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RNA-Directed Actions of 8-Chloro-Adenosine in Multiple Myeloma Cells

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ABSTRACT

The purine analogue, 8-chloro-adenosine (8-Cl-Ado), induces apoptosis in a number of multiple myeloma (MM) cell lines. This ribonucleoside analogue accumulates as a triphosphate and selectively inhibits RNA synthesis without perturbing DNA synthesis. Cellular RNA is synthesized by one of three polymerases (Pol I, II, or III); thus, the inhibition of one or more RNA polymerases may be mediating 8-Cl-Ado cytotoxicity. Here, we have addressed this question by dissecting the RNA-directed actions of 8-Cl-Ado in MM cells. Differential alterations in [^{3}H]uridine incorporation were found in the three major classes of RNA after a 20-h exposure with 10 μM 8-Cl-Ado. The synthesis rate of Pol III transcripts, 5 S and tRNA, remained unchanged, whereas Pol I-mediated rRNA synthesis decreased by ~20%. In contrast, mRNA synthesis, which is transcribed by Pol II, rapidly declined within 4 h and reached a 50% decrease, which was maintained for 20 h. Parallel to RNA synthesis inhibition, 8-Cl-Ado was maximally incorporated in the mRNA (>13 nmol/mg RNA), which was 5-fold higher than the tRNA and rRNA incorporation. Electrophoretic and radiographic analysis of newly synthesized and processed [^{14}C]uridine-labeled transcripts indicated that the analogue blocks transcription elongation. Consistent with that result, high-performance liquid chromatography analysis of micrococcal nuclease and spleen phosphodiesterase-digested RNA demonstrated that the analogue incorporation is at the 3′ terminus. In conclusion, our data demonstrate that in MM cells, 8-Cl-Ado is preferentially incorporated into mRNA, suggesting a propensity toward Pol II, and inhibits RNA synthesis by premature transcriptional chain termination.

INTRODUCTION

8-Cl-cAMP[1] is cytotoxic to many tumor types (1–8). Although it has been used in the clinic, there is increasing evidence that its metabolite, the nucleoside analogue 8-Cl-Ado, actually exerts the lethal activity. Several lines of evidence indicate that 8-Cl-Ado is generated from 8-Cl-cAMP extracellularly via the enzymatic actions of serum PDE and 5′-nucleotidase (3, 9–11). Pharmacokinetic investigations during a Phase I study of 8-Cl-cAMP in breast cancer patients provided additional evidence for the conversion of 8-Cl-cAMP to 8-Cl-Ado (12). Studies using an adenosine kinase-deficient cell line additionally demonstrated the need of this enzyme and accumulation of the triphosphorylated metabolite, 8-Cl-ATP, for both 8-Cl-cAMP- and 8-Cl-Ado-induced cytotoxicity (13). Taken together, these investigations have established that 8-Cl-cAMP serves as a prodrug of 8-Cl-Ado, which acts as a purine nucleoside analogue.

8-Cl-Ado has a strong potential for use as an effective agent in the therapy of MM. MM is a uniformly fatal indolent B-cell malignancy that responds poorly to chemotherapy, including purine nucleoside analogues. In preclinical and clinical settings, neither cladribine nor fludarabine displayed any cytotoxic activity in this disease, whereas both analogues are effective in treating other indolent B-cell malignancies (14, 15). In contrast, our previous studies demonstrated that 8-Cl-Ado mediates cytolysis in a variety of MM cell lines, including lines that are resistant to conventional chemotherapeutic agents (9). Hence, at present, 8-Cl-Ado is the only purine analogue that exhibits efficacy in MM preclinical model systems. This is believed to be due to its uniqueness both for metabolism and mechanism of actions.

