Published in final edited form as:

Leuk Lymphoma. 2012 October ; 53(10): 2024–2032. doi:10.3109/10428194.2012.678003.

Multifaceted actions of 8-amino-adenosine kill BCR-ABL positive cells

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Abstract

Survival of chronic myelogenous leukemia (CML) cells is dependent on the BCR-ABL kinase, the activity of which is contingent on the level of BCR-ABL protein and the availability of ATP. We hypothesized that 8-aminoadenosine (8-amino-Ado) mediated reduction in cellular ATP level and inhibition of mRNA synthesis leading to a decrease in protein level would result in a multifaceted targeting of BCR-ABL

Using K562 cells, we demonstrate that there was a dose and time-dependent increase in 8-amino-ATP, accompanied by >95% decline in the endogenous ATP pool. In parallel, 8-amino-Ado inhibited RNA synthesis, and resulted in depletion of *BCR-ABL* transcript. Consistent with this, BCR-ABL and ABL protein levels were also decreased. These effects were associated with initiation of cell death as visualized by PARP cleavage, decreased clonogenicity, and greater than additive interaction with imatinib. In imatinib-sensitive and resistant KBM5 cells, 8-amino-Ado treatment augmented imatinib effect on growth inhibition.

Keywords

ATP analog; BCR-ABL; apoptosis; transcription; 8-amino-adenosine

Introduction

Chronic myelogenous leukemia (CML) is a pluripotent hematopoietic stem cell disorder. A unique but common feature of CML is the Philadelphia chromosome (Ph) [1–3]. The Ph chromosome is generated by the reciprocal translocation of chromosome 9 and chromosome 22. At the breakpoints on these chromosomes are the *C-ABL* and the *BCR* gene,

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respectively. The molecular consequence of the 9;22 translocation is the *BCR-ABL* oncogene where the *CABL* gene is under the transcription control of the *bcr* locus [4]. In CML this oncogene encodes a 210 kDa BCR-ABL protein with dysregulated tyrosine kinase activity [1]. The constitutively active BCR-ABL kinase interacts with many proteins and results in the activation of many signaling pathways involved in proliferation and resistance to apoptosis [5–9].

Clinical and laboratory investigations have demonstrated that survival of CML cells is dependent on the BCR-ABL fusion protein [10,11]. The activity of BCR-ABL is dependent on the formation and stability of *BCR-ABL* transcript [12,13], the level of BCR-ABL protein [14,15], and phosphorylation of this protein which is dependent on the availability of ATP, which serves as a phosphate donor [16]. Since the BCR-ABL kinase acts as a survival factor for CML cells, disrupting this mechanism should lead to cell death.

The proof-of-concept studies for inhibition of BCR-ABL kinase leading to death of Ph+ CML cell lines have identified imatinib mesylate (STI-571, Gleevec). Clinical success of this agent has established the utilization of this strategy for patients with early phase chronic CML disease [17]. However, for accelerated phase and blast crisis CML, imatinib mesylate has limited activity [18]. In addition, several mechanisms of resistance to this kinase inhibitor have been identified which include increased expression of *BCR-ABL* mRNA and protein [19–21], mutation in BCR-ABL kinase [22–24] and presence of additional survival pathways [25,26]. These observations emphasize the need for additional strategies for the treatment of CML.

Previous studies in our group using human leukemia, lymphoma and myeloma cell lines have demonstrated that adenosine (Ado) analogs such as 8-chloro-Ado and 8-amino-Ado (Figure 1) accumulate as their respective triphosphates, which is associated with a decrease in endogenous ATP pool [27–30]. Mechanistic investigations demonstrated that 8-Cl-ATP incorporated into different RNA species with maximum insertion in mRNA leading to an inhibition of mRNA synthesis [31].

Based on these observations, we hypothesized that the reduction in cellular ATP level by 8-amino-Ado and inhibition of mRNA synthesis leading to a decrease in the levels of *BCR-ABL* transcripts and protein would result in a multifaceted targeting of BCR-ABL. The present investigation was conducted to test this hypothesis and the consequences of this targeting of BCR-ABL on the survival of Ph+ cells.

