotic proteins suggests that the inactivation of these proteins by dephosphorylation at early time points may be an important mechanistic step in 8-NH₄-Ado-induced programmed cell death.

MAPks are signaling modules regulated through a threetiered phosphorylation cascade, which coordinates diverse extracellular stimuli and regulates fundamental cellular processes, including changes in gene expression, proliferation, differentiation, cell cycle arrest, and apoptosis. MAPks are inactivated when dephosphorylated at threonine and/or tyrosine residues by cellular phosphatases (12, 13). The Akt kinase pathway is another signaling cascade that plays a pivotal role in cell growth and survival. Akt kinase is a serine/threonine kinase activated by both phosphatidylinositol 3-kinase (PI3K)-dependent and PI3K-independent mechanisms and negatively regulated by Src homology-2 domain-containing inositol phosphatase-1/2 (SHIP 1/2) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN). Akt can either negatively or positively regulate downstream targets by altering their enzymatic activity or cellular localization. Akt substrates are involved in several cellular processes, including regulation of protein synthesis, metabolism, homeostasis, cell cycle, cell survival and growth, and apoptosis (14, 15).

Materials and Methods

Cell Culture

The MM.1S and MM.1R cell lines were developed previously in our laboratory (16, 17). The original cell line (MM.1) was established from the peripheral blood of a multiple myeloma patient treated with steroid-based therapy. A steroid-sensitive clone (MM.1S) was isolated, and subsequently, a steroid-resistant variant (MM.1R) was developed by chronic exposure to glucocorticoids. RPMI-8226 cells and the multidrug-resistant derivative MDR10V multiple myeloma cells were obtained from Dr. William S. Dalton (H. Lee Moffitt Cancer Center, Tampa, FL; ref. 18). Cells were grown in RPMI 1640 (Invitrogen, Baltimore, MD) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL fungizone in a 37°C incubator with 5% CO₂.

Drugs and Chemicals

8-NH₄-Ado was purchased from R.I. Chemicals, Inc. (Orange, CA) and 8-Cl-Ado was from BioLog (La Jolla, CA). Cytarabine was obtained from Sigma (St. Louis, MO). Fludarabine was purchased from Berlex Laboratories (Alameda, CA) as a sterile, lyophilized powder that was dephosphorylated to its nucleoside, 2-fluoro-9-β-D-arabinofuranosyladenine, for in vitro studies. Gemcitabine was obtained from Eli Lilly and Co. (Indianapolis, IN). The kinase inhibitors SB202190 and SB203580 were purchased from Sigma. PD98059, U0126, and LY294002 were obtained from Calbiochem (San Diego, CA). Okadaic acid was purchased from Alexis Biochemicals (San Diego, CA).

Cell Proliferation Assay

The MTS assay was done as described previously (19). Briefly, multiple myeloma cells were cultured into 96-well plates at a concentration of 25,000 cells per well and incubated with the 8-NH₄-Ado for 72 hours. Cell proliferation was determined using the MTS Cell Titer 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.

