Mechanisms of Cell Death of Chronic Lymphocytic Leukemia Lymphocytes by RNA-Directed Agent, 8-NH₂-Adenosine

Kumudha Balakrishnan,¹ William G. Wierda,² Michael J. Keating,² and Varsha Gandhi¹,²

Abstract

Purpose: To determine if RNA-directed nucleoside analogue, 8-NH₂-adenosine, induces cell death and if that is accompanied with transcription inhibition of the key survival factors of chronic lymphocytic leukemia (CLL) cells.

Experimental Design: Primary lymphocytes from CLL patients were incubated with 10 μmol/L 8-NH₂-adenosine for 2, 4, and 6 or 8 hours. The accumulation of analogue triphosphate and the decline in endogenous ATP pool were analyzed by high-performance liquid chromatography. Inhibition of global RNA and protein synthesis was measured and correlated with specific decline in transcript and protein levels of MCL-1, XIAP, and BCL-2, the key survival factors of CLL. These biochemical and molecular end points were related to cell death of these quiescent lymphocytes.

Results: In vitro incubations of CLL lymphocytes with 8-NH₂-adenosine resulted in rapid but heterogeneous accumulation of 8-NH₂-ATP (390-680 μmol/L), with a concomitant decline in endogenous ATP (median, >50% by 4 hour). Global RNA synthesis was decreased in all samples and was associated with a decline in MCL-1, XIAP, and BCL-2 transcripts. There was a parallel decrease in the protein level of MCL-1 and XIAP but not BCL-2. These biochemical changes were accompanied by apoptosis.

Conclusion: The evidence of CLL cell death with complementary changes in the expression of survival proteins provides a molecular rationale for using 8-NH₂-adenosine as a therapeutic agent for this indolent leukemia.

B-cell chronic lymphocytic leukemia (B-CLL) is the commonest hematologic malignancy in the adult, representing nearly 23% of all leukemias (1). The disease involves a continuous increase in lymphocyte counts due to defects in the apoptotic machinery rather than increased cell proliferation (2). It is characterized by the accumulation of resting, long-lived, CD5⁺ B cells in blood, which express high levels of the antiapoptotic proteins such as MCL-1 and BCL-2 (3). Despite the efficacy of alkylating agents and purine nucleoside analogues in the treatment of this disease, CLL remains incurable. For this reason, the development of new drugs or combination regimens is ongoing (4, 5).

It is critical to understand the biology of CLL for development of new agents. In this respect, several studies have shown that CLL is either slow-growing or nongrowing cellular population (6). The basic defect in CLL is one of apoptosis and has been attributed to alterations in the BCL-2 family members (7–11). Generally, there is a fine balance in the expressions of proapoptotic and antiapoptotic BCL-2 family proteins in cells, and the net effect of the opposing forces controls cellular proliferation, differentiation, and apoptosis (12, 13). This balance is lost in CLL with an overexpression of BCL-2 and MCL-1. The proto-oncogene BCL-2, which delays apoptosis, has been documented to be highly expressed in most B-CLL (13). Although a translocation of the gene occurs infrequently in this disease (14), BCL-2 overexpression has been shown to be related to DNA hypomethylation (7). Overexpression of BCL-2 is associated with resistance to chemotherapy and inhibition of apoptosis (15). When cultured in vitro, CLL cells with high BCL-2 levels survived longer than those with low levels, suggesting an inherent survival advantage by BCL-2.

Like other BCL-2 family members, MCL-1 is an oncogene that promotes tumorigenesis and protection against apoptosis and drug resistance of malignant cells. MCL-1 was initially identified in the human myeloid leukemia cell line, ML-1; however, later, it was discovered that antiapoptotic activity of MCL-1 seems important in a variety of tumors (16–18). MCL-1 expression is required to maintain viability and promote cell differentiation, whereas MCL-1 down-regulation induces apoptosis (19). Deletion of MCL-1 prevents embryonic development (20), whereas overexpression of MCL-1 in transgenic mice enhances hematopoietic cell growth and survival in the specific myeloid lineage (21). This antiapoptotic molecule is also required for B- and T-cell development and maintenance (22).
Therefore, the wide expression of MCL-1 in human tissues and its effects on apoptosis, differentiation, and cell cycle confirm its crucial role in cellular homeostasis; conversely, dysfunction of MCL-1 regulation, expression, or degradation is observed in various diseases (23, 24).

