8-Amino-Adenosine Activates p53-Independent Cell Death of Metastatic Breast Cancers

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Abstract
8-Amino-adenosine (8-NH2-Ado) is a ribose sugar nucleoside analogue that reduces cellular ATP levels and inhibits mRNA synthesis. Estrogen receptor-negative (ER–) metastatic breast cancers often contain mutant p53; therefore, we asked if 8-NH2-Ado could kill breast cancer cells without activating the p53-pathway. Regardless of the breast cancer subtype tested or the p53 status of the cells, 8-NH2-Ado was more cytotoxic than either gemcitabine or etoposide. 8-NH2-Ado treatment inhibited cell proliferation, activated cell death, and did not activate transcription of the p53 target gene p21 or increase protein levels of either p53 or p21. This occurred in the estrogen receptor-positive (ER+) MCF-7 cells that express wild-type p53, the ER+ T47D cells that express mutant p53, and the ER– MDA-MB-468 cells or MDA-MB-231 cells that both express mutant p53. 8-NH2-Ado induced apoptotic death of MCF-7 cells and apoptosis was not inhibited by knockdown of functional p53. Moreover, the pan-caspase inhibitor Z-VAD blocked the 8-NH2-Ado–induced MCF-7 cell death. Interestingly, 8-NH2-Ado caused the MDA-MB-231 cells to detach from the plate with only limited evidence of apoptotic cell death markers and the cell death was not inhibited by Z-VAD. Inhibition of MDA-MB-231 cell autophagy, by reduction of ATG7 or 3-methyladenine treatment, did not block this 8-NH2-Ado–mediated cytotoxicity. Importantly 8-NH2-Ado was highly cytotoxic to triple-negative breast cancer cells and worked through a pathway that did not require wild-type p53 for cytotoxicity. Therefore, 8-NH2-Ado should be considered for the treatment of triple-negative breast cancers that are chemotherapy resistant. Mol Cancer Ther; 11(11); 2495–504. ©2012 AACR.
that does not activate the p53 pathway might be more effective.

Unique and promising RNA-directed treatments for myeloma are the nucleoside analogs 8-chloro-adenosine (currently in phase I trials) and 8-NH₂-Ado (11, 12). Importantly, 8-NH₂-Ado is more cytotoxic to myeloma cancer cells than to normal human lymphocytes (13, 14). Moreover, 8-NH₂-Ado is an RNA-directed nucleoside analog that inhibits transcription and polyadenylation (12, 15). Adenosine analogues accumulate in cells as triphosphates and decrease the endogenous ATP pool (12). Preclinical studies on multiple myelomas have been very encouraging (13, 14, 16, 17). 8-Chloro-adenosine has recently proved cytotoxic to breast cancer cells through depletion of cyclin E (11). However, no study has investigated whether 8-NH₂-Ado can effectively kill breast cancer cells without activating a p53 pathway. We have investigated whether p53 function is required in breast cancer cells without requiring functional p53. We predicted that 8-NH₂-Ado would inhibit the growth of breast cancer cells without requiring functional p53. In this report, we show that 8-NH₂-Ado is more effective than gemcitabine and etoposide at inhibiting the growth of multiple breast cancer subtypes, including triple-negative cell lines. Furthermore, we show that 8-NH₂-Ado induces p53-independent cell death that can proceed through an apoptotic pathway as well through a novel cytotoxic pathway that does not require autophagy or necrosis.

Materials and Methods

Cell culture

All the cell lines used for this study were obtained from American Type Culture Collection (ATCC). The authors did not carry out further authentication. MDA-MB-231 cells (p53 mutant 280, Arg–Lys), T47D (p53 mutant 194, Leu–Phe), MDA-MB-468 (p53 mutant 273, Arg–His), and MCF-7 (p53 wild-type) from ATCC were grown in Dulbecco’s Modified Eagle Medium (DMEM) medium (Mediatech), 10% FBS (Gemini), and 50 U/mL of penicillin–streptomycin (Mediatech) at 5% CO₂ in a humidified incubator. Clonal MCF-7 line D11 with inducible (Tet-on) shRNA for p53 was established and characterized in our laboratory (18). To induce shRNA expression, D11 cells were treated with 2 μg/mL doxycycline for 6 days. The MCF7.beclin1 clone (19) was a generous gift from Beth Levine (UT Southwestern Medical Center, Dallas, TX) and was grown in the absence of tetracycline to induce Beclin 1 expression for 5 days.