Immunoblotting Analysis

Cells (5 × 10⁶) were treated with 10 µmol/L 8-NH₄-Ado for the indicated times and harvested. Cell pellets were washed with cold PBS (8.1 g NaCl, 1.14 g Na₂HPO₄, 0.22 g KCl, 0.25 g/L KH₂PO₄, 0.22 g sodium pyrophosphate, 500 µmol/L phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 10% glycerol) at 4°C for 1 hour. Lysates were centrifuged at 9,000 × g at 4°C for 1 minute and the supernatants were collected and stored at −20°C. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Protein at a concentration of 30 µg was mixed with sample buffer [125 mmol/L Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 500 µmol/L phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 10% glycerol] at 4°C for 1 hour. Lysates were centrifuged at 9,000 × g for 1 hour. Lysates were centrifuged at 9,000 × g for 1 hour and the supernatants were collected and stored at −20°C. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Protein at a concentration of 30 µg was mixed with sample buffer [125 mmol/L Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 500 µmol/L phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 10% glycerol] at 4°C for 1 hour. Lysates were centrifuged at 9,000 × g for 1 hour and the supernatants were collected and stored at −20°C. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Cells were grown in RPMI 1640 (Invitrogen, Baltimore, MD) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL fungizone in a 37°C incubator with 5% CO₂. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Protein was fractionated on a precast 8% to 16% Tris-glycine gel (Invitrogen/Novex, Carlsbad, CA). Protein at a concentration of 30 µg was mixed with sample buffer [125 mmol/L Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 500 µmol/L phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 10% glycerol] at 4°C for 1 hour. Lysates were centrifuged at 9,000 × g for 1 hour and the supernatants were collected and stored at −20°C. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Protein was fractionated on a precast 8% to 16% Tris-glycine gel (Invitrogen/Novex, Carlsbad, CA). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). Following protein transfer, membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween 20 and incubated with the primary antibody overnight at 4°C and subsequently with horseradish peroxidase–linked secondary antibody (Amer sham, Arlington Heights, IL). Blots were developed using Enhanced Chemiluminescence Plus Western Blotting Detection reagent (Amersham) and the signal was visualized with X-ray film (Hyperfilm, Amersham).

For reprobing purposes, blots were stripped using Restore Western Blot Stripping Buffer from Pierce Biotechnology (Rockford, IL). Phospho–MAPK kinase (MEK) 3/6 (Ser³⁸⁰/Ser³⁸²), phospho-p38 (Thr¹⁸⁶/Tyr¹⁸⁷), phospho–activating transcription factor-2 (Thr⁶⁹/Thr⁷¹), phospho-c-Raf (Ser²⁵⁹), phospho–MAPK/ERK kinase (MEK) 1/2 (Ser²³⁷/Ser²⁴²), total MEK1/2, phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), total ERK1/2, phospho–p90 ribosomal S6 kinase (Ser²³⁷), total ribosomal S6 kinase, phospho–phosphoinositide-dependent kinase-1 (Ser³⁴⁵), total phosphoinositide-dependent kinase-1, phospho–PTEN (Ser³⁸⁰), total PTEN, phospho-Akt (Ser⁴⁷³), total Akt, phospho–glycogen synthase kinase 3β (Ser²⁸), total

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glycogen synthase kinase-3β, phospho–Forkhead homologue in rhabdomyosarcoma (FKHR)–like 1 (Thr325)/FKHR (Thr385), and phospho–FKHR (Ser256) primary antibodies were obtained from Cell Signaling Technology (Beverly, MA). Total MKK3, total MKK6, total p38, total activating transcription factor-2, total c-Raf, total FKHR, total FKHR-like 1, phospho–c-Jun NH2-terminal kinase (JNK), and total JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-3, caspase-9, and poly(ADP-ribose) polymerase (PARP) antibodies were obtained from PharMingen (San Diego, CA). Anti-MAPK phosphatase 1 (MKP1) was from Upstate (Lake Placid, NY). Anti-caspase-8 mouse serum was a generous gift of Dr. Marcus Peter (Ben May Institute for Cancer Research, University of Chicago, Chicago, IL).

**Flow Cytometry**

To determine the distribution of cells within the cell cycle, 1 × 10^6 MM.1S cells were pelleted (500 × g for 5 minutes at 4°C), washed twice in ice-cold PBS, 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 μg/mL propidium iodide, 5 μg/mL DNase-free RNase). The stained cells were analyzed using an Epics Profile II flow cytometer (Coulter Electronics, Inc., Hialeah, FL).