When MCL-1 is conditionally removed from lymphocytes, there is a profound reduction in both B and T lymphocytes and cytokine (interleukin-7)–mediated survival response was also lost in these lymphocytes (22). Down-regulation of MCL-1 using antisense oligonucleotides induced apoptosis (18). Moreover, the induction of apoptosis with chemotherapy in CLL cells in vitro has been associated with down-regulation of MCL-1, but not BCL-2, suggesting that this feature may be required for drug activity (25–27). Finally, MCL-1 may have a role in drug resistance in CLL, as patients clinically resistant to chemotherapy seem to have higher pretreatment leukemic cell levels of MCL-1 than those who are sensitive to chemotherapy (2). Taken together, these reports suggest that BCL-2 and MCL-1 are potential targets for development of CLL therapeutics.

Based on this background, we hypothesized that targeting the expression levels of MCL-1 and BCL-2 could induce cytotoxicity in CLL lymphocytes. In nondonivating lymphocytes, global inhibition of RNA synthesis could lead to a decline in transcripts of antiapoptotic genes and proteins. This diminution of antiapoptotic proteins involved in the survival of these cells would induce cell death. Using this strategy, previously, we have shown that RNA-directed chlorinated adenosine analogue, 8-Cl-adenosine (28), induced cell death in multiple myeloma (29) and primary CLL lymphocytes (30). Recent report has shown that the second-generation of adenosine analogue, 8-NH₂-adenosine (Fig. 1), was more potent than 8-Cl-adenosine (31).

In the present study, using primary leukemic lymphocytes obtained from patients with CLL, we show the cytotoxic effect of 8-NH₂-adenosine. The sequential events leading to CLL cell death were an accumulation of analogue triphosphate, decrease in cellular bioenergy, inhibition of RNA and protein synthesis, and a decline in transcriptional and translational levels of antiapoptotic proteins such as MCL-1 and XIAP.

**Patients and Methods**

**Drugs and chemicals.** 8-NH₂-adenosine was purchased from RI Chemical, Inc. (Orange, CA). For high-performance liquid chromatography standards, triphosphates of these analogues were custom synthesized by Bio Log (La Jolla, CA). [³H]Uridine and [³H]leucine were purchased from Moravek Biochemicals (Brea, CA; specific activity, 41.2 and 120 Ci/mmol, respectively).

![Fig. 1. Structure of 8-NH₂-adenosine.](image-url)
Table 1. Patient characteristics: the in vitro studies carried out in CLL lymphocytes (n = 5)

<table>
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</table>

probes for MCL-1 were purchased from Applied Biosystems Assays on Demand program and the BCL-2, XIAP, and 18S primers and probes were purchased from their “Predeveloped Assay Reagents.” The relative gene expression levels were quantitated by using the ΔΔCt method (33) normalizing to 18S. The results are presented as a percentage of the gene expression level in the untreated sample from the same patient.

**Immunoblot analysis.** Cells were lysed on ice for 20 minutes in lysis buffer containing 25 mmol/L HEPES (pH 7.5), 300 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.5% sodium deoxycholate, 20 mmol/L glycerophosphate, 1% Triton X-100, 0.1% SDS, 0.2 mmol/L EDTA (pH 8), 0.5 mmol/L DTT, 1 mmol/L sodium orthovanadate (pH 10), and protease inhibitor. Cells were centrifuged at 14,000 g for 15 minutes at 4°C, and the supernatant was stored at −80°C until use. Protein content was determined using detergent-compatible protein assay kit according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). Aliquots (30 μg) of total cell protein were boiled with Laemmli sample buffer and loaded onto 8% to 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Osmonics Labstore, Minnetonka, MN). Membranes were blocked for 1 hour in PBS Tween containing 5% nonfat dried milk and then incubated with primary antibodies for 2 hours followed by species-specific horseradish peroxidase–conjugated secondary antibody (diluted 1: 5,000) for 1 hour. The blots were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce Biotechnology, Inc., Rockford, IL) and normalized to the actin levels (obtained from Sigma, St. Louis, MO) in each extract. Rabbit polyclonal antibody to MCL-1 (sc-819), mouse monoclonal antibody to XIAP (BD Biosciences/Transduction Laboratories, Lexington, KY), and mouse monoclonal antibody to BCL-2 (sc-509) from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal antibody to poly(ADP-ribose) polymerase (PARP) from BD PharMingen International (San Diego, CA) were used to detect these proteins in each sample.