Materials and Methods

Cell line

K562 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (GIBCO, Grand Island, NY) in incubator at 37°C in 5% CO₂. KBM5[32] and imatinib-resistant KBM5 cells (KBM5/STI)[33] were kindly provided by Dr. M. Andreeff (Department of Leukemia, MD Anderson Cancer Center). KBM5/STI were derived through chronic exposure to imatinib and acquisition of T315I BCR-ABL point

mutation. Cells were routinely tested for *Mycoplasma* using a commercially available kit and following the manufacturer's procedure (Mycotect Kit, Invitrogen, Grand Island, NY).

Materials

8-amino-Ado was obtained from R.I. Chemicals, Inc. (Orange, CA). 8-amino-ATP was custom synthesized by Biolog Life Sciences institute (Bremen, Germany). All other chemicals were reagent grade.

Accumulation of 8-amino-ATP and decline of ATP

Exponentially growing cells were incubated with 8-amino-Ado for the indicated concentrations and times. Nucleotides were extracted from the cell pellet using the perchloric acid method [34] and separated by high-pressure liquid chromatography (HPLC) as described before [30]. The absorbance of elements from anion exchange were monitored at a wavelength of 254 nm. The intracellular concentrations of nucleotides and 8-amino-ATP were calculated from a given number of cells of a determined mean cell volume (Coulter channelizer, Coulter Electronics, Inc.). This calculation assumes the nucleotides are uniformly distributed in the total cell volume.

Effect of 8-amino-Ado on RNA synthesis

Cells were incubated with varying concentrations of 8-amino-Ado and for various times. [³H]uridine (1 µCi/ml) (Moravsek Biochemicals, Brea, CA) was added to each aliquot 30 minutes before removal from culture, and the aliquot was incubated for an additional 30 minutes. The cells were collected on multiscreen GV filters under vacuum (multiscreen assay system, Millipore Corp., Bedford, MA) and washed two times each with ice-cold PBS and ice-cold perchloric acid, and finally with 70% ethanol. The amount of RNA synthesis was determined by measuring the amount of radioactivity retained in the acid-insoluble material on the filters using scintillation counting. These data were expressed as a percentage of control (untreated) value of cells.

BCR-ABL gene expression using real-time RT PCR

RNA was extracted from K562 cells at various time points after 8-amino-Ado treatment using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's procedure. The RNA was quantitated with the RiboGreen RNA Quantification kit (Molecular Probes, Eugene, OR). The number of *BCR-ABL* transcripts was determined by real-time reverse transcription polymerase chain reaction (RT-PCR) using an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA). Equivalent amounts of RNA (50 ng) were assayed using the TaqMan One-Step RT-PCR kit and MultiScribe reverse transcriptase (Applied Biosystems) as described by the manufacturer. The *BCR-ABL* specific primers (Sigma Genosys, Woodlands, TX) and probe (Applied Biosystems) were as follows: bcr-b2 primer ACAGCATTCCGCTGACCAT; abl-a2 primer CGAGCGGCTTCACTCAGA; probe 6FAM-CCTGAGGCTCAAAGTCAGATGCTACTGG-TAMRA. Using RNA standards described above, the levels of Bcr-Abl expression were quantitated. For normalization purposes, 18S rRNA levels were also assayed using commercially available primers and probe (Applied

Biosystems). Changes in the expression of *BCR-ABL* transcript were quantitated by using the C_t method.

Western blot analysis

Cells were pelleted at various times during incubation with 8-amino-Ado and were lysed as previously described.[35] Equal amounts of protein were loaded onto an 8% or 4–12% gel and subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF-membranes (Millipore, Bedford, MA) or nitrocellulose (Bio-Rad, Hercules, CA). Membranes were blocked with 5% milk in PBS-T buffer (PBS buffer and 0.1% Tween 20) or Odyssey blocking buffer (LI-COR Inc., Lincoln, NE) as previously described.[35], then incubated with the following antibodies: c-Abl (Santa Cruz Biotechnology, Santa Cruz, CA), PARP (BD Pharmingen, San Diego, CA), β -actin (Sigma-Aldrich, St. Louis, MO). Membranes were washed three times with PBS-T and then incubated for one hour either with secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ) or infrared-label secondary antibodies (LI-COR). After washing the membranes again three times with PBS-T, protein bands were visualized either by the SuperSignal West Dura enhanced chemiluminescence (ECL) Western blotting detection system (Pierce Biotechnology, Rockford, IL) or using a LI-COR Odyssey Infrared Imager as described.[35]. Protein levels were quantified by densitometry or infrared imager and normalized to β -actin.