**ATP Depletion Assay**

MM.1S cells were grown in dextrose-free RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone. Cellular ATP levels were manipulated by the addition of either antimycin A (2 μmol/L, a mitochondrial inhibitor) or 2-deoxy-D-glucose (5 mmol/L, an inhibitor of glycolysis) from Sigma with and without varying concentrations of dextrose. Six different metabolic conditions were examined: (a) antimycin A without dextrose, (b) antimycin A plus 0.25 mmol/L dextrose, (c) antimycin A plus 1 mmol/L dextrose, (d) antimycin A plus 10 mmol/L dextrose, (e) 2-deoxy-D-glucose without dextrose, and (f) 2-deoxy-D-glucose plus 10 mmol/L dextrose. Control cells were not subjected to ATP depletion; dextrose (10 mmol/L) was added to dextrose-free RPMI 1640. Endogenous ATP was measured in a luciferase-based assay using the ATP determination kit from Molecular Probes (Eugene, OR) and the levels corresponding to each treatment were normalized to untreated controls (20).

**Results**

**8-NH2-Ado Causes Loss of Phosphorylation Key Signaling Molecule**

In MM.1S cells, the purine nucleoside analogue 8-NH2-Ado (10 μmol/L) induces apoptosis as evidenced by an increase in Annexin V binding, a decrease in mitochondrial membrane potential, and an increase in cells with a sub-G1 DNA content (11). To investigate whether the 8-NH2-Ado-induced loss of cell viability involves modulation of growth regulatory signaling pathways, we studied the activation of key signaling molecules by assessing their phosphorylation status. MM.1S cells were treated with 10 μmol/L 8-NH2-Ado for the indicated times and whole cell extracts were assessed for phosphorylation changes by immunoblotting analysis.

**p38 MAPK Pathway.** p38 MAPK is activated by its upstream activating kinases MKK3 and/or MKK6. Immunoblot analysis revealed that 8-NH2-Ado treatment induces dephosphorylation of MKK3/6 over time. Phosphorylated MKK3/6 protein levels decrease significantly by 2 hours of 8-NH2-Ado treatment and are negligible by 6 hours of treatment. p38 phosphorylation levels are dramatically reduced by 1 hour of drug treatment, with no appreciable phosphorylation after 2 hours. The phosphorylation status of the p38 substrate activating transcription factor-2 is also compromised, with levels of phosphorylated protein decreasing considerably by 2 hours of treatment (Fig. 1A). Total protein levels for all the proteins assessed in this MAPK module remain unchanged.

**ERK1/2 Pathway.** Although there is dramatic dephosphorylation of ERK1/2, the phosphorylation levels of other components of the ERK pathway are not similarly affected by 8-NH2-Ado treatment. The phosphorylation levels of the upstream ERK1/2-activating kinases MEK1/2 seem to increase, not decrease, on drug treatment, while total MEK1/2 protein levels do not change. Phosphorylation of the ERK1/2 kinases, however, decreases significantly by 30 minutes of 8-NH2-Ado treatment and declines to negligible levels by 2 hours, while total ERK1/2 levels remain unchanged. Whereas total protein levels are unaffected, 8-NH2-Ado treatment seems to modestly decrease the phosphorylation level of the ERK1/2 substrate p90 ribosomal S6 kinase, but this effect is not as dramatic as that observed with ERK1/2 or components of the p38 MAPK pathway (Fig. 1B).

**c-Jun NH2-Terminal Kinase.** JNK or stress-activated kinases form one subfamily of the MAPK group of serine/threonine protein kinases and are involved in cellular processes, such as apoptosis. However, unlike the other MAPK proteins p38 and ERK, JNK phosphorylation is unaffected by 8-NH2-Ado treatment (Fig. 1C).

**Akt Kinase Pathway.** Total and phosphorylated levels of the Akt regulatory protein phosphoinositide-dependent kinase-1 remain unchanged; however, the Akt kinase dramatically loses phosphorylation on 8-NH2-Ado treatment. Phospho-Akt levels decrease significantly by 2 hours of treatment and eventually decline further to negligible levels. The downstream targets of Akt are also similarly affected. Members of the Forkhead family of transcription factors undergo dramatic loss of phosphorylation, whereas total protein levels do not change. FKHR-like 1 phosphorylation decreases dramatically by 2 hours of drug treatment, with no appreciable phosphorylation at 4 and 6 hours. FKHR phosphorylation is also similarly affected (data not shown). Phospho–glycogen synthase kinase-3β levels diminish by 2 hours of 8-NH2-Ado treatment and are negligible by 6 hours (Fig. 1D).
To ascertain whether the changes in phosphorylation levels of these key signaling molecules is a direct result of cell death, parallel cultures were assessed for cellular viability by cell cycle analysis. Cells undergoing apoptosis have reduced DNA content caused by cleavage and loss of small DNA fragments. Therefore, apoptotic cells are identified as those cells in the sub-G₁ fraction of the cell cycle. This analysis revealed no differences between the sub-G₁ fraction of untreated cells and cells treated with 8-NH₂-Ado for up to 4 hours, indicating that the loss of phosphorylation observed by immunoblotting was not due to a concomitant loss of cell viability (Fig. 2).