**Results**

**Accumulation of 8-NH2-ATP and decline in cellular bioenergy.** CLL lymphocytes from different patients showed heterogeneity for accumulation of 8-NH2-ATP. However, in all, during the 8-NH2-adenosine incubation, there was a gradual accumulation of 8-NH2-ATP. At 2 hours, the triphosphate accumulation was median 380 μmol/L, at 4 hours it increased to 526 μmol/L, and at 6 or 8 hours it further increased to 680 μmol/L (Fig. 2A). Concomitant with the accumulation of analogue triphosphate, there was a decline in cellular bioenergy in these leukemic lymphocytes (Fig. 2B). At start, CLL lymphocytes from these patients had 1,900 to 3,400 μmol/L endogenous ATP. At 4 hours, all samples showed a significant decrease (1,200-1,800 μmol/L) in ATP concentration. Taking the starting value of ATP as 100% the decline at 6 or 8 hours was >70%. When the relationship between endogenous ATP pool and the accumulation of 8-NH2-ATP was studied, it was identified that there was a weak linear relationship (r = 0.58, P = 0.02; data not shown) between ATP concentration and analogue triphosphate accumulation.

When CLL lymphocytes were incubated with different concentration of 8-NH2-adenosine (0, 3, 10, 30, and 100 μmol/L), there was a dose-dependent accumulation of 8-NH2-ATP (n = 2). For the first patient, the values were 133, 522, and 872 μmol/L at 10, 30, and 100 μmol/L of 8-NH2-adenosine. For the second patient, at these concentrations, the 8-NH2-ATP levels were 755, 1,565, and 2,382 μmol/L, respectively (data not shown).

**Inhibition of RNA and protein synthesis by 8-NH2-adenosine.** Previous studies using exponentially growing cell lines have shown that 8-NH2-adenosine is an RNA-directed nucleoside analogue (31). To determine inhibition of RNA synthesis by 8-NH2-adenosine in quiescent CLL cells, lymphocytes from patients were assessed for [3H]uridine incorporation. Cells from all five patients showed inhibition of RNA synthesis.

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**Fig. 2.** Plots of accumulation of 8-NH2-ATP (A) and depletion of ATP pool (B) with the treatment of 8-NH2-adenosine in primary CLL cells from five patients. The CLL lymphocytes were incubated with 10 μmol/L 8-NH2-adenosine for 2, 4, and 6 or 8 hours and PCA-extracted nucleotides were analyzed by high-performance liquid chromatography.
starting at 2 hours. After this point, however, there were variations between patients (Fig. 3A). When the same cells were analyzed for other macromolecule-like protein synthesis using \[^{3}H\]leucine incorporation assay, three of five patients showed a significant decline in the synthesis of protein, whereas cells from two patients showed no discernable decrease in protein synthesis (Fig. 3B). In contrast to RNA and protein, there was no effect on DNA synthesis by 8-NH\(_2\)-adenosine (data not shown). This was expected, as CLL lymphocytes are quiescent and not replicating DNA. The variation among patient samples for the extent of inhibition of RNA and protein syntheses could be due to differences in the intracellular levels of 8-NH\(_2\)-ATP. To determine this relationship, these data were compared and plotted (Fig. 3C). There was a significant linear relationship between the concentrations of 8-NH\(_2\)-ATP and inhibition of RNA synthesis at all time points in these five patients (\(r = 0.67, P = 0.005\)). Similar correlations were made between intracellular level of 8-NH\(_2\)-ATP and inhibition of protein synthesis; however, this was very weak and statistically not significant (\(r = 0.40, P = 0.12\); data not shown).