Clonogenic assay

K562 cells were incubated with various concentrations of 8-amino-Ado for 24 hours. The cells were washed twice in PBS and resuspended in fresh RPMI 1640 medium supplemented with 10% FBS. 300 or 3000 cells were suspended in 2 mL medium containing 0.85% nutrient agar (Becton Dickinson, Sparks, MD), Iscove's medium [1 mL of medium containing 17.67g Iscove's Modified Dulbecco's medium (Gibco Invitrogen, Carlsbad, CA), 1.51 g NaHCO_3 , and 5% glutamine (Invitrogen) in 500 mL deionized H_2O], and 20% FBS in 6-well culture plates. After 10–14 days of incubation, colonies (defined as groups of >50 cells) were counted microscopically in each well.

Combination treatment

Exponentially growing cultures of K562, KBM5 or KBM5/STI cells were incubated for 24 or 48 hours with 100 nM 8-amino-Ado alone, 300 nM imatinib alone, or both agents in combination. The cell concentration was measured using a Coulter channelizer and compared with untreated control cells.

Cord Blood CD34 positive cells—Cord Blood units (CB) were provided under University of Texas MD Anderson Cancer Center IRB-approved protocols. CB mononuclear cells (CB MNCs) were prepared by centrifugation on ficoll layers (Histopaque 1077, Sigma) at $400 \times g$ 20 minutes then collecting the interface cells. Washed CB MNCs were incubated in Clinimacs buffer (PBS EDTA buffer, Miltenyi Biotec) containing CD34 microbeads (CD34 reagent, Miltenyi Biotec), 0.5% Human Serum Albumin (HSA, Baxter Healthcare) and an FC blocker (Gammagard, Baxter Healthcare) for 30 minutes on ice. After incubation,

CB CD34 positive cells were selected by magnetic activated cell sorting (MACS, LS column, Miltenyi Biotec) according to the manufacturer's instructions.

Apoptosis assay—Cells (1×10^6) were treated with DMSO alone, 100 nM 8-amino-Ado alone, 300 nM imatinib alone, or both agents in combination for 48 h. Cells were washed, then resuspended in 100 μ L of Annexin binding buffer (Roche, Indianapolis, IN), mixed with 5 μ L of annexin-FITC solution (BD Pharmingen) and 5 μ L of propidium iodide (PI; 50 μ g/mL, Sigma-Aldrich) solution. At least 10,000 cells were measured per sample using a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 (GraphPad Software Inc.) and *p*-values were obtained using the paired one-tailed Student's *t*-test.

Results

Accumulation of 8-amino-ATP and effect on cellular ATP pool

Exponentially growing K562 cells were incubated for 4 hours with 0.1, 1, 10, and 30 μ M 8-amino-Ado. 8-amino-Ado accumulates as 8-amino ATP in a dose-dependent manner (Figure 2). At 10 μ M 8-amino-Ado, 8-amino-ATP concentration is $\sim$ 5 mM, which is higher than the endogenous ATP pool in untreated cells which is $\sim$ 4 mM. 8-amino-ATP accumulation appears to be saturated by 10 μ M 8-amino-Ado.

In parallel to accumulation of analog triphosphates, there was a decline in endogenous level of ATP pool. The starting level of intracellular ATP pool is between 4 and 4.5 mM. There was a moderate effect of 1 μ M 8-amino-Ado on the ATP pool, however majority of the ATP pool disappeared at 10 and 30 μ M 8-amino-Ado and only 15% and 5% of ATP remained at these concentrations, respectively (Figure 2).