Effect of 8-NH₂-Ado on Phosphorylation of p38 MAPK in Various MM Cell Lines

The effect of 8-NH₂-Ado treatment on phosphorylation levels was assessed in additional myeloma cell lines to determine whether the drug-induced alterations in protein phosphorylation occur in multiple cell lines or are limited to the MM.1S myeloma cell line. RPMI-8226 parent myeloma cells, the multidrug-resistant derivative MDR10V cells, and the glucocorticoid-resistant MM.1R cells are all affected by the cytotoxic ability of 8-NH₂-Ado (11). Phosphorylation levels of p38 were assessed in these cell lines in response to 8-NH₂-Ado treatment and found to decrease in a dose-dependent manner, whereas total p38 levels remain unchanged (Supplementary Data). The data suggest that 8-NH₂-Ado-induced loss of protein phosphorylation is not restricted to the MM.1S myeloma cell line.

8-NH₂-Ado and Loss of Phosphorylation in Myeloma

Figure 1. 8-NH₂-Ado induces loss of phosphorylation of MAPK signaling molecules and Akt kinase pathway components. A, components of the p38 MAPK pathway; B, components of the ERK1/2 pathway; C, JNK; D, components of Akt kinase pathway. MM.1S cells were exposed to 10 μmol/L 8-NH₂-Ado for the indicated times, after which cells were lysed as described in Materials and Methods. Protein (30 μg) was separated by gel electrophoresis, transferred to PVDF membrane, and probed with the indicated phosphorylation-specific antibodies. Blots were stripped and reprobed with the corresponding total protein antibodies to ensure that drug treatment does not affect total protein levels and to ensure equal loading and transfer. Representative experiments; two additional studies yielded equivalent results.

Figure 2. 8-NH₂-Ado does not affect cell viability at early time points. MM.1S cells were incubated with 10 μmol/L 8-NH₂-Ado for the indicated times. Cells were harvested and fixed as described in Materials and Methods. DNA content was stained with propidium iodide and analyzed for cell cycle by flow cytometry. Representative experiment; two additional studies yielded equivalent results.

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4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org).
Endogenous ATP was measured in a luciferase-based assay and the levels corresponding to each treatment are shown as a percentage of untreated control (Fig. 4). For the purpose of comparison, it is important to note that a 2-hour exposure to 8-NH₂-Ado decreases endogenous ATP to 65% of control (ref. 11; Supplementary Data). Extracts from each condition were immunoblotted against phospho-p38 (Fig. 4), phospho-ERK1/2, and phospho-Akt (data not shown) and did not reveal a decrease in phosphorylation. These results indicate that the effect of 8-NH₂-Ado on phosphorylation of p38, ERK, Akt, and other proteins in the kinase modules is not simply the result of decreased endogenous ATP levels.

Effect of 8-NH₂-Ado on Cellular Phosphatases

One possible mechanism for the decrease in phosphorylation of the kinase molecules and their substrates is an increase in the activity of the phosphatase(s) that regulates them. To test this hypothesis, we assessed the levels of MKP1, a dual-specificity phosphatase that can act to dephosphorylate p38 (24, 25). However, by immunoblotting, there was no 8-NH₂-Ado-stimulated change in MKP1 levels, suggesting that this phosphatase is unlikely to be involved (Fig. 5A).