**Effect of 8-NH\(_2\)-adenosine on MCL-1, XIAP, and BCL-2 mRNA transcript.** The global RNA synthesis inhibition in CLL lymphocytes by 8-NH\(_2\)-adenosine should affect the synthesis of new transcripts. This should result in a decline in transcripts with a short half-life such as MCL-1 and XIAP. To investigate this, primary CLL cells from patients were incubated with 8-NH\(_2\)-adenosine for 2, 4, and 6 or 8 hours and total RNA was isolated. The MCL-1 transcript level was declined to 25% to 75% at 2 hours. At 4 hours, either there was an additional decrease or the levels remained constant (Fig. 4A). At 6 or 8 hours in some patients, the transcript levels increased; however, they were still lower than the starting value. The decrease in MCL-1 transcript level was correlated with either 8-NH\(_2\)-ATP or inhibition of RNA synthesis. In either case, there was no relationship between these two variables (\(r = 0.30-0.35, P = 0.18-0.26\); data not shown).

When CLL lymphocytes were tested for XIAP transcripts of another short-lived protein, all patients showed a response as the levels of XIAP decreased. The decline in transcripts were between 77% and 22% at 2 hours; at 4 hours, it further decreased to 62% to 18%; at 6 or 8 hours, the transcript level either increased or remained constant (Fig. 4B). Previous studies showed that BCL-2 is also responsible for the survival of CLL lymphocytes. To investigate if 8-NH\(_2\)-adenosine has any

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**Fig. 3.** Effect of 8-NH\(_2\)-adenosine on macromolecule synthesis. CLL lymphocytes from five patients were incubated with 10 \(\mu\)mol/L 8-NH\(_2\)-adenosine for 2, 4, and 6 or 8 hours and assessed for either \[^{3}H\]uridine incorporation to measure RNA synthesis (A) or \[^{3}H\]leucine incorporation to measure protein synthesis (B). Data from (A) and Fig. 2A were plotted to determine the relationship between intracellular level of 8-NH\(_2\)-ATP and inhibition of RNA synthesis (C).
effect on BCL-2 transcript level, primary CLL lymphocytes were measured for BCL-2 mRNA levels. As with MCL-1, for BCL-2 transcripts, there was heterogeneity among patients. Three patients showed a response as the levels of BCL-2 decreased. For the remaining two patients, either there was minor or no discernable decrease in the BCL-2 transcript levels (Fig. 4C).

**Effect of 8-NH₂-adenosine on MCL-1, XIAP, and BCL-2 protein levels.** To determine if the decline in the transcripts of antiapoptotic proteins results in knocking down the protein, expression of MCL-1, XIAP, and BCL-2 proteins were measured by immunoblot analysis. As shown in Fig. 5A (one representative patient 4), there was a time-dependent decrease in MCL-1 and XIAP protein expression levels. In contrast, there was no significant change in BCL-2 protein expression. Similar studies in cells from five patients were done and quantitated by densitometry analysis for MCL-1, XIAP, and BCL-2 protein expression (Fig. 5B-D). The decrease in MCL-1 protein expression levels was between 50% and 80% in these lymphocytes by 6 or 8 hours of drug incubations. For XIAP, the decrease in protein expression levels was between 60% and 80% in these lymphocytes. In contrast, there was no significant decrease in the expression of BCL-2 protein levels in all five patients studied.

**Induction of apoptosis in chronic lymphocytic leukemia lymphocytes.** Treatment of CLL lymphocytes with 10 μmol/L 8-NH₂-adenosine leads to an increase in the time-dependent cleavage of PARP in all five patients. For illustrative purposes, immunoblot of PARP from one representative patient (patient 4) is shown (Fig. 6). The cleavage of PARP protein was observed starting at 2 hours in this patient but occurred by 4 hours in all other patients. When these protein lysates were tested for caspase-3 activation, there was a time-dependent increase in cleaved caspase-3, which increased by 2-fold at 4 hours and 3-fold at 6 hours (data not shown).