To determine the kinetics of 8-amino-Ado metabolism in K562 cells, exponentially growing cells were incubated with 0.1, 1, 10, and 30 μ M 8-amino-Ado for 24 hours and nucleotide concentrations were assayed every 2 hours for 12 hours and at 24 hours (Figure 3). There was a time-dependent accumulation of 8-amino-ATP, which reached 125–150 μ M after 12 – 24 hour treatment with 0.1 μ M 8-amino-Ado (Figure 3A). With 1 μ M 8-amino-Ado treatment, 8-amino-ATP further accumulated to between 1200–2700 μ M after 12 and 24 hours. 8-amino-ATP accumulated to over 4 mM with both 10 and 30 μ M 8-amino-Ado, and the concentration of 8-amino-ATP achieved was higher than the cellular ATP pool in untreated K562 cells ($\sim$ 4 mM). The accumulation of 8-amino-ATP reached a plateau by 10 hours and the levels remained constant through 24 hours (Figure 3A).

Similar to analog triphosphate accumulation, the depletion of the cellular ATP pool was also time-dependent. With both 10 and 30 μ M 8-amino-Ado, ATP levels rapidly decreased. The maximum decline in ATP pool was achieved at 10 hours, with the greatest decrease seen with 10 μ M (15% of control) and 30 μ M (5% of control) 8-amino-Ado (Figure 3B).

8-amino-Ado inhibition of total RNA synthesis

Due to a decline in the ATP pool and accumulation of analog triphosphate and its incorporation into RNA [29,31] we hypothesized that RNA synthesis would be affected by 8-amino-Ado. In order to evaluate the effect of 8-amino-ATP on global RNA synthesis in K562 cells, radioactive uridine incorporation into RNA was measured. At 1, 10, and 30 μ M 8-amino-Ado, there was a dose-dependent decrease in uridine incorporation (Figure 4). Kinetically the RNA synthesis decreased during the first 8 to 10 hours of incubation with 8-amino-Ado and then remained constant through 24 hours. At 24 hours, RNA synthesis inhibition was decreased by 20% at 1 μ M and by 76% at 10 μ M. The maximal effect was seen with 30 μ M 8-amino-Ado; compared to untreated cells RNA synthesis was inhibited by more than 95%.

8-amino-Ado induces a decline in *BCR-ABL* transcript in K562 cells

Due to the inhibition of total RNA synthesis induced by 8-amino-Ado treatment, endogenous short-lived mRNA transcripts such as *BCR-ABL* would get depleted over time. To quantitate the endogenous level of *BCR-ABL* mRNA and its decline after treatment with 8-amino-Ado, RNA from untreated and 8-amino-Ado-treated cells was extracted and analyzed by real-time RT-PCR assay. Since 10 and 30 μ M 8-amino-Ado resulted in greater than 80% inhibition of total RNA synthesis, relative transcript levels were measured with these concentrations. Under both these conditions, there was a steady decline in the level of *BCR-ABL* mRNA, which was detectable throughout the within 6 hours (Figure 5). By 24 hours, the endogenous level of *BCR-ABL* mRNA was reduced by 60 and 80% at 10 and 30 μ M exogenous drug, respectively.

Decrease in total *BCR-ABL* protein levels with 8-amino-Ado

To determine if the reduction in *BCR-ABL* transcript number translates into a decrease in *BCR-ABL* protein, we performed immunoblot analysis using cell lysates from K562 cells treated with 10 and 30 μ M 8-amino-Ado. We focused on these concentrations since treatment with 10 and 30 μ M 8-amino-Ado decreased the endogenous level of *BCR-ABL* transcripts. Proteins were detected using a specific anti-abl antibody and normalized using β -actin as a loading control (Figure 6). Following treatment with 10 μ M 8-amino-Ado, qualitative data suggested that there was a decline in both *BCR-ABL* and Abl total protein levels. The average protein levels from four independent experiments were measured quantitatively via densitometry, and a decline in *BCR-ABL* protein level was detected after 4 hours (Table 1), which was statistically significant ($p = 0.048$). The decrease in the amount of *BCR-ABL* protein was maintained up to 12 hours, however the results were statistically significant only until 10 hours. There was also a reduction in normalized Abl protein levels detected after 4 hours of treatment, which was statistically significant ($p = 0.026$), with further reduction after 6–10 hours of treatment. In contrast to *BCR-ABL*, Abl protein levels did not appear to return to control levels at 24 hours, however these results were not statistically significant.