Reagents

Etoposide, propidium iodide, doxycyclin, trypan blue solution, and MTT) were purchased from Sigma. Gemcitabine and 8-NH₂-Ado were provided by Dr. Steve Rosen (Robert H. Lurie Comprehensive Cancer Center, Chicago, IL). The activity of the 8-NH₂-Ado varied in the 2 batches donated to the laboratory. Therefore, the treatments to achieve similar inhibition of growth ranged from 10 to 15 μmol/L. The general Caspase Inhibitor Z-VAD-FMK was purchased from R&D Systems (FMK001). The Image-iT LIVE Lysosomal and Nuclear Labeling Kit (Molecular Probes I34202) was used to detect autophagy in the cells. Cell Death Detection ELISA was purchased from Roche (11 544 675 001). Apoptosis detection was done with ApoScreen Annexin V-FITC Kit (Southern Biotech 10010-02). 3-Methyladenine (3-MA) and Necrostatin 1 were purchased from Sigma.

MTT analysis, flow cytometry, and apoptosis detection by Histone: DNA complexes

MTT. Toxic effects of the drugs were determined by measuring the mitochondrial activity of each cell line using the tetrazolium dye-based microtitration assay to measure mitochondrial dehydrogenases activity (20). Cells were seeded at 1.25 × 10⁵ cells per well in 12-well plates and allowed to attach overnight. Cells were then treated with the drugs as indicated in the figures at the concentrations shown. The absorbance was quantified by measuring the absorbance at 550 nm (the 620 nm absorbance was subtracted for background). All MTT data are represented as mitochondrial dehydrogenase activity as percentage of a dimethyl sulfoxide (DMSO) vehicle-treated control.

Flow cytometry. The Annexin V-FITC reagent was used according to manufacturer’s protocol after 16 hours of drug treatment. Cells were detached with trypsin, washed twice with PBS, and resuspended in the binding buffer provided by the manufacturer with the addition of 10 μL ApoScreen Annexin V-FITC and incubated for 10 minutes. This step was followed by the addition of 10 μL of propidium iodide and then flow cytometry was conducted. Fluorescence-activated cell sorting (FACS) analysis was carried out using on a BD Bioscience FACS scan.

Enrichment factor detection. The apoptosis detection kit used anti-histone antibody and anti-DNA antibody to detect the values of histones associated with DNA in the cytoplasm as an indication of apoptosis. Cytoplasmic extract was prepared after the cells were treated with drugs for 24 hours in a 12-well plate. The plate was spun down at 1,500 × g for 10 minutes and the cells were resuspended in 500 μL of lysis buffer and incubated for 30 minutes at room temperature. The cells were resuspended and spun in an eppendorf tube at 13,000 rpm for 10 minutes and the supernatant was collected and used for the ELISA procedure at a 1:3 dilution. The ELISA steps were carried out as described by the manufacturer’s direction with slight modifications. The final step was modified to stop color development by the addition of 100 μL of 5% SDS. Detection was carried out at 415 nm against a substrate solution blank. Enrichment factor = mU of the drug treated (dying/dead cells)/mU of the corresponding control.

Quantitative reverse transcription PCR

Standard procedures from the manufacturers were used for these assays. RNA was isolated using QIAshredder...
columns and RNeasy Mini Kit (Qiagen). Five micrograms of RNA was used for cDNA synthesis using high capacity cDNA Archive Kit reagents (Applied Biosystems). One hundred and fifty nanograms of cdNA was combined with TaqMan Universal Master Mix and Applied Biosystems Assays on Demand primers/probes for p21 (Hs00355782_m1) or actin (4352935E). PCR reaction was carried out in 7500 Sequence Detection System (Applied Biosystems) and actin was used as the normalizer.

Whole-cell protein extract
Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% NP-40, 0.5% deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 50 mmol/L Tris-Cl pH8) with 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 8.5 µg/mL aprotinin, and 2 µg/mL leupeptin and incubated on ice for 20 minutes with periodic vortexing. Pellets were then centrifuged at 9,300 x g for 20 minutes. The supernatants were collected and kept at −80°C for future analysis.