**Effect of in vitro culturing of chronic lymphocytic leukemia lymphocytes.** For all the abovementioned variables, quiescent CLL cells were cultured in artificial in vitro conditions. To rule out the possibility that the decrease in ATP pool, RNA synthesis, and induction of cell death was due to culturing, untreated CLL
cells from three patients were analyzed for these end points. The ATP levels varied between 95 ± 3% and 119 ± 6% after 0, 2, 4, and 24 hours of in vitro culturing of cells, suggesting no decline in the bioenergy. Similarly, RNA synthesis was not affected by in vitro culturing as at 4 hours the inhibition of RNA synthesis was similar to that in the matched 0-hour sample for all three patients (99.7%, 85.3%, and 105% of respective control value). When CLL primary cells were incubated for 0, 2, and 4 hours in culture medium without 8-NH₂-adenosine, there was no PARP cleavage seen (data not shown), suggesting that the induction of cell death in CLL lymphocytes is in response to drug treatment. Culturing for longer time (24 hours), however, resulted in some PARP cleavage in these lymphocyte extracts.

**Discussion**

Whereas patients with early-stage indolent leukemia survive a median of 11 years, patients with advanced-stage B-CLL have a median survival of 1.5 to 3 years with few therapeutic options that affect overall survival. Therefore, new therapeutic strategies are needed for patients with B-CLL. For many years, alkylating agents either alone or in combination has been the treatment of choice for B-CLL (34). Recent studies have shown that the nucleoside analogues produce a higher complete remission rate and more sustained remissions than that observed with typical alkylating agents (35, 36). In the last few years, purine nucleoside analogues such as fludarabine, has changed the

![Fig. 5. Effect of 8-NH₂-adenosine on expression of antiapoptotic proteins in CLL lymphocytes.](image-url)

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treatment possibilities of B-CLL (37–39) either as monotherapy or in combination with alkylating agents.

Unlike cell lines, CLL primary cells are quiescent and hence DNA is not replicating. The deleterious effects of alkylating agents do not require DNA replication. Hence, it is easy to understand the mechanism of actions of DNA damaging agents in killing CLL cells. Non-DNA-directed actions have been reported for fludarabine also. The antileukemia activity of fludarabine in B-CLL lymphocytes was associated with the inhibition of RNA synthesis due to preferential incorporation of this analogue into mRNA with resulting premature termination of the transcript (40). Huang et al. (41) showed that termination of mRNA transcription and the consequent depletion of proteins required for cell survival may be a novel biochemical mechanism of action of fludarabine in CLL cells. Consistent with this strategy, inhibition of RNA synthesis by α-amanitin or actinomycin D was closely correlated with cytotoxicity in CLL cells (41). Other DNA-directed drugs have also been found to target RNA in B-CLL cells (42). Therefore, it seems that non-DNA-directed approaches work effectively in killing quiescent CLL cells. Consistent with this observation, C-8-substituted adenosine analogues are effective in inducing cell death in CLL lymphocytes (ref. 30 and present work).

The two key features of CLL biology are having a non-proliferative status of leukemic lymphocytes and having an overabundance of survival proteins such as MCL-1 and BCL-2. Nonetheless, all cells, either proliferating or quiescent, with or without the continued synthesis of other proteins, require active energy production and RNA transcription for the synthesis of housekeeping enzymes and other proteins essential for their existence. Therefore, perturbing bioenergy and inhibiting RNA synthesis may impose a lethal effect on quiescent cells. The decline in ATP synthesis and intracellular energy charge seem to play a key role in the fundamental cellular processes (43, 44). Our data in CLL lymphocytes show that there was a time-dependent decline in the endogenous level of ATP (Fig. 2B) during 8-NH₂-ATP accumulation (Fig. 2A). This change in bioenergy may affect nuclear transcription and cellular translation initiating cell death.

