8-amino-adenosine Activates p53-Independent Cell Death of Metastatic Breast Cancers

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Running title: 8-amino-adenosine induces p53-independent cell death
Abstract:

8-amino-adenosine (8-NH$_2$-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-NH$_2$-Ado could kill breast cancer cells without activating the p53-pathway. Regardless of the breast cancer sub-type tested, or the p53 status of the cells, 8-NH$_2$-Ado was more cytotoxic than either gemcitabine or etoposide. 8-NH$_2$-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+ T47-D cells that express mutant p53, and the ER-MDA-MB-468 cells or MDA-MB-231 cells that both express mutant p53. 8-NH$_2$-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-NH$_2$-Ado induced MCF-7 cell death. Interestingly 8-NH$_2$-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 3MA treatment, did not block this 8-NH$_2$-Ado mediated cytotoxicity. Importantly 8-NH$_2$-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-NH$_2$-Ado should be considered for the treatment of triple negative breast cancers that are chemotherapy resistant.
Introduction:

Treatment of metastatic and triple negative breast cancer remains a challenge in the clinical setting. Gemcitabine is a nucleoside analogue that is approved by the FDA to be used in combination with paclitaxel for the first-line treatment of patients with metastatic breast cancer after they have received anthracycline chemotherapy. Gemcitabine activates DNA damage pathways that signal to the tumor suppressor p53 (1), and a functional p53 pathway improves gemcitabine cytotoxicity (2). The p53 protein is a tumor suppressor that activates transcription of many downstream target genes and thereby controls cell growth and cell death (3). However, in 70 percent of human cancers the $p53$ gene is found mutated (4). In breast cancer, $p53$ is often found mutated in triple negative and metastatic tumors (5). Therefore identifying nucleoside analogue chemotherapeutic treatments that can kill breast cancers independently of activating the p53-pathway is an important aspect of improving cancer treatments. The identification of drugs that can inhibit the growth of triple negative breast cancers with mutant $p53$ is an understudied area. Therefore we have worked to identify a compound that inhibits breast cancer proliferation regardless of the hormone receptor status or the mutant $p53$ status. We hypothesized that the nucleoside analogue, 8-amino-adenosine (8-NH$_2$-Ado) would inhibit breast cancer cell proliferation independently of the p53 pathway.

In normal cells wild-type p53 is the guardian of the genome, however inactivation of p53 through either mutation or interactions with oncogenic proteins,
plays a large role in tumor promotion (4). Currently breast cancers resistant to chemotherapy are treated with drugs that activate DNA damage pathways (6). DNA damaging drugs activate the p53-pathway and studies indicate that wild-type p53 can be responsible for the vast number of side effects associated with the death of normal cells during DNA damage (7). Recent work with a panel of breast cancer cell lines suggests that combining the DNA-damaging agent gemcitabine with a PARP inhibitor (that blocks DNA repair and inhibits all cell checkpoints) enhances the cytotoxic outcome on cancer cells (8-10). However a chemotherapeutic nucleoside that does not activate the p53 pathway might be more effective.

Unique and promising RNA-directed treatments for myeloma are the nucleoside analogues 8-chloro-adenosine (currently in phase I trials) and 8-NH$_2$-Ado (11, 12). Importantly, 8-NH$_2$-Ado is more cytotoxic to myeloma cancer cells than to normal human lymphocytes (13, 14). Moreover, 8-NH$_2$-Ado is an RNA-directed nucleoside analogue 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 if 8-NH$_2$-Ado can effectively kill breast cancer cells without activating a p53 pathway. We have investigated if p53 function is required for 8-NH$_2$-Ado to inhibit breast cancer cell proliferation. We predicted that 8-NH$_2$-Ado would inhibit the growth of breast cancer cells without requiring functional p53. In
this report we demonstrate that 8-NH$_2$-Ado is more effective than gemcitabine and etoposide at inhibiting the growth of multiple breast cancer subtypes, including triple negative cell lines. Furthermore we demonstrate that 8-NH$_2$-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. The authors did not carry out further authentication. MDA-MB-231 cells (*p53* mutant 280, Arg to Lys), T47D (*p53* mutant 194, Leu to Phe) MDA-MB-468 (*p53* mutant 273, Arg to His) and MCF-7 (*p53* wild type) from American Type Culture Collection (ATCC) were grown in DMEM medium (Mediatech), 10% FBS (Gemini) and 50 units/ml of penicillin-streptomycin (Mediatech) at 5% CO₂ 37°C 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 (DOX) for 6 days. The MCF7.*beclin1* clone (19) was a generous gift from Beth Levine and was grown in the absence of tetracycline to induce Beclin 1 expression for five days.

**Reagents**

Etoposide, Propidium Iodide, Doxycyclin, Trypan Blue solution, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Gemcitabine and 8-NH₂-Ado were provided by Dr. Steve Rosen. The activity of the 8-NH₂-Ado varied in the two batches donated to the laboratory. Therefore the treatments to achieve similar inhibition of growth ranged from 10 µM to 15 µM. 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.
Death Detection ELISA was purchased from Roche (11 544 675 001). Apoptosis detection was performed with ApoScreen Annexin V-FITC Kit (Southern Biotech 10010-02). 3-Methyladenine 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 micro-titration assay to measure mitochondrial dehydrogenases activity (20). Cells were seeded at 1.25 x 10^5 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 is represented as mitochondrial dehydrogenase activity as % of a DMSO vehicle treated control. **Flow Cytometry:** The Annexin V-FITC reagent was used according to manufacturer’s protocol following 16 hours of drug treatment. Cells were detached with trypsin, washed twice with PBS, and re-suspended in the binding buffer provided by the manufacturer with the addition of 10 μl ApoScreen Annexin V-FITC and incubated for 10 min. This step was followed by the addition of 10 μl of propidium iodide and then flow cytometry was performed. 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 1500 x g for 10 min. and the cells were re-suspended in 500 μl of lysis buffer and incubated for 30 min. at room temperature. The cells were re-suspended and spun in an eppendorf tube at 13,000 rpm for 10 min. 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 (qRT-PCR)**

Standard procedures from the manufacturers were used for these assays. RNA was isolated using QIAshredder columns and RNeasy Mini Kit (Qiagen). 5 μg of RNA was used for cDNA synthesis using High Capacity cDNA Archive Kit reagents (Applied Biosystems). 150 ng 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 extraction

Cells were lysed in RIPA buffer (0.1% SDS, 1% NP-40, 0.5% Deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-Cl pH8) with 1 mM PMSF, 8.5 µg/ml Aprotinin and 2 µg/ml Leupeptin and incubated on ice for 20 min. with periodic vortexing. Pellets were then centrifuged at 9,300 x g for 20 min. The supernatants were collected and kept at -80°C for future analysis.

Western blot

50 µg of protein extract were separated by 4-12% SDS-PAGE (Invitrogen) and electro-transferred to nitrocellulose or PVDF membrane. Immunoblotting was done with p53 monoclonal antibody supernatants (pAb421, pAb240 and pAb1801), LC3B (Cell Signaling 2775), PARP (BD Bioscience 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 HRp-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 performed according to manufacturer’s protocol and images were taken on 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% confluency with 100 nM of *atg7* siRNA smart pool or non-targeted siRNA from Dharmacon for 6 hrs using Lipofectamine 2000 (Invitrogen) as per manufacturers 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 15μM 8-NH$_2$-Ado in fresh DMEM with 10% FBS was then carried 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-NH$_2$-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 pre-clinical studies. We used a well-defined set of breast cancer cell lines ranging
from tumorigenic to highly metastatic and compared their sensitivity to 8-NH$_2$-Ado to the two DNA-damaging drugs (gemcitabine and etoposide). The cell lines examined were MCF-7, MDA-MB-231, T47-D and MDA-MB-468. These four cell lines are well described for their $p53$ mutations (5). MCF-7 cells have wild type p53, while MDA-MB-231, T47-D and MDA-MB-468 cells have mutant p53. Additionally, MCF-7 and T47-D cell lines are both estrogen and progesterone receptor positive. We compared the outcome of cells treated with 8-NH$_2$-Ado to that observed with the standard chemotherapeutics gemcitabine and etoposide. Gemcitabine is a DNA-directed nucleoside analogue that causes replication stalling and single stranded breaks in the DNA while etoposide is a topoisomerase inhibitor that causes double stranded breaks to the DNA (1). In contrast, 8-NH$_2$-Ado is an RNA-directed nucleoside analogue (12). Treatments were carried out with relevant concentrations for gemcitabine (8) and etoposide (18) in breast cancer and for 8-NH$_2$-Ado in myeloma (13). Treatment with 10 μM 8-NH$_2$-Ado approached the IC-50 dosage for MCF-7 cells and surpassed that IC-50 value for the three cell lines with mutant p53 (MDA-MB-231, T47-D and MDA-MB-468 cells) (Figure 1). The MTT proliferation assay monitors a cell population’s response to external factors including cell growth and cell death. In all instances with 8-NH$_2$-Ado the cells detached from the plate (most likely indicating cell death, see Figure 4A for representative images). Moreover, in all cases 8-NH$_2$-Ado treatment was far more cytotoxic than either etoposide or gemcitabine (Figure 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 to the MDA-MB-231 cell line. These two 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), while 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 targeted destruction by its negative regulator Mdm2 (23). Following DNA damage the levels of wild-type p53 protein increase due to post-translational 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 following etoposide and gemcitabine treatment (Figure 2A, lanes 2 & 4); however no wild-type p53 protein increase was detected following 8-NH₂-Ado treatment (Figure 2A, compare lanes 1 & 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 (Figure 2A, lanes 5 through 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-NH$_2$-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 (Figure 2). This indicated that 8-NH$_2$-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 (Figure 2). Gemcitabine treatment of both cell lines caused a robust increase in $p21$ transcript without a significant change in p21 protein (Figure 2). In support of our p53-independent hypothesis, no signaling to p53 was evident in 8-NH$_2$-Ado treated cells, while 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-NH$_2$-Ado induces significant apoptotic death of MCF-7 cells.**

To determine the signal transduction pathways activated following treatment of the cells with 8-NH$_2$-Ado we examined cell death markers associated with apoptosis. We assessed the cleavage of poly-ADP-ribose polymerase (PARP), the enrichment of cytoplasmic histone-associated DNA fragments, and Annexin V staining (Figure 3). We detected PARP cleavage in
MCF-7 cells treated with 8-NH₂-Ado (Figure 3A, compare lane 3 to lanes 1, 2, and 4), as well as an increase in apoptosis associated enrichment factor, which detects cytoplasmic histones attached to fragmented DNA (Figure 3B) and a reproducibly robust increase in Annexin V staining (Figure 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 (Figure 3A, compare lane 6 to lanes 4, 5, and 7). Moreover, in the MDA-MB-231 cells, we detected a reproducibly low increase in apoptosis enrichment factor and Annexin V staining (Figures 3B and 3C), suggesting that the death of these cells was through a non-apoptotic 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 if 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 (Figure 4A). Moreover, simultaneous addition of Z-VAD blocked the cleavage of PARP (Figure 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 (Figures 4A and 4B). Our data indicate that the 8-NH₂-Ado induced
death of MCF-7 cells was caspase dependent while 8-NH$_2$-Ado induced death of MDA-MB-231 cells was caspase-independent.

**The p53 protein is not required for 8-NH$_2$-Ado induced MCF-7 cell death.**

To confirm that the killing ability of 8-NH$_2$-Ado did not require functional p53 we tested if 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 (Figure 5A). No detectable change in 8-NH$_2$-Ado induced PARP cleavage, or MCF-7.shp53 clone viability was caused by the reduction in p53 protein (Figure 5). In combination with the data demonstrating that 8-NH$_2$-Ado did not activate the wild-type p53-pathway (Figure 2), these results support the conclusion that 8-NH$_2$-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-NH$_2$-Ado increased autophagy in MDA-MB-231 cells until the point of cell death.
8-NH$_2$-Ado increases the autophagy markers in MDA-MB-231 cells but does not require autophagy for cytotoxicity.

While 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 two different autophagic markers in the cells before and after 8-NH$_2$-Ado treatment. 8-NH$_2$-Ado treatment reduces glucose consumption and myeloma cells counteract this stress by increasing autophagy (16). It is presumed that autophagy is a pro-survival 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 an 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-NH$_2$-Ado and a slight increase in LC3-II in the treated MCF-7 cells (Figure 6A, compare lanes 1 and 2 to 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).

While 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-NH$_2$-Ado induces autophagic cell death. We therefore asked how increased expression of the autophagy inducing protein, Beclin-1, in MCF-7 cells influenced the 8-NH$_2$-Ado induced death pathway. We used MCF7.beclin1 clones which
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 (Figure 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 over-expressing MCF-7 cells with 8-NH₂-Ado to determine if this influenced the MCF-7 cell death. While 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 (Figure 6A, compare lanes 3 and 4 to 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 (Figure 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 pharmacological treatment using 3-Methyladenine (3MA) or by siRNA atg7 mediated knockdown (Figures 6B-6F). Simultaneous addition of 8-NH₂-Ado with 10 mM of 3MA did not influence the PARP western blot pattern (Figure 6B). The treatment with 3MA slightly decreased the viability as assessed by the MTT
assay and the appearance of floating cells (Figures 6C and 6D). We also knocked down atg7 to inhibit autophagy and saw no increase in MTT activity (Figures 6E and 6F). 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 pharmacological 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 antitumor 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 due to 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 8-NH$_2$-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-NH$_2$-Ado is a possible therapeutic option for cancers with mutant p53 as well as for certain cancers lacking functional apoptotic pathways.

Surprisingly 8-NH$_2$-Ado treatment of different breast cancer cell lines inhibited cancer cell growth by different pathways. 8-NH$_2$-Ado treatment was unable to activate wild-type p53. This is consistent with 8-NH$_2$-Ado working through an RNA-mediated signal transduction pathway. 8-NH$_2$-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-NH$_2$-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-NH$_2$-Ado induced unequivocal apoptosis independently of p53. However in MDA-MB-231 cells 8-NH$_2$-Ado induced a signal transduction pathway towards 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 therapeutics 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 demonstrated that the 8-NH₂-Ado inhibited the proliferation of four different breast cancer cell lines better than the commonly used therapeutics 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 following 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-NH$_2$-Ado induced cell death of MDA-MB-231 cells did not require autophagy. Inhibition of autophagy by 3MA or knockdown of atg7 did not inhibit the MDA-MB-231 cell death. This indicates that 8-NH$_2$-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-NH$_2$-Ado regardless of the p53 status, or the estrogen receptor status, makes this nucleoside analogue an attractive choice for patients who fail prior therapies. Most recently 8-NH$_2$-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-NH$_2$-Ado inhibits cell growth without increasing annexin positivity suggesting a mechanism other that apoptosis (39). Based on our breast cancer studies with 8-NH$_2$-Ado we conclude that this nucleoside analogue 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 nM levels of drug are achieved in plasma (39). This suggests feasibility of using these drugs at the needed dosage in human patients. Additionally previous studies show that 8-NH$_2$-Ado is not cytotoxic to normal lymphocytes (13, 14). When we tested reduction mammoplasty cells they were sensitive to 8-NH$_2$-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-NH$_2$-Ado as an agent to treat aggressive breast cancer.

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References

**Figure 1:** 8-NH$_2$-Ado is more cytotoxic than are standard chemotherapeutic drugs to breast cancer cell lines with either wild-type or mutant p53. Breast cancer cell lines with either wild-type p53 (Panel A: MCF-7) or oncogenic mutant p53 (Panel B: MDA-MB-231, Panel C: T47-D, and Panel D: MDA-MB-468) were incubated with etoposide (ETOP), 8-NH$_2$-Ado (8AA), or gemcitabine (GEM); Panel E shows structures of compounds used (obtained from chemicalbook.com). All drug treatments were carried out at a concentration of 10 μM for 24 hrs. Sensitivity of the cells to the three drugs was assessed by MTT assay based on mitochondrial dehydrogenase activity. Standard error bars represent three independent experiments. Results were normalized to the DMSO treatment.

**Figure 2:** In contrast to standard chemotherapeutics, 8-NH$_2$-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 μM etoposide (ETOP), 8-NH$_2$-Ado (8AA), or gemcitabine (GEM), for 24 hrs. 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-MB231 cells (as indicated). Quantitative real-time PCR (qRT-PCR) was carried out to determine the fold increase of p21 mRNA following drug treatment relative to the DMSO treated control in B) MCF-7 cells and C) MDA-MB-231 cells. Normalized to actin and DMSO vehicle cells.
Figure 3: 8-NH$_2$-Ado induces robust apoptotic cell death in MCF-7, but not in MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were treated with 10 μM etoposide (ETOP), 8-NH$_2$-Ado (8AA), or gemcitabine (GEM), for 24 hrs. A) western blot analysis was used to assess PARP cleavage and actin protein from whole cell lysates of MCF-7 and MDA-MB231 cells (as indicated). B) An enzyme-linked immuno-absorbent assay (ELISA) for the quantitative, in vitro, determination of cytoplasmic histone-associated DNA fragments was carried out. The values were scored as enrichment factor for the comparison of absorbance from the drug treatment of MCF-7 and MBA-MB-231 cells versus the control DMSO vehicle treated cells. Increased enrichment factor values in the histogram serve as an indication of increased apoptosis. C) To assess for early stage apoptosis, MCF-7 and MDA-MB-231 cells were treated with 10 μM 8-NH$_2$-Ado for 16 hours, scraped from the plate for harvesting and stained with Annexin V and propidium iodide. The bottom right quadrant shows the early apoptotic cells that are Annexin V positive. In MCF-7 cells (shown on the bottom) there were 4.1% early Annexin V positive cells before treatment and 25.2% positive cells after treatment. In MDA-MB-231 cells (shown top) there were 1.7% early Annexin V positive cells before treatment and 6.4% after treatment.

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 μM 8-NH$_2$-Ado for 24 hrs in the presence or absence of 50 μM Z-VAD, the pan-caspase
inhibitor added to the cell growth media. A) Phase contrast microscopy at 20X magnification demonstrated 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-NH₂-Ado. B) Western blot analysis of the protein extract from MCF-7 and MDA-MB-231 cells treated with 8-NH₂-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: The p53 protein is not required for 8-NH₂-Ado induced MCF-7 cell death. The MCF-7 p53 shRNA 2120 clone D11 was treated with 2 µg/ml doxycycline (+ DOX) for six days of induced shRNA expression and maximum reduction of p53. Cells with or without p53 knockdown were treated with 10 µM 8-NH₂-Ado for 24 hrs. 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-NH₂-Ado with or without p53 knockdown was assessed by MTT assay of live cells based on mitochondrial dehydrogenase activity and is shown % of DMSO treated control. C) The structure of DOX was obtained from chemicalbook.com.

Figure 6: 8-NH₂-Ado increases the autophagy markers in MDA-MB-231 cells but does not require autophagy for cytotoxicity MCF-7 and MDA-MB-231
cells were treated with 15 μM 8-NH₂-Ado for 24 hrs. A) Western blot analysis was used to compare cell extracts from MDA-MB-231, MCF-7, or MCF7. beclin 1 cells treated with 15 μM 8-NH₂-Ado for 24 hrs for PARP cleavage, Beclin 1, and LC3 and actin. B) MDA-MB-231 cells were treated with the autophagy inhibitor 3MA along with the addition of 8-NH₂-Ado and PARP was examined by western blot. C) The MTT assay was use to determine MDA-MB-231 mitochondrial activity following 8-NH₂-Ado with or without the addition of 3MA. D) Phase contrast microscopy at 20X magnification demonstrated no reversion of 8-NH₂-Ado induced death of MDA-MB-231 cells with prior treatment with 3MA. E) Western blot analysis was used to assess ATG7 and PARP levels in non-targeted siRNA or atg7 siRNA with and without 8-NH₂-Ado treatment in MDA-MB-231 cells. F) The MTT assay was used to determine MDA-MB-231 mitochondrial activity following 8-NH₂-Ado with or without the addition of non-targeted siRNA or atg7 siRNA. G) The structure of 3MA was obtained from chemicalbook.com.
FIGURE 1

A. MCF-7

B. MDA-MB-231

C. T47-D

D. MDA-MB-468

E. Structures of Etoposide, RAA, and GEM.
FIGURE 2

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FIGURE 3

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Molecular Cancer Therapeutics

8-amino-adenosine Activates p53-Independent Cell Death of Metastatic Breast Cancers

Alla Polotskaia, Sandy Hoffman, Nancy L Krett, et al.

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