Western blot analysis
Fifty micrograms of protein extract were separated by 4% to 12% SDS-PAGE (Invitrogen) and electrotransferred to nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Immunoblotting was done with p53 monoclonal antibody supernatants (pAb421, pAb240, and pAb1801), LC3B (Cell Signaling; 2775), PARP (BD Biosciences; 51-6639GR), p21 (Ab-1 Oncogene Research Science; OP64), Beclin 1 (Novus Biologicals; 110-87318), ATG-7 (Cell Signaling; 8558), and actin (Sigma; A2066). The membranes were then incubated in anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma) and the signals were visualized by chemiluminescence.

Lysosomal staining
Labeling of the live cells adherent to the cover slips with red-fluorescent LysoTracker dye and blue-fluorescent Hoechst dye was conducted according to manufacturer's protocol and images were taken on an Olympus fluorescent microscope.

RNA interference and transfections
For siRNA experiments, MDA-MB-231 cells were seeded in media without penicillin–streptomycin and allowed to attach overnight. Cells were transfected at 60% confluence with 100 nmol/L of atg7 siRNA smart pool or nontargeted siRNA from Dharamacon for 6 hours using Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol. At the end of the incubation period, fresh DMEM with 10% FBS was added and the cells were allowed to grow for 48 hours. Drug treatment with 15 µmol/L 8-NH2-Ado in fresh DMEM with 10% FBS was then carried out for 24 hours. Cells were harvested by scraping into the media, washed with PBS, and lysed in RIPA buffer for protein analysis or analyzed on the plate by MTT analysis for proliferation.

Results
8-NH2-Ado is more cytotoxic than standard chemotherapeutic drugs to breast cancer cell lines regardless of p53 or estrogen receptor status
Cell culture studies are an excellent first-line indication of the efficacy of chemotherapeutic drugs for particular cancer subtypes. Therefore, cell culture studies with well-defined cell lines with defined genotypes are ideal for first line preclinical studies. We used a well-defined set of breast cancer cell lines ranging from tumorigenic to highly metastatic and compared their sensitivity with 8-NH2-Ado and with the 2 DNA-damaging drugs (gemcitabine and etoposide). The cell lines examined were MCF-7, MDA-MB-231, T47-D, and MDA-MB-468. These 4 cell lines are well described for their p53 (MDA-MB-231, T47-D, and MDA-MB-468) and etoposide or gemcitabine (18) in breast cancer and for 8-NH2-Ado (12). Treatments were carried out with relevant concentrations for gemcitabine (8) and etoposide (18) in breast cancer and for 8-NH2-Ado in myeloma (13). Treatment with 10 µmol/L 8-NH2-Ado approached the IC50 dosage for MCF-7 cells and surpassed the IC50 value for the 3 cell lines with mutant p53 (MDA-MB-231, T47-D, and MDA-MB-468 cells; Fig. 1). The MTT proliferation assay monitors cell populations’ response to external factors including cell growth and cell death. In all instances with 8-NH2-Ado, the cells detached from the plate (most likely indicating cell death, see Fig. 4A for representative images). Moreover, in all cases, 8-NH2-Ado treatment was far more cytotoxic than either etoposide or gemcitabine (Fig. 1). This strongly suggested that functional p53 was not required for cell death induction. To examine if the mechanism of action of 8-NH2-Ado was p53-independent, we focused on comparing the MCF-7 cell line with the MDA-MB-231 cell line. These 2 cell lines were uniquely sensitive to 8-NH2-Ado. MDA-MB-231 cells are metastatic and express gain-of-function oncogenic mutant p53 that blocks the p53-family member protein p63 (21), whereas MCF-7 cells have wild-type p53 and are not metastatic (22).

In contrast to standard chemotherapeutics, 8-NH2-Ado does not activate the p53 pathway
Wild-type p53 is normally found at low levels in dividing cells due to the targeted destruction by its negative regulator Mdm2 (23). After DNA damage, the levels of wild-type p53 protein increase due to posttranslational modifications that block the interaction of p53 with Mdm2 (24). High levels of oncogenic variant p53 protein are a
hallmark of cancer cells containing genetic point mutations in the p53 gene that results in stable p53 protein (25). We detected an increase in wild-type p53 protein in MCF-7 cells after etoposide and gemcitabine treatment (Fig. 2A, lanes 2 and 4); however, no wild-type p53 protein increase was detected after 8-NH₂-Ado treatment (Fig. 2A, compare lanes 1 and 3). Furthermore, MDA-MB-231 cells contained high levels of stable mutant p53 before drug treatment and this protein level was not affected by etoposide, 8-NH₂-Ado, or gemcitabine treatment (Fig. 2A, lanes 5–8).