8-Cl-Ado resembles a classical nucleoside analogue, which must be converted to its phosphorylated form before incorporation into nucleic acids or for its actions on other cellular targets (10, 13). Nonetheless, this analogue differs from fludarabine and cladribine for its metabolism to the phosphorylated form. In contrast to these analogues, which use deoxycytidine kinase for activation, 8-Cl-Ado uses adenosine kinase for the intracellular metabolism to 8-Cl-AMP. The high specific activity of this enzyme and substantial substrate specificity for 8-Cl-Ado results in near millimolar levels of 8-Cl-ATP in MM cells. Furthermore, parallel to 8-Cl-ATP accumulation, the endogenous ATP pool decreases. Finally, as oppose to other purine analogues, which primarily act on DNA, 8-Cl-Ado inhibits RNA synthesis without any affect on DNA synthesis (13). Hence, 8-Cl-Ado seems to be a unique purine nucleoside analogue with effective cytotoxicity in MM cells.

Although the metabolic uniqueness of 8-Cl-Ado has been described in detail, little is known about its actions on RNA synthesis. To increase our understanding of the unique actions of this analogue, the present study focuses on analyzing its RNA-mediated activity in MM cells. We have investigated in detail the effects of 8-Cl-Ado on total RNA synthesis, the synthesis rate of the various RNA species, and the RNA species specificity of its action. We have also determined incorporation of this analogue and amount in the various RNA species, site of incorporation into RNA, and consequence of this incorporation.

MATERIALS AND METHODS

Cell Culture. The MM.1S cell line was derived from a patient with MM (4, 16) and was obtained from the laboratory of Drs. Nancy Krett and Steven Rosen (Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL). The cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum in the presence of 5% CO_2 at 37°C. Cells were routinely tested for *Mycoplasma* infection using a commercially available kit (Gen-Probe Inc., San Diego, CA) as per the manufacturer's procedure.

Materials. 8-Cl-Ado was obtained from Dr. Vishnuvajjala Rao (Drug Development Branch, National Cancer Institute, Bethesda, MD). For HPLC analyses, 8-Cl-ATP, 8-Cl-AMP, and 8-Cl-inosine were custom-synthesized by Bio Log (La Jolla, CA). Deoxycoformycin was obtained from Dr. V. N. Narayanan (National Cancer Institute). [2-^{3}H]8-Cl-Ado, [5, 6-^{3}H]uridine (~40 Ci/mM, 1 mCi/ml), and [^{14}C]uridine (~400 mCi/mmol; 0.1 mCi/ml) were obtained from Moravek Biochemicals (Brea, CA). All of the other chemicals were reagent grade.

8-Cl-ATP Accumulation and Effect on ATP Pool. MM cells were incubated with 10 μM 8-Cl-Ado and aliquots (5×10^6 to 2×10^8 cells) were removed at the indicated times. Nucleotides were extracted using perchloric acid, and the extracts were neutralized with KOH as described previously (17, 18), and stored at −20°C until analyzed. The neutralized extracts were applied

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[1] The abbreviations used are: 8-Cl-cAMP, 8-Cl-cyclic AMP; 8-Cl-Ado, 8-chloroadenosine; MM, multiple myeloma; sRNA, low molecular weight 5 S and tRNA; Pol I, II, or III, DNA-dependent RNA polymerase I, II, or III; 8-Cl-A(RNA), 8-Cl-Ado incorporated RNA; PDE, phosphodiesterase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; RR, ribonucleotide reductase; HPLC, high-performance liquid chromatography.

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to an anion-exchange Partisil-10 SAX column, and the nucleotide triphosphates were separated as described previously (13). The column eluate was monitored by UV absorption at 256 nm, and the nucleoside triphosphates were quantitated by electronic integration with reference to external standards. 8-Cl-ATP was identified by comparing its retention profile and absorption spectrum with those of an authentic standard. The intracellular concentration of nucleotides contained in the extract was calculated from a given number of cells of a determined mean volume. This calculation assumed that the nucleotides were uniformly distributed in a total cell volume. The lower limit of sensitivity of this assay was 10 pmol in an extract of 5×10^6 cells corresponding to a cellular concentration of 1 μM.