In addition, the effect of 8-NH₂-Ado treatment on PTEN, which encodes a key phosphatase involved in the negative regulation of the PI3K/Akt signaling pathway (26, 27), was assessed (Fig. 5B). Like MKP1, total and phospho-PTEN levels are unaltered by 8-NH₂-Ado treatment. Although subcellular location plays a major role in regulation of PTEN function, phosphorylation of the COOH-terminal domain has also been shown to negatively regulate phosphatase activity (28, 29). Therefore, unchanged phospho-PTEN levels indicate that this phosphatase is not involved in the drug-mediated effect on protein phosphorylation.

Effect of Other Nucleoside Analogues on Phosphorylation Levels

Not only does 8-NH₂-Ado induce a novel cellular effect by significantly altering the phosphorylation levels of key signaling molecules, but it also seems to be unique among other nucleoside analogues, both pyrimidine and purine, in its ability to do so. Although a congener of 8-NH₂-Ado, 8-Cl-Ado, induces apoptosis in multiple myeloma cells (21), a time course of 10 μmol/L 8-Cl-Ado treatment in MM.1S cells does not reveal any effect on the phosphorylation status of p38 (Fig. 3A), ERK1/2, or Akt kinase (data not shown). Another purine analogue, fludarabine, and the pyrimidine analogues cytarabine and gemcitabine are also cytotoxic to multiple myeloma cells (22, 23). However, when used at a 10 μmol/L concentration in MM.1S cells for 4 hours, a time and a concentration at which 8-NH₂-Ado causes a dramatic loss of phosphorylation of these kinases, they do not cause a decrease in the phosphorylation of p38 (Fig. 3B), ERK1/2, or Akt (data not shown).

ATP Depletion of MM.1S Cells

Because 8-NH₂-Ado causes dramatic shifts in endogenous ATP pools (ref. 11; Supplementary Data), the decrease in available ATP may have an effect on kinases or phosphatases, ultimately affecting the phosphorylation of important signaling pathways in cells. To test if decreases in ATP alone are sufficient to cause the observed decreases in phosphorylation, we manipulated cellular ATP levels by the addition of either antimycin A, which inhibits the electron transport chain, or 2-deoxy-D-glucose, which inhibits glycolysis, and achieved a graded ATP depletion by introducing increasing concentrations of dextrose. Endogenous ATP was measured in a luciferase-based assay and the levels corresponding to each treatment are shown as a percentage of untreated control (Fig. 4). For the purpose of comparison, it is important to note that a 2-hour exposure to 8-NH₂-Ado decreases endogenous ATP to 65% of control (ref. 11; Supplementary Data). Extracts from each condition were immunoblotted against phospho-p38 (Fig. 4), phospho-ERK1/2, and phospho-Akt (data not shown) and did not reveal a decrease in phosphorylation. These results indicate that the effect of 8-NH₂-Ado on phosphorylation of p38, ERK, Akt, and other proteins in the kinase modules is not simply the result of decreased endogenous ATP levels.

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In a parallel approach to testing the involvement of cellular phosphatases, we treated MM.1S cells with varying concentrations of the phosphatase inhibitor okadaic acid in combination with 8-NH2-Ado for 4 hours to assess whether the serine/threonine protein phosphatases PP2A and PP1 are involved. Cell extracts immunoblotted against phospho-p38 and total p38 antibodies showed that in the presence of 8-NH2-Ado there is a partial recovery of phosphorylation at a concentration of 30 nmol/L okadaic acid (Fig. 5C). Additionally, treatment of MM.1S cells with okadaic acid significantly delays 8-NH2-Ado-induced loss of p38 phosphorylation. A time course of MM.1S cells treated with 10 μmol/L 8-NH2-Ado and 30 nmol/L okadaic acid reveals that in the presence of okadaic acid the decrease in phospho-p38 levels is delayed and still present at 6 hours, in contrast to MM.1S cells treated with 8-NH2-Ado alone (Fig. 5D). The 30 nmol/L concentration of okadaic acid in cells is indicative of selective inhibition of PP2A over PP1 (30–32), suggesting that activation of PP2A may play a role in the 8-NH2-Ado-induced decrease in phosphorylation of p38.