Cytotoxicity of 8-amino-Ado

To determine if the decline in BCR-ABL protein was associated with cell death, PARP cleavage, a hallmark of apoptosis, was analyzed in K562 cells treated with 10 μ M 8-amino-Ado. The 85 kDa cleavage product appeared at 4 hours while the 116 kDa full protein declined with incubation with 10 μ M 8-amino-Ado (Figure 7A). Therefore K562 cells exposed to 8-amino-Ado underwent cell death via apoptotic pathways.

To further establish the cytotoxic effect of this compound, we performed colony formation assays on K562 cells incubated with various concentrations of 8-amino-Ado for 24 hours. There was a dose-dependent effect on the inhibition of the clonogenic potential of K562 cells with an IC_{50} of 0.3 μ M (Figure 7B). Treatment with 3 μ M 8-amino-Ado resulted in greater than 95% inhibition of the clonogenicity of K562 cells.

Combination of 8-amino-Ado with imatinib

Since 8-amino-Ado treatment resulted in decreased BCR-ABL protein levels and cellular ATP pool, we evaluated whether 8-amino-Ado enhances imatinib effects. Cells were treated with either 100 nM 8-amino-Ado alone, 300 nM imatinib alone, or simultaneous combination of both agents. As shown in Figure 8A, there was an average of 33% and 45% decline in cell growth in K562 cells following single agent 8-amino-Ado or imatinib treatment, respectively. When used in combination, 8-amino-Ado and imatinib resulted in further inhibition of cell growth, and there was a 71% decrease compared with control. Based on fraction analysis, expected cell death with both agents is 63%, however 71% decline was observed suggesting a greater than additive interaction.

We conducted experiments in imatinib-sensitive (KBM5) and resistant (KBM5/STI) cell lines to evaluate whether 8-amino-Ado would have an effect in additional cell lines as well as imatinib-resistant CML cell line. Cells were incubated with either drug alone or in combination, and cell growth was measured after 24 and 48 hours. There was little or no change in cell growth (<5%) with 8-amino-Ado treatment as a single agent in either cell line after 24 or 48 hours (Figure 8B), however 8-amino-Ado did appear to augment imatinib activity in KBM5 cells. The average decrease in cell growth in KBM5 cells after 48 hours were: 8-amino-Ado alone (4%), imatinib alone (32%) and in combination (47%), which is greater than the expected additive amount (35%). In parallel, there was an increase in PARP cleavage in the combination treatment compared with single agent alone after 24 h (Supplemental Figure 1). Interestingly in the imatinib-resistant line, KBM5/STI, 8-amino-Ado also appeared to affect imatinib activity, albeit at a lower level. After 48 hours, there was no effect on KBM5/STI from 8-amino-Ado alone, however the averaged decrease in cell growth were; imatinib alone (8%) and in combination (15%); expected additive effect is 8%. While modest, our data indicate that the effects of 8-amino-Ado and imatinib in combination are greater than additive in imatinib-resistant KBM5/STI cells.

To evaluate potential cytotoxicity of this combination on normal cells, CD34+ cells were isolated from cord blood and evaluated with the same concentration of drug as in the previous experiment. In cells obtained from three different blood samples, there was little induction of cell death as measured by annexin-V staining in the CD34+ or CD34- fractions

that were treated with either drug alone or in combination (Figure 8C), thus the combination strategy of 8-amino-Ado and imatinib may be therapeutically appealing.

Discussion

The success of imatinib in the clinic has been limited in later phases of CML with acquired drug resistance [36] and next generation kinase inhibitors such as nilotinib [37] and dasatinib [38] were developed. While these agents have been effective in early stage CML, amplification of the *BCR-ABL* transcript and/or protein levels and mutation of the oncogene ultimately result in resistance to treatment, underscoring the need for other therapies that target the BCR-ABL kinase. The survival of the BCR-ABL positive CML cells depends on the BCR-ABL kinase activity, which is dependent on *BCR-ABL* mRNA transcript levels, protein translation, phosphorylation, and presence of phosphate donor (i.e. ATP). Disruption of any of these factors should translate in inhibition of Ph⁺ cell survival, and each one of these strategies has been either postulated, tested, or validated in the clinic.

To reduce *BCR-ABL* mRNA levels, strategies have included targeting the *BCR-ABL* transcription using antisense oligonucleotides [39–41] or via cleavage of *BCR-ABL* transcript [42] by ribozymes. To reduce BCR-ABL protein levels, agents such as homoharringtonin, also known as omacetaxine [43,44] and arsenic trioxide [45] have been utilized. Although several of these studies demonstrated that these agents alone induced cell death or enhanced cell death response in combination with imatinib, it was also observed that due to the long half-life of BCR-ABL protein that these approaches were not optimal [39,41,46]. To circumvent this challenge, a combination targeting both *BCR-ABL* mRNA and protein has also been suggested [43], and preliminary data during a clinical trial using combination of homoharringtonine with imatinib demonstrated effectiveness in patients with advanced and/or imatinib-resistant CML.[47]

The Ado analog used in the present study combines several of these strategies by targeting both transcription and ATP depletion. Previous work in our group has shown that the adenosine analogs such as 8-chloro- and 8-amino-Ado cause a time-dependent decrease in RNA synthesis in human multiple myeloma [29,30,48,49] and mantle cell lymphoma cell lines [28]. 8-amino-Ado effect was maximal on RNA polymerase II and mRNA synthesis, which may be due to incorporation followed by premature chain termination [31], potentially altered structural conformation of the analog in RNA possibly prohibiting further polymerization [50], or polyadenylation inhibition by the active metabolites 8-Cl-ATP and 8-amino-ATP [50,51]. ATP depletion may be due to modulation of ATP synthase activity. [52]

The present study demonstrates that 8-amino-Ado is a potent inhibitor of RNA synthesis in K562 cells (Figure 4). The mRNA of *ABL* and *BCR-ABL* contain AU-rich elements (ARE) in the 3' untranslated region [53], which contribute to the degradation of short-lived mRNA. Accordingly, the half-life of the *BCR-ABL* mRNA is approximately 2 hours [54]., therefore inhibition of RNA synthesis would impact steady-state levels of this short-lived transcript. Many regulated processes, such as cell growth and signal transduction, are mediated by transient RNAs and stabilization of these RNAs can lead to disease caused by prolonged

response [55]. Hence a tight control for the degradation of these transcripts is necessary for normal biological and physiological processes of a cell. A general transcription inhibitor such as flavopiridol or roscovitine can selectively target tumor cells because some cancer cells are more dependent on inducible genes, such as genes involved in cell cycle control and apoptosis, which usually have labile RNAs [56]. The cytotoxic effects of 8-amino-Ado in CML cells may be due to the destabilizing *BCR-ABL* transcript levels, which are necessary for the continued survival of Bcr-Abl positive cells. Consequently, the decline in the BCR-ABL and ABL proteins (Figure 6 and Table 1) may be an indirect effect due to decreased transcript levels of these two genes. Since the half-lives of these proteins are 24 to 48 hours [16], the effect of 8-amino-Ado on protein levels was not as dramatic as on mRNA.

In addition to inducing a decline in the *BCR-ABL* mRNA and protein levels, there is an accumulation of 8-amino-ATP (Figure 3A) and a reduction in the endogenous ATP levels in K562 cells (Figure 3B). The decline in cellular ATP pools acts to further inhibit RNA synthesis because ATP is both a precursor for RNA synthesis as well as a necessary energy co-factor. In parallel, since imatinib acts as a competitive inhibitor of the ATP binding site of BCR-ABL [57], reducing the levels of its natural substrate, ATP, may compromise BCR-ABL kinase activity. Concentrations of 0.5 mM ATP has been used for BCR-ABL in vitro kinase assays, and we demonstrate here that treatment with 10 μ M 8-amino-Ado decreases intracellular ATP concentrations from 4 mM to less than 0.5 mM (Figure 3B). Thus 10 and 30 μ M 8-amino-Ado could be sufficient to inhibit constitutive BCR-ABL kinase activity in K562 cells due ATP depletion, which could also potentiate imatinib activity. These combined effects of targeting both RNA synthesis and depletion of ATP may be responsible for the cytotoxicity of 8-amino-Ado in BCR-ABL positive K562 cells, which exhibit features of apoptosis such as PARP cleavage (Figure 7A) and impaired the clonogenic survival (Figure 7B).

In addition to these above-mentioned actions, our recent investigations have suggested that 8-amino-Ado treatment impaired glucose consumption by regulating glucose transporters in myeloma cell lines [58]. In mantle cell lymphoma, this analogue inhibited several survival kinases such as AKT and ERK [28]. Additionally, 8-amino-ATP may have an effect on the BCR-ABL kinase activity. These pathways, while not evaluated in the current work, may potentiate imatinib-mediated effects.

Clinical resistance to imatinib has been a feature of T315I mutation[59], which prevents inhibitor binding into BCR-ABL catalytic site via steric hindrance. Our results in the parental KBM5 and T315I mutation-harboring imatinib resistant cell line KBM5/STI suggest that addition of 8-amino-Ado in combination may be a strategy to inhibit BCR-ABL signaling. The effects of 8-amino-Ado and imatinib were greater than additive in the sensitive KBM5 cell line (Figure 8B), which was consistent with our hypothesis. Interestingly, treatment with either 8-amino-Ado or imatinib alone had minimal effect on the resistant KBM5/STI cell growth, however, combination treatment resulted in growth inhibition (Figure 8B). Although there was growth inhibition and PARP cleavage in treated KBM5 cells, there was no significant increase in annexin-positivity detected by flow cytometry (data not shown), which suggests a mechanism other than apoptosis. Given that this analog modulates triphosphate levels, a distinct possibility could involve AMP-activated

kinase, which is a central inducer for autophagy (reviewed in [60]). Indeed, we have previously shown in myeloma cells that 8-amino-Ado treatment reduced glucose consumption and activated autophagy [58]. Further studies are needed to determine if induction of autophagy is also observed in CML cells treated with 8-amino-Ado.

While it is early to move this combination strategy to clinic, the feasibility of such an approach exists. First, the concentrations of 8-amino-Ado used in the present investigations may be physiologically achievable. For example, during the Phase I investigations of 8-Cl-Ado, a related congener, at the current dose of 100 mg/m²/d x 5 day schedule as high as 500 nM levels of drug are achieved in plasma and the dose is still being escalated (Gandhi, unpublished data). Second, steady-state concentration of imatinib in plasma at the standard dose of 400 mg/d is 2 μ M [61]; greater than what was used in the current investigations. Third, previous studies have demonstrated that 8-amino-Ado was not cytotoxic to normal lymphocytes obtained from healthy donors[28]. Finally, our current studies with CD34+ and CD34- cells suggest minimal toxicity to these hematopoietic cells (Figure 8C) providing a therapeutic index.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a Lymphoma SPORE (CA136411) grant from the National Cancer Institute, Department of Health and Human Services.

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Figure 1.
Structure of 8-amino-Ado

Figure 2. Dose-dependent accumulation of 8-amino-ATP and decline of intracellular ATP in K562 cells

Exponentially growing cultures of K562 cells were incubated with the indicated concentrations of 8-amino adenosine for 4 hours. The nucleotides were extracted using PCA extraction technique and analyzed by HPLC. White bars, ATP; black bars, 8-amino-ATP. Error bars, SD.

Figure 3. Time-dependent accumulation of 8-amino ATP and decrease of intracellular ATP in K562 cells

Exponentially growing cultures of K562 cells were incubated with 0.1 (■), 1 (▲), 10 (▨), or 30 (◆) μM 8-amino-Ado for up to 24 hours. Nucleotides were extracted with perchloric acid every 2 hours up to 12 hours and at 24 hours. Levels of 8-amino-ATP (A) and intracellular ATP (B) were then analyzed by HPLC. *Data points*, mean of two independent experiments.

Figure 4. Inhibition of RNA synthesis in K562 cells by 8-amino-Ado treatment

K562 cells were exposed to 1 (▲), 10 (◻), and 30 (◆) μM 8-amino-Ado for the indicated timepoints. [^3H]-uridine was added to the cell cultures during the last 30 minutes of incubation. RNA synthesis was measured by the amount of [^3H]-uridine incorporation and expressed as percent of control cells. *Data points*, mean of 3 (for 1 and 30 μM) or 5 (10 μM) independent experiments; error bars, SEM.

Figure 5. 8-amino-Ado induces a decline in Bcr-Abl transcript in K562 cells

RNA was isolated from K562 cells incubated with 10 (○) and 30 (◆) μM 8-amino-Ado for the indicated timepoints. The amount of *BCR-ABL* mRNA present was measured by real-time RT PCR using primers specific to the *BCR-ABL* fusion region. The data was expressed as a percent of control untreated cells. *Data points*, mean of three independent experiments; error bars, SEM.

Figure 6. Bcr-Abl protein levels decrease in response to 8-amino-Ado treatment in K562 cells
Immunoblot analysis of Bcr-Abl in K562 cells treated with 10 μ M 8-amino-Ado for 12 hours. β -Actin was used as a loading control.

Figure 7. Cytotoxic effects of 8-amino-Ado in K562 cells

A, Exponentially growing cultures of K562 cells were incubated for 12 hours with 10 μM 8-amino-Ado and aliquots were removed every 2 hours. Cellular proteins were extracted and analyzed by Western blot for PARP. The 85 kDa PARP cleavage product is evident as early as 4 hours. β -Actin was used as a loading control.

B, K562 cells were incubated for 24 hours with various concentrations of 8-amino-Ado. The cells were washed twice and then 300 or 3000 cells were plated in agar. After an incubation of 10–14 days, colonies were counted under a microscope, and clonogenicity was expressed as a percentage of control untreated cells clonogenic potential. 8-amino-Ado inhibits clonogenic survival of K562 cells with an IC_{50} of 0.3 μM . K562 cells completely lose clonogenic potential with 3 μM 8-amino-adenosine. *Data points*, mean of five independent experiments conducted in triplicate.

Figure 8. Inhibition of K562 cell growth by 8-amino-Ado and imatinib

A, Exponentially growing cultures of K562 cells were incubated for 24 hours with control DMSO (black bar), 100 nM 8-amino-Ado alone (grey bar), 300 nM imatinib alone (white bar), or both agents in combination (hatched bar), and the cell concentration was measured. The data shown is a mean of two independent experiments conducted in triplicate $\pm$ SEM.

B, Exponentially growing cultures of KBM5 or KBM5/STI cells were incubated for 48 hours with control DMSO (black bar), 100 nM 8-amino-Ado alone (grey bar), 300 nM imatinib (white bar) alone, or both agents in combination (hatched bar), and the cell concentration was measured after 24 and 48 hours. The data shown is a mean of four independent experiments $\pm$ SEM.

C, Separated CD34⁺ and CD34⁻ cells isolated from cord blood were incubated for 48 hours with control DMSO (black bar), 100 nM 8-amino-Ado alone (grey bar), 300 nM imatinib (white bar) alone, or both agents in combination (hatched bar). Annexin V binding assay was used for apoptosis assay as described under *Material and Methods*, after 24 and 48 hours. The data shown is from cells isolated from 3 different blood specimens.

Table 1

Quantitative analysis of Bcr-Abl and Abl protein decline after treatment with 8-amino-Ado

Protein level compared to β -actin Mean $\pm$ SD				
Hours	Bcr-Abl	<i>p</i> value	Abl	<i>p</i> value
0	0.6 $\pm$ 0.3		0.5 $\pm$ 0.4	
2	0.7 $\pm$ 0.5	0.224	0.5 $\pm$ 0.3	0.433
4	0.3 $\pm$ 0.3	0.048 *	0.3 $\pm$ 0.3	0.026 *
6	0.2 $\pm$ 0.1	0.026 *	0.2 $\pm$ 0.1	0.049 *
8	0.2 $\pm$ 0.1	0.042 *	0.1 $\pm$ 0.1	0.047 *
10	0.2 $\pm$ 0.1	0.029 *	0.1 $\pm$ 0.1	0.041 *
12	0.3 $\pm$ 0.2	0.105	0.1 $\pm$ 0.1	0.065

* statistically significant