To further examine the p53-independent signaling of 8-NH₂-Ado, we asked if drug treatment could increase the level of the cyclin-dependent kinase inhibitor p21. We reproducibly observed that 8-NH₂-Ado treatment reduced the level of p21 protein in MCF-7 cells and decreased the p21 transcript level in both MCF-7 and MDA-MB-231 cells (Fig. 2). This indicated that 8-NH₂-Ado did not activate a DNA damage response in either cell line and blocked the transcription of a key cell-cycle inhibitor. This was in stark contrast to the outcomes observed with etoposide or gemcitabine treatment, both of which activated the transcription of p21. As expected, etoposide mediated a robust increase in p21 protein and transcript in MCF-7 cells and surprisingly increased p21 protein and transcript levels in MDA-MB-231 cells (Fig. 2). Gemcitabine treatment of both cell lines caused a robust increase in p21 transcript without a significant change in p21 protein (Fig. 2). In support of our p53-independent hypothesis, no signaling to p53 was evident in 8-NH₂-Ado-treated cells, whereas both etoposide and gemcitabine caused DNA damage signaling that could function through wild-type p53 in MCF-7 cells as well as through an alternative pathway in the MDA-MB-231 cells, perhaps through the p53 family member p73.

8-NH₂-Ado induces significant apoptotic death of MCF-7 cells

To determine the signal transduction pathways activated after treatment of the cells with 8-NH₂-Ado, we examined cell death markers associated with apoptosis. We assessed the cleavage of PARP, the enrichment of cytoplasmic histone-associated DNA fragments, and Annexin V staining (Fig. 3). We detected PARP cleavage in MCF-7 cells treated with 8-NH₂-Ado (Fig. 3A, compare lane 3 with lanes 1, 2, and 4), as well as an increase in apoptosis-associated enrichment factor, which detects cytoplasmic histones attached to fragmented DNA (Fig. 3B) and a reproducibly robust increase in Annexin V staining (Fig. 3C). In MDA-MB-231 cells, we observed a reduction in PARP (most likely through degradation) and found no detectable cleavage product after treatment with 8-NH₂-Ado (Fig. 3A, compare lane 6 with lanes 4, 5, and 7).
and 7). Moreover, in the MDA-MB-231 cells, we detected a reproducibly low increase in apoptosis enrichment factor and Annexin V staining (Fig. 3B and C), suggesting that the death of these cells was through a nonapoptotic pathway.

The pan-caspase inhibitor Z-VAD blocks 8-NH₂-Ado induced MCF-7 death, but not MDA-MB-231 cell death

To further examine the apoptotic cell death of MCF-7 cells, we asked whether the observed cell death could be inhibited with the pan-caspase inhibitor Z-VAD-FMK (Z-VAD). The change in morphology of 8-NH₂-Ado-treated MCF-7 cells (including the floating cell phenotype) was completely blocked when Z-VAD was simultaneously added to the treatment protocol (Fig. 4A). Moreover, simultaneous addition of Z-VAD blocked the cleavage of PARP (Fig. 4B, compare lanes 2 and 3). However, in MDA-MB-231 cells, the simultaneous addition of Z-VAD did not inhibit 8-NH₂-Ado-induced floating cells or PARP reduction (Fig 4A and B). Our data indicate that the 8-NH₂-Ado–induced death of MCF-7 cells was caspase dependent, whereas 8-NH₂-Ado induced death of MDA-MB-231 cells was caspase independent.

The p53 protein is not required for 8-NH₂-Ado–induced MCF-7 cell death

To confirm that the killing ability of 8-NH₂-Ado did not require functional p53, we tested whether the knockdown of p53 by inducible shRNA would influence the apoptosis outcome of MCF-7 cells. We have previously reported the construction of an inducible shRNA p53 knockdown cell line of MCF-7 cells (18). We used this MCF-7.shp53 2120 clone (D11) for this study. Knockdown of p53 was induced by the addition of doxycycline (Fig. 5A). No detectable change in 8-NH₂-Ado–induced PARP cleavage or MCF-7. shp53 clone viability was caused by the reduction in p53 protein (Fig. 5). In combination with the data showing that 8-NH₂-Ado did not activate the wild-type p53 pathway (Fig. 2), these results support the conclusion that 8-NH₂-Ado–induced apoptotic cell death of MCF-7 cells is p53 independent. However, this did not address the mechanism of cell death occurring in the MDA-MB-231 cells. Previous studies have detected that some novel chemotherapeutic drugs induce autophagic cell death of MDA-MB-231 cells (26, 27). We addressed the possibility that 8-NH₂-Ado increased autophagy in MDA-MB-231 cells until the point of cell death.