Effect of 8-NH2-Ado on Caspase Activation and PARP Cleavage

In MM.1S cells, 8-NH2-Ado treatment activates the effector caspases, caspase-8 and caspase-9, as measured by a fluorometric assay (11). By immunoblotting, cleaved and activated caspase-8 and caspase-9 occurred between 2 and 4 hours of 10 μmol/L 8-NH2-Ado treatment. Cleavage of the universal caspase substrate PARP also occurs starting at 2 hours of drug treatment (Fig. 6). These markers of apoptosis temporally follow the loss of phosphorylation of the signaling kinases.

Effect of Kinase Inhibitors on 8-NH2-Ado-Mediated Cell Cytotoxicity

Cell proliferation assays were done to investigate whether kinase inhibitors can modulate the effects of 8-NH2-Ado.
8-NH2-Ado on cellular viability. MM.1S cells were treated with varying doses of the p38 kinase inhibitors SB202190 and SB203850, the ERK1/2 inhibitors PD98059 and U0126, and the PI3K inhibitor LY294002 alone and with 10 μmol/L 8-NH2-Ado. In cell viability assays, the combination of 10 μmol/L 8-NH2-Ado and the kinase inhibitors does not result in synergy to increase the cytotoxic effects of 8-NH2-Ado, nor do the kinase inhibitors diminish the cytotoxic effect of 8-NH2-Ado (data not shown).

Discussion

There will be ~15,000 new cases of multiple myeloma diagnosed in 2004 (1). Multiple myeloma remains an incurable malignancy in need of new therapeutic approaches. 8-NH2-Ado, a promising option, belongs to the cytotoxic nucleoside analogue class of drugs. Nucleoside analogues are antimetabolites that interfere with the synthesis of nucleic acids. These agents can exert their cytotoxic activity by being incorporated into and altering the DNA and RNA macromolecules themselves, by interfering with various enzymes involved in the synthesis of nucleic acids, or by modifying the metabolism of physiologic nucleosides (33).

8-NH2-Ado induces cell death in vitro in several myeloma cell lines. Cell death occurs by apoptosis as measured by increased Annexin V binding, decreased mitochondrial membrane potential, increased activity of both caspase-8 and caspase-9, and PARP cleavage. 8-NH2-Ado is efficiently phosphorylated intracellularly to the active triphosphate form (8-amino-ATP) by adenosine kinase, dissipates cellular bioenergy by causing a decrease in endogenous ATP pools, and specifically inhibits both DNA and RNA synthesis, making it an exciting candidate for clinical use (11). In addition, 8-NH2-Ado exerts a differential effect on normal versus malignant cells. Normal lymphocytes accumulate much lower levels of 8-amino-ATP, exhibit only a modest decrease in endogenous ATP pools, and are less sensitive to the toxic effects of 8-NH2-Ado (11).

Here, we report the distinctive effect of 8-NH2-Ado on the phosphorylation status of several cellular proteins. The changes in phosphorylation of key signal transduction molecules as a result of treatment with 8-NH2-Ado is a novel effect for a nucleoside analogue drug and seems to be unique to 8-NH2-Ado among this class of drugs. We detected a dramatic decrease in the phosphorylation of p38 MAPK, ERK1/2, and Akt kinase, as well as other components of these pathways, over time with no concomitant changes in the total protein levels. It is important to note that all the signaling molecules evaluated exhibited high basal phosphorylation levels. This is not a serum-induced effect, as serum-starved MM.1S cells also express high baseline protein phosphorylation levels (data not shown), suggesting instead the possible constitutive activation of an upstream component(s). The kinetics of dephosphorylation of MKK3/6, the MKKs upstream of p38, are slower than p38, indicating that there may be another p38-activating protein that is being affected at an earlier time. In addition, phosphorylation levels of MEK1/2, the kinases responsible for activating ERK1/2, exhibit an increase rather than a decrease on 8-NH2-Ado treatment, implying that the 8-NH2-Ado-mediated effect on phosphorylation seems to target ERK1/2 directly and not through the upstream MEK1/2 proteins. Similarly, in the Akt pathway, 8-NH2-Ado treatment affects Akt kinase directly and eventually its downstream targets rather than an upstream element.