Measurement of dATP Pool. Nucleotides were extracted using 60% methanol for determination of cellular dATP pool. The DNA polymerase assay as modified by Sherman and Fyfe (19) was used to quantitate dATP in the cell extracts. The Klenow fragment of DNA polymerase I lacking exonuclease activity (United States Biochemical Corporation, Cleveland, OH) was used to start a reaction in a mixture that contained 100 mM HEPES buffer (pH 7.3), 10 mM $MgCl_2$, 7.5 μg of BSA, and synthetic oligonucleotides of defined sequences as templates annealed to a primer, [^{3}H]dTTP and either standard dATP or the extract from 1 or 2×10^6 MM cells before and after 8-Cl-Ado treatment. Reactants were incubated for 1 h and applied to filter discs; after they were washed, the radioactivity on the discs was determined by liquid scintillation counting and compared with that in the standard dATP samples.

Inhibition of Total RNA Synthesis. Cells were incubated with 10 μM 8-Cl-Ado for up to 12 h. One h before removal of the aliquot, 2 μCi/ml [^{3}H]uridine was added to these cultures, and incubation was continued for an additional hour in a multiscreen assay system (Millipore Corp., Bedford, MA). The cells were then collected on multiscreen-GV filters under vacuum and washed four times each with ice-cold 8% trichloroacetic acid, water, and 100% ethanol. The radioactivity in the acid-insoluble material retained on the filters was measured by scintillation counting and expressed as the percentage of control (untreated) value of cells.

Quantitative Real Time RT-PCR. MM.1S cells were harvested after treatment with 10 μM 8-Cl-Ado for various amounts of time, and the RNA was extracted with RNAzol B (Tel-test, Friendswood, TX) as described (20). The cell culture and RNA extraction was performed in triplicate. The gene expression levels of M1 and M2 ribonucleotide reductase (RR) subunits as well as GAPDH was measured using the TaqMan RT-PCR on an ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA). Cell equivalent amounts of RNA, ~200 ng of the untreated RNA sample, were assayed in duplicate using the TaqMan PCR Core kit and murine leukemia virus reverse transcriptase (Applied Biosystems) as described by the manufacturer. The specific probe and primer for *M1* an *M2* were as follows: M1-forward primer 5′-TGAAACTATTTATTATGGTGCTCTGGAA-3′; M1-reverse primer 5′-GCTTTGGATACTCCCGAGAGG-3′; M1-probe 5′-6-carboxyfluorescein-CTGCTCCTTGGCAAGGTCACCAGCCTA-TAMRA-3′; M2-forward primer 5′-TGAGCCGCTGCTGAGAGAA-3′; M2-reverse primer 5′-TAGCTCATGGTACTATAGACCGTC-3′; and M2-probe 5′-6-carboxyfluorescein-ACCCCCGCCGCTTTGTCATCTTC-TAMRA-3′. The *GAPDH* specific probe and primers are as described (21). The gene expression levels were quantitated using standard curves generated from known dilutions of untreated MM.1S cell RNA (22).

Analysis of Specific RNA-Species Synthesis. MM.1S cells were cultured for 4, 8, 12, and 20 h with 10 μM 8-Cl-Ado. During the last hour of culture, the RNA was labeled by adding 1 μl/ml [5, 6-^{3}H]uridine. For the 20-h time point, the RNA was separated into r, m, and sRNA as described (23). For the remaining time points, the mRNA was similarly separated, whereas the remainder of the sample was not subjected to ion exchange chromatography as described. Instead, after precipitation and extraction with RNAzol B, the sample was resuspended in H_2O and assessed directly. The majority of the RNA from the RNAzol extraction is rRNA. Two μl of each RNA sample was assessed for tritium incorporation using a scintillation counter (Perkin-Elmer Life Sciences Inc., Boston, MA). The cell culturing, RNA species isolation, and measurement of tritium incorporation were performed in triplicate for each time point.

RNA Incorporation. Cells were treated with 10 μM [2-^{3}H]8-Cl-Ado for 20 h, and lysates were enriched for r, m, and sRNA (23). Two μl of each RNA sample was assessed for tritium incorporation using a scintillation counter (Perkin-Elmer Life Sciences Inc.). The cell culturing, RNA species isolation, and measurement of tritium incorporation was performed in triplicate.