8-NH₂-Ado increases the autophagy markers in MDA-MB-231 cells but does not require autophagy for cytotoxicity

Although autophagy is initially a cell survival pathway where cytosolic components are encapsulated in double-membrane vesicles, it can also be used as a death pathway if pushed to cannibalize the cell (28, 29). We examined the increase of 2 different autophagic markers in the cells before and after 8-NH₂-Ado treatment. 8-NH₂-Ado treatment reduces glucose consumption and myeloma cells counteract this stress by increasing autophagy (16). It is presumed that autophagy is a prosurvival response of the myeloma cells and not a cell death mechanism. During autophagy, cytoplasmic LC3-I is modified to become LC3-II (30). The processing of LC3-I to LC3-II is visible as a more quickly migrating form on SDS-PAGE. However, the increase in LC3-II can be assessed as a marker for induction of autophagy or inhibition of autophagosome clearance (31, 32). We saw an increase in LC3-II in MDA-MB-231 cells treated with 8-NH₂-Ado and a slight increase in the treated MCF-7 cells (Fig. 6A, compare lanes 1 and 2 with lanes 3 and 4). We also observed an increase in acidic organelles in the treated MDA-MB-231 cells using LysoTracker Red staining (data not shown).

Although the appearance of increased LC3-II indicates that during the cell death there is an associated autophagy response, it cannot be used to claim that 8-NH₂-Ado

Figure 2. In contrast to standard chemotherapeutics, 8-NH₂-Ado does not increase protein levels of either p53 or p21 or transcription of the p53 target gene p21. MCF-7 and MDA-MB-231 cells were treated with 10 μmol/L etoposide (ETOP), 8-NH₂-Ado (8AA), or gemcitabine (GEM) for 24 hours. A, Western blot analysis was used to assess the level of p53, p21, and actin protein from whole-cell lysates of MCF-7 and MDA-MB-231 cells (as indicated). Quantitative real-time PCR was carried out to determine the fold increase of p21 mRNA after drug treatment relative to the DMSO-treated control in MCF-7 cells (B) and MDA-MB-231 cells (C). Normalized to actin and DMSO vehicle cells.
induces autophagic cell death. We therefore asked how increased expression of the autophagy inducing protein, Beclin-1, in MCF-7 cells influenced the 8-NH₂-Ado–induced death pathway. We used MCF7

beclin1 clones that were a generous gift from Beth Levine (19). The increase in Beclin-1 expression in MCF-7 cells did not cause an increase in LC3-II (Fig. 6A, lanes 5 and 6). Importantly, MCF-7 cells have low expression of the autophagy regulating protein, Beclin 1 (19). The introduction of exogenously expressed Beclin 1 into MCF-7 cells promotes autophagy, inhibits cellular proliferation, and blocks the tumorigenesis of these cells in nude mice (19). We treated Beclin 1 overexpressing MCF-7 cells with 8-NH₂-Ado to determine whether this influenced the MCF-7 cell death. Although 8-NH₂-Ado induced PARP cleavage in MCF-7 cells, we found that Beclin 1 overexpression in MCF7

beclin1 clones blocked the 8-NH₂-Ado PARP cleavage (Fig. 6A, compare lanes 3 and 4 with lanes 5 and 6). The MCF7

beclin1 cells were resistant to 8-amino-adenosine–mediated apoptosis as indicated by a lack of floating cells (data not shown) and no evident PARP cleavage (Fig. 6A, lanes 5 and 6). However, even though PARP cleavage was not apparent, 8-NH₂-Ado–treated MCF7

beclin1 cells still exhibited decreased proliferation and reduced viability (seen by MTT and trypan blue exclusion, data not shown). This suggested that autophagy accompanied the 8-NH₂-Ado–induced cell death but did not assist the death or make it more aggressive.

To further examine the influence of autophagy on 8-NH₂-Ado–induced cell death, we observed treated MDA-MB-231 cells during inhibition of autophagy by pharmacologic treatment using 3-MA or by siRNA atg7–mediated knockdown (Fig. 6 B–F). Simultaneous addition of 8-NH₂-Ado with 10 mmol/L of 3-MA did not influence the PARP Western blot pattern (Fig. 6B). The treatment with 3-MA slightly decreased the viability as assessed by the MTT assay and the appearance of floating cells (Fig. 6C and D). We also knocked down atg7 to inhibit autophagy and saw no increase in MTT activity (Fig. 6E and F). The knockdown of atg7 increases MCF-7 cells resistance to photodynamic therapy suggesting that it helps the cells to die (33). However, 8-NH₂-Ado treatment caused a p53-independent cell death that was associated with autophagy but was not assisted by autophagy. This corresponds with the previous data showing 8-NH₂-Ado induces autophagy as a survival response (16). Furthermore, we examined if the death was caused by necrosis by pretreating the MDA-MB-231 cells with a pharmacologic inhibitor called necrostatin 1. This also did not inhibit the 8-NH₂-Ado induced cell death (data not shown).

Discussion

Apoptosis is a well-described cell death pathway but the relationship between autophagy and cell death is controversial (34). The autophagy gene beclin 1 is a haploinsufficient tumor suppressor and increased expression of Beclin 1 in MCF-7 cells promotes autophagy and inhibits the formation of human breast tumors in mouse models (35). Autophagic cell death is sometimes described as programmed cell death II, and has been
suggested as a new target for cancer therapy (36). However, it has been clearly documented that autophagy maintains cellular homeostasis and can have both anti-tumor and tumor-promoting properties (37). Recently, autophagy has been shown to promote ras-driven tumor growth (38). 8-NH$_2$-Ado has previously been associated with a survival program that is initiated because of metabolic dysfunction and this survival program happens alongside the induction of apoptosis (16). In this study, we investigated if p53 function played a part in 8-NH$_2$-Ado–induced cell death. Importantly, we have discovered that 8-NH$_2$-Ado inhibits the growth of multiple breast cancer lines without engaging a functional p53 program. Moreover, we have made the important observation that

![Figure 4](https://example.com/figure4.png)

Figure 4. The pan-caspase inhibitor Z-VAD blocks 8-NH$_2$-Ado–induced PARP cleavage. MCF-7 and MDA-MB-231 cells were treated with 10 μmol/L 8-NH$_2$-Ado for 24 hours in the presence or absence of 50 μmol/L Z-VAD, the pan-caspase inhibitor added to the cell growth media. A, phase contrast microscopy at ×20 magnification showed a reversion of MCF-7 cells, but not MDA-MB-231 cells, to the DMSO vehicle–treated cell morphology (with fewer floating cells) when Z-VAD was added with 8-NH$_2$-Ado. B, Western blot analysis of the protein extract from MCF-7 and MDA-MB-231 cells treated with 8-NH$_2$-Ado and Z-VAD was used to determine if Z-VAD addition reversed the PARP cleavage. Lanes are as indicated in the figure. C, the structure of Z-VAD was obtained from chemicalbook.com.

![Figure 5](https://example.com/figure5.png)

Figure 5. The p53 protein is not required for 8-NH$_2$-Ado–induced cell death. The p53 shRNA 2120 clone D11 was treated with 2 μg/mL doxycycline (+DOX) for 6 days of induced shRNA expression and maximum reduction of p53. Cells with or without p53 knockdown were treated with 10 μmol/L 8-NH$_2$-Ado for 24 hours. A, Western blot analysis was used to assess the level of p53, PARP, and actin protein from whole-cell lysates of the clonal p53 shRNA MCF-7 cells as indicated. B, sensitivity of the cells to 8-NH$_2$-Ado with or without p53 knockdown was assessed by MTT assay of live cells based on mitochondrial dehydrogenase activity and is shown as percentage of DMSO-treated control. C, the structure of DOX was obtained from chemicalbook.com.
8-NH₂-Ado can initiate a caspase-dependent cell death pathway in MCF-7 cells and a caspase-independent cell death pathway in MDA-MB-231 cells. This suggests that 8-NH₂-Ado is a possible therapeutic option for cancers with mutant p53 as well as for certain cancers lacking functional apoptotic pathways.

Surprisingly, 8-NH₂-Ado treatment of different breast cancer cell lines inhibited cancer cell growth by different pathways. 8-NH₂-Ado treatment was unable to activate wild-type p53. This is consistent with 8-NH₂-Ado working through an RNA-mediated signal transduction pathway. 8-NH₂-Ado is known to target the cancer cells through the inhibition of transcription as well as through ATP depletion (39). In our hands, common signal transduction outcomes of 8-NH₂-Ado treatment were the inhibition of p21 transcription and the increase in LC3-II.

However, when we compared the induction of cell death in MCF-7 cells and MDA-MB-231 cells, we observed the engagement of different signal transduction pathways. In MCF-7 cells, 8-NH₂-Ado induced unequivocal apoptosis independently of p53. However, in MDA-MB-231 cells, 8-NH₂-Ado induced a signal transduction pathway toward death that was p53 independent and difficult to define. Inhibiting autophagy or necrosis did not block this death pathway.

Important for future therapeutic consideration is the fact that 8-NH₂-Ado inhibited breast cancer cell proliferation and induced breast cancer cell death without requiring the activation of wild-type p53. The ability of 8-NH₂-Ado to use RNA-dependent cell killing signal transduction pathways is an excellent strategy for treating cancers resistant to the current therapeutic options that...
depend on DNA damage. It is important to determine therapies that can be equally effective on heterogeneous tumors that have lost their functional p53 pathway, their DNA damage pathway, or their apoptotic pathway. The molecular mechanism of action of 8-NH2-Ado fits this paradigm and would increase the chances of eradicating hard to kill cancers. We showed that the 8-NH2-Ado inhibited the proliferation of 4 different breast cancer cell lines better than the commonly used therapeutic gemcitabine and etoposide. Moreover, the cytotoxicity of the drug was not influenced by the presence of wild-type or mutant p53.

MCF-7 cells are an example of cancer cells with deficient autophagy and apoptotic pathways but they were highly sensitive to 8-NH2-Ado. This MCF-7 cancer cell line is well documented for being haploinsufficient for the autophagy gene beclin 1 (19). Interestingly, increased expression of Beclin 1 in MCF-7 beclin1 cells inhibited their apoptosis after 8-NH2-Ado treatment but did not influence the ability of the drug to block MCF-7 beclin1 proliferation. MDA-MB-231 cells undergo autophagic cell death (with an associated increase in Beclin 1 protein) when treated with an indole-3-carbinol metabolite (40); however, we saw that 8-NH2-Ado–induced cell death of MDA-MB-231 cells did not require autophagy. Inhibition of autophagy by 3-MA or knockdown of atg7 did not inhibit the MDA-MB-231 cell death. This indicates that 8-NH2-Ado does not induce cell death by autophagy but rather that autophagy accompanies the cell death.

The efficacy of breast cancer cell killing by 8-NH2-Ado regardless of the p53 status, or the estrogen receptor status, makes this nucleoside analogue an attractive choice for patients who failed previous therapies. Most recently, 8-NH2-Ado was suggested as a therapeutic to inhibit BCR-ABL mRNA and protein levels in imatinib-resistant cancers (39). This combination treatment with imatinib and 8-NH2-Ado inhibits cell growth without increasing Annexin positivity suggesting a mechanism other that apoptosis (39). On the basis of our breast cancer studies with 8-NH2-Ado, we conclude that this nucleoside analog should be investigated further as a therapeutic option for breast cancers that have failed other treatments. The RNA-directed nucleoside analogue 8-chloro-adenosine is currently in phase I clinical trials and as high as 500 nmol/L levels of drug are achieved in plasma (39). This suggests feasibility of using these drugs at the needed dosage in human patients. In addition, previous studies show that 8-NH2-Ado is not cytotoxic to normal lymphocytes (13, 14). When we tested reduction mammoplasty cultures (13, 14). When we tested reduction mammoplasty cells, they were sensitive to 8-NH2-Ado in the culture setting but these cells were very difficult to grow and therefore were not a good indicator of overall cytotoxicity to a patient (Polotskaia and Bargonetti, unpublished data).

The outcomes of 8-chloro-adenosine studies should help to pave the way for the consideration of 8-NH2-Ado as an agent to treat aggressive breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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