Although several cellular proteins are affected, the phosphorylation status of several signaling molecules, including JNK, protein kinase C, and signal transducers and activators of transcription proteins, is unaltered with 8-NH2-Ado treatment (data not shown), indicating that the decrease in phosphorylation caused by 8-NH2-Ado is not a global event but a specific effect of biological consequence observed in several different myeloma cell lines.

MAPKs are a family of critical cellular signaling molecules regulated through a three-tiered cascade (12, 13). In general, activation of ERK1/2 has been linked to cell survival, whereas JNK and p38 are linked to induction of apoptosis. However, this is an oversimplification and the actual biological role of each MAPK module is highly dependent on cell type and context and the differential expression of the module’s component isoforms. For instance, whereas activation of p38 has been linked to the induction of apoptosis in several cell types (12, 34), the inactivation of p38α in knockout mice results in embryonic lethality (35, 36). In multiple myeloma, interleukin-6 (IL-6) secreted by bone marrow stromal cells and insulin-like growth factor-I (IGF-I) have been reported to activate multiple MAPK cascades that in turn promote multiple myeloma cell proliferation (37, 38). Antisense experiments have shown that MEK/ERK activation is essential for IL-6-mediated multiple myeloma cell proliferation (39). Furthermore, p38 MAPK inhibition by a chemical inhibitor (VX-745) seems to block IL-6 and vascular endothelial growth factor (VEGF) secretion from bone marrow stromal cells and subsequent paracrine myeloma cell growth in the bone marrow (40). It is known that IL-6 promotes myeloma cell growth, survival, and drug resistance, whereas VEGF induces myeloma cell migration (37, 41). Therefore, the ability of 8-NH2-Ado to induce loss of phosphorylation and inhibition of p38 MAPK and the MEK/ERK cascade may be of significant clinical relevance.

The serine/threonine kinase Akt, which is activated mainly by the upstream PI3K, also plays an important role in cell survival, and overexpression of Akt isoforms has been observed in several cancers (15). Aberrant expression of Akt has been detected in bone marrow biopsies of multiple myeloma patients and in multiple myeloma cell lines (42). In addition, it has been reported that IL-6 secreted by bone marrow stromal cells activates the PI3K/Akt pathway, which promotes cell survival and...
proliferation in multiple myeloma, whereas IGF-I has been shown to induce adhesion, migration, and proliferation in human myeloma cells via activation of β1 integrin and PI3K/Akt signaling (37, 43).

Akt substrates include those involved in regulation of growth and apoptosis. The Akt substrate glycogen synthase kinase-3β is upstream of metabolic responses and is involved in the regulation of proliferative and antiapoptotic pathways. The enzymatic activity of glycogen synthase kinase-3β isosforms is inhibited by Akt-mediated phosphorylation (44). The Forkhead family of transcription factors, also known as the Foxo protein family, are Akt substrates that have been well documented to play a role in programmed cell death. The Forkhead proteins are sequestered in the cytoplasm by 14-3-3 proteins when phosphorylated by Akt, preventing them from fulfilling their function as proapoptotic transcription factors (14, 15). In myeloma cells, IGF-I protects cells from glucocorticoid-induced apoptosis by activating the PI3K pathway and inducing the phosphorylation and activation of the Forkhead family member, FKHR-like 1. Inhibition of FKHR-like 1 results in the loss of ability to inhibit cellular proliferation and induce apoptosis in myeloma cells (45). Therefore, decreased phosphorylation of Akt induced by 8-NH2-Ado treatment may lead to decreased phosphorylation of downstream substrates, ultimately contributing to apoptosis.

The decreased phosphorylation of the kinases that we have observed cannot be attributed to loss of endogenous ATP levels that occurs on 8-NH2-Ado treatment of MM.1S cells, as cells depleted of ATP independent of 8-NH2-Ado do not exhibit a loss of p38 or Akt phosphorylation. Rather, a slight increase in p38 and Akt phosphorylation is observed, consistent with previous reports in the literature of antimycin A treatment in other cell lines (46, 47), and in phospho-p38, consistent with the fact that the p38 MAPK signaling module is stress responsive. 8-NH2-Ado-induced loss of phosphorylation of p38 may be due to either a decrease in upstream kinase activity or an increase in phosphatase activity. Phosphatase families that regulate the MAPKs include the serine/threonine-specific protein phosphatases (PP1, PP2A, PP2B, and PP2C), tyrosine-specific phosphatases, and dual-specificity phosphatases, also termed MKPs (48). Our results show that the dual-specificity phosphatase family member MKP1 is not involved but that PP2A may play a role in the observed 8-NH2-Ado-induced decrease in phosphorylation. Although okadaic acid inhibits both PP1 and PP2A, the concentration of okadaic acid (30 nmol/L) that blunts 8-NH2-Ado-induced decrease in phosphorylation of p38 in MM.1S cells has been reported to have a higher specificity for PP2A (31, 32). However, because okadaic acid treatment does not allow for full recovery of phosphorylation levels decreased by 8-NH2-Ado and only delays the ability of 8-NH2-Ado to induce dephosphorylation, albeit dramatically, there may be other factors involved in this phenomenon. Furthermore, the Akt phosphatase PTEN, which has been implicated in the negative regulation of the PI3K/Akt cascade in myeloma cells (26, 49), is not involved in this 8-NH2-Ado-mediated effect on phosphorylation, because total PTEN and phospho-PTEN levels are unchanged with drug treatment. Additional studies are required to further dissect the mechanism of action of 8-NH2-Ado-mediated loss of protein phosphorylation. Because its congener compound, 8-Ci-Ado, does not exert a similar effect on protein phosphorylation, it is possible that the 8-NH2-Ado’s cellular metabolite, 8-amino-ATP, may have intrinsic "phosphatase" activity based on its structure, which allows it to bind to and dephosphorylate certain proteins preferentially.

The cleavage of pro-caspase-8 and pro-caspase-9 to generate the active caspase fragments, and cleavage of the universal caspase substrate PARP, establishes a temporal link between 8-NH2-Ado-induced loss of protein phosphorylation and 8-NH2-Ado-mediated apoptosis in myeloma cells. In the same period, 8-NH2-Ado treatment causes decreased DNA and RNA synthesis (11) in a parallel pathway leading to apoptosis. Further correlative evidence for the functional significance of 8-NH2-Ado-induced loss of protein phosphorylation is provided by the observation that p38 levels decline on 8-NH2-Ado treatment of PC-3 prostate cancer cells, which also undergo apoptosis in response to the drug (data not shown). However, phospho-p38 levels are not altered on 8-NH2-Ado treatment in a negative control pancreatic cancer cell line, which is not subject to cytotoxicity by 8-NH2-Ado (data not shown).

Protein phosphorylation is an important post-translational modification involved in intracellular signal relay. It plays an important role in the regulation of function of a significant percentage of human gene products, and abnormal signaling due to aberrant phosphorylation has been implicated in several disease states, including myeloma. Therefore, a new focus of drug development is to target dysregulated protein kinases (50). The distinctive effect of 8-NH2-Ado on the phosphorylation status of cellular signaling proteins is a novel phenomenon for a nucleoside analogue drug and seems to be unique to 8-NH2-Ado among this class of drugs. In addition to its effect on DNA and RNA synthesis, the ability of 8-NH2-Ado to regulate key signaling molecules makes it an exciting and promising candidate for drug development and clinical use in the treatment of multiple myeloma.

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mediated inhibition of the G1-S transition in mammary carcinoma cells and 8-NH2-cAMP induce cell death independently of cAMP kinase-


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