Transcript Analysis of [^{14}C]Uridine-Labeled RNA. Exponentially growing MM.1S cells were centrifuged at $430 \times g$ for 5 min and concentrated to 5×10^6 cells/ml before treatment with 10 μM 8-Cl-Ado for the indicated times. During the last hour of treatment, cells were labeled with 5 μM [^{14}C]uridine for 1 h followed by a 1-h 5 μg/ml actinomycin D chase to allow processing of the labeled transcripts. Total RNA was isolated, and 2 μg RNA samples were electrophoresed on a 4% formaldehyde/1% agarose gel, visualized by ethidium bromide staining, and quantitated using Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, NY). The RNA was transferred to Hybond-N + nylon membrane (Amersham Biosciences, Piscataway, NJ) in 0.1 N sodium hydroxide, 3 M sodium chloride, and 0.3 M sodium citrate using a vacuum blotter (Bio-Rad, Hercules, CA). The newly synthesized and processed ^{14}C-labeled RNA was visualized by autoradiography and quantitated using a Storm 840 PhosphorImager and Image Quant 5.0 software (Amersham Biosciences). The quantitative results were expressed as a percentage of the ratio of 28 S to 18 S in the untreated cells.

HPLC Analysis of 8-Cl-Ado RNA Incorporation. For this purpose, [2-^{3}H]8-Cl-Ado was HPLC purified to 97%. Cells were incubated with HPLC purified 8-Cl-Ado for 8 h, and total RNA was extracted. The RNA was degraded to internal nucleotides and 3′-terminal nucleosides by the sequential action of micrococcal nuclease and spleen PDE (Sigma-Aldrich Corp., St. Louis, MO) as described (24) in the absence or presence of 250 pmol deoxycoformycin, an adenosine deaminase inhibitor. For separation of nucleosides and nucleotides, the digests were applied to a YMC-Pack ODS-AQ S-5 120A reverse-phase column (4.6 × 250 mm; YMC, Inc., Tokyo, Japan). Two different elution approaches were used, both with a flow rate of 1.5 ml/min for 50 min on a Waters 600E System Controller (Waters Corp.). The samples were eluted with a linear gradient of: (*a*) methanol; or (*b*) ammonium acetate and methanol as described by Rodriguez *et al.* (21) and Gandhi *et al.* (25), respectively. The column eluate was monitored by online scintillation counting and UV absorption at 256 nm. The retention profile of the radioactive peak of the digest was compared with those of authentic standards as well as with liberated ^{3}H products from snake venom PDE digestion of [^{3}H]8-Cl-A(RNA). The digestion pattern of this enzyme results in both terminal and internal nucleotides to be liberated as a monophosphate.

RESULTS

Kinetics of 8-Cl-ATP Accumulation, ATP Decline, and Changes in RNA Synthesis Levels. To gain insight into the mechanism of 8-Cl-Ado-mediated cytotoxicity of MM cells, we chose to examine its effects in the myeloma cell line, MM.1S. DNA fragmentation analysis has demonstrated previously that the apoptotic effects of 8-Cl-Ado on MM.1S cells are evident as early as 16 h after treatment (9, 10). To assess the kinetics of the metabolic effects of 8-Cl-Ado, the time requirements for 8-Cl-ATP accumulation, alterations in the endogenous ATP and dATP pool, and RNA synthesis levels were compared. The results show that the response is rapid (Fig. 1); within 2 h the accumulation of 8-Cl-ATP is >100 μM, whereas both RNA synthesis and ATP levels decrease by 25%. In addition, there was a time-dependent increase in 8-Cl-ATP accumulation, whereas the ATP and RNA synthesis levels continue to decline. In contrast to the reduction in the ATP pool, no changes in the dATP levels were detected.

In general, ribonucleotide diphosphates can be converted to deoxyribonucleotide diphosphates by the actions of RR and then accumulate as the deoxy form of the triphosphate. However, in 8-Cl-Ado treated MM cells, the phosphorylated metabolite, 8-Cl-dATP, was not detected. This conversion may be hampered if there is a deficiency in the RR enzyme levels due to an inhibition of the RR subunit gene expression by the action of 8-Cl-Ado. To address this question, we examined the mRNA levels of both the *M1* and *M2* subunits of RR by real-time RT-PCR. The mRNA levels for both *M1* and *M2* over a 12-h period of 8-Cl-Ado treatment ranged between 90% and 100% of the levels in untreated cells (data not shown), and were similar to changes

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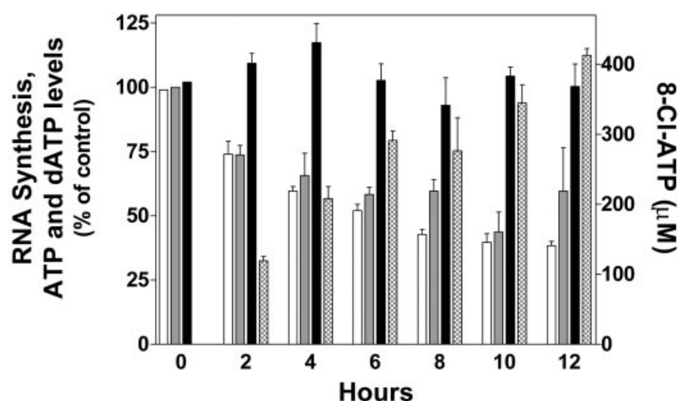

Fig. 1. Accumulation of 8-Cl-ATP and effects on RNA synthesis, ATP, and dATP pools. MM.1S cells were treated with 10 μM 8-Cl-Ado for the indicated times, and aliquots were processed to measure the levels of 8-Cl-ATP (▨), and changes in ATP (□) and dATP (■) pools. Additional aliquots were treated with [^{3}H]uridine to quantitate RNA synthesis (▤).

observed in the *GAPDH* levels used as a control. These results indicate that the lack of deoxy-8-Cl-Ado metabolites is not due to an inhibition of RR gene expression.

Effects of 8-Cl-Ado on the Synthesis of Different RNA Species. Inhibitors of RNA synthesis are known to act both ubiquitously on all of the RNA polymerases, *i.e.,* cordycepin (26), or preferentially on a particular polymerase, *i.e.,* α-amanitin (27), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (28). To better understand the mechanism by which 8-Cl-Ado inhibits RNA synthesis (Fig. 1), we examined its effects on the synthesis of the three major RNA species, rRNA, mRNA, and sRNA, as a reflection of the activity of their predominant polymerase, Pol I, II, and III, respectively. Treatment with 8-Cl-Ado had a differential effect on the synthesis of the three RNA species (Fig. 2). Whereas the synthesis of sRNA remained unchanged, that of rRNA decreased to ~80% of the control value after 20 h. In contrast, the synthesis of mRNA was affected the most, because it was inhibited by >35% at this time point.

Because the species-specific effect of 8-Cl-Ado was primarily in mRNA and rRNA, we additionally examined their synthesis inhibition at 4, 8, and 12 h of 8-Cl-Ado treatment. The results show that the decline in the mRNA synthesis was rapid with ~40% decrease of [^{3}H]uridine incorporation by 4 h as compared with the untreated control cells (Fig. 3) and reached >50% inhibition by 8 h. The inhibition of rRNA followed a similar pattern of inhibition, although with ~10% less inhibition as seen with mRNA.

Incorporation of 8-Cl-Ado into RNA. To understand the mechanism for the inhibition of RNA synthesis, incorporation of 8-Cl-ATP into RNA was assessed and quantitated. Fig. 4 shows that the analogue incorporated into all three of the RNA species, indicating that 8-Cl-ATP serves as a substrate for all three of the polymerases. The analogue was preferentially incorporated into mRNA (>13 nmol/mg RNA) compared with rRNA (~3 nmol/mg RNA) and sRNA (~2 nmol/mg RNA). Incorporation into the sRNA was somewhat surprising, because this population showed little to no synthesis inhibition although its polymerase still uses 8-Cl-ATP. The preferential incorporation of 8-Cl-Ado in mRNA was consistent with the greater inhibition of mRNA synthesis compared with other RNA species.

Premature Termination of Transcription by 8-Cl-Ado. Because the analogue is incorporated into RNA, we wanted to explore possible modes by which it may be inhibiting RNA synthesis, *i.e.,* chain termination on incorporation or slowing polymerase activity while still allowing transcription to proceed after incorporation. To address these possibilities we first examined the effects of 8-Cl-Ado on the synthesis of a large transcript, the rRNA transcript, which can be readily visualized.

The rRNA gene is transcribed as a large 45 S ribosomal precursor, which is subsequently trimmed and spliced through several nuclease cleavage steps. The intermediate cleavage steps and products are variable and cell-type-dependent but eventually form the 18 S, 5.8

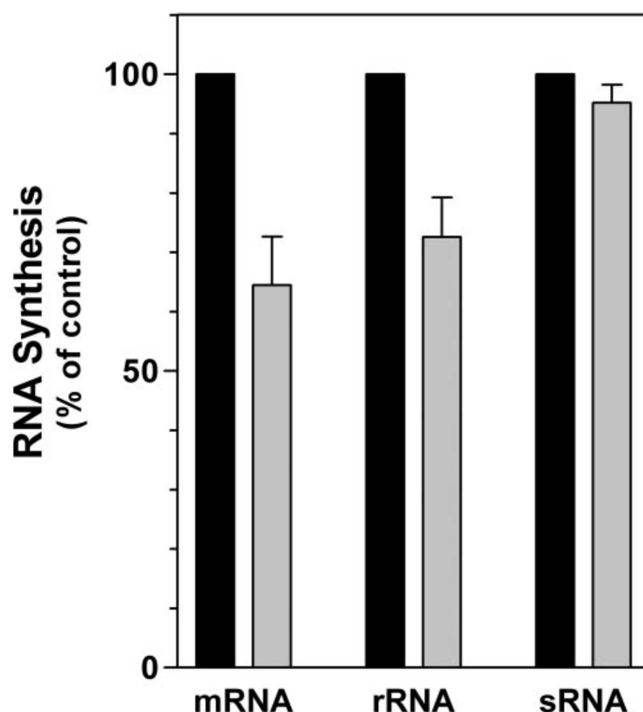

Fig. 2. Differential inhibition of the synthesis of the three RNA classes. *A,* relative change in m, r, and sRNA synthesis after 8-Cl-Ado treatment. MM.1S cells, untreated (■) or treated with 10 μM 8-Cl-Ado for 20 h (▤), were incubated with [^{3}H]uridine during the final h, pelleted, lysed, and the various RNA species were isolated. The levels of tritium incorporation are reported as a percentage of the incorporation in the RNA species from untreated cells. The average absolute [^{3}H]uridine incorporation values in untreated cells were 7.9×10^4 dpm/μg/h for mRNA, 6.4×10^3 dpm/μg/h for rRNA, and 3.2×10^3 dpm/μg/h for tRNA.

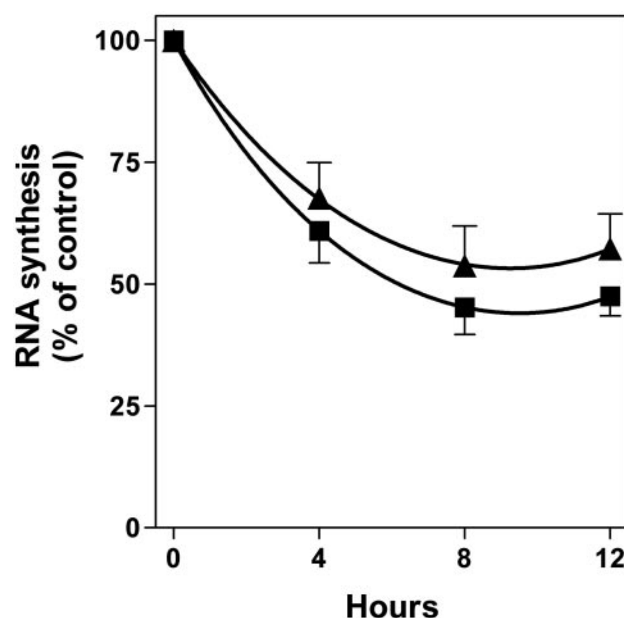

Fig. 3. Time-dependent effects of 8-Cl-Ado on mRNA and rRNA synthesis. As in Fig. 3, [^{3}H]uridine incorporation was measured in m, ■, and rRNA, ▲, with the 8-Cl-Ado treatment times indicated.

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