Consistent with this postulate and previously identified actions of 8-NH₂-adenosine on general RNA synthesis (31) and polyadenyllic acid formation (45), global RNA synthesis inhibition was observed in leukemic lymphocytes of these patients (Fig. 3A). Whereas general inhibition of RNA synthesis is an important mechanism, loss of specific transcripts might be critical. The inhibition of RNA synthesis by 8-NH₂-adenosine in CLL cells may eventually deplete functional mRNA level. Although RNA synthesis may be inhibited shortly after cells are exposed to 8-NH₂-adenosine, the preexisting RNA in the cells may still be able to support protein synthesis for a certain period. Thus, RNA transcript with a relatively short elimination time would be affected by 8-NH₂-adenosine to a much greater extent than the RNA transcripts with slow turnover rates. The elimination rate of MCL-1, XIAP, and BCL-2 transcripts are <3, <6, and >10 hours, respectively. In agreement with this physiologic turnover rate of these transcripts, when the antiapoptotic mRNA transcripts of MCL-1, XIAP, and BCL-2 were measured, there was a significant decline in MCL-1 and XIAP transcripts compared with BCL-2 transcripts (Fig. 4A-C).

When protein synthesis was measured by [³²P]leucine incorporation it was noted that a general (or global) inhibition of protein synthesis was observed in three patients (Fig. 3B). This effect on protein synthesis will further reduce expression level of MCL-1 protein even if MCL-1 transcript levels are relatively less affected. Because certain protein molecules can be severely depleted even when the overall protein synthesis activity is only moderately reduced, it is important not to underestimate the biological significance of a partial inhibition of protein synthesis. For instance, if a critical survival factor is depleted, the cells are likely to die despite the continued synthesis of other proteins. The survival factor for CLL is MCL-1. MCL-1 differs from BCL-2 at the NH₂ terminus, where it has a unique amino acid segment that contains PEST motifs (sequences rich in proline, glutamate, serine, and threonine; refs. 19, 46). These PEST motifs likely contribute to the fact that the half-life of MCL-1 is ∼1 hour compared with 10 to 14 hours for BCL-2 protein (19). When CLL lymphocytes from five patients were incubated with 8-NH₂-adenosine, immunoblot analysis showed significant decline in the expression of MCL-1 and XIAP (Fig. 5A-C). In contrast, there was no down-regulation in the expression of BCL-2 protein (Fig. 5D), suggesting that fast half-life of protein may be required to observe drug-induced decline in protein expression levels. This is consistent with other studies (18) showing that specific depletion of MCL-1 mRNA and protein, in the absence of changes in cellular levels of BCL-2, results in a rapid entry into apoptosis. Such results are obtained in CLL cells by transcription inhibitors such as 8-Cl-adenosine (30), 8-NH₂-adenosine (present study), and flavopiridol (26, 47, 48) or translation inhibitor such as cycloheximide (49). Direct evidence for the role of MCL-1 as survival protein comes by a rapid down-regulation of MCL-1 protein levels by antisense oligonucleotides and the coincident induction of apoptosis in B-lymphoma, B-CLL, and multiple myeloma cell lines (16, 18, 50, 51).

Failed programmed cell death (apoptosis) of the leukemia cells is currently considered the principal mechanism of pathogenesis of CLL and this has been attributed to an overexpression of antiapoptotic protein like MCL-1. It is likely then that the reduction in the level of this survival protein should initiate CLL cell death. In contrast, decrease in MCL-1 protein level would not affect the survival of normal cells. Hence, dependency of CLL cells on MCL-1 would lead to selectivity of global transcription inhibitors and such strategy would be context dependent. Our data show that the adenosine analogue under study induces apoptosis in CLL lymphocytes (Fig. 6). Hence, the evidence of apoptotic CLL cell death in
response to 8-NH2-adenosine, with complementary changes in the expression of survival proteins regulating cell propensity to undergo apoptosis, provides a molecular rationale for using 8-NH2-adenosine as a therapeutic agent for CLL.

Acknowledgments

We thank Min Du for analysis of 8-NH2-ATP and ATP using high-performance liquid chromatography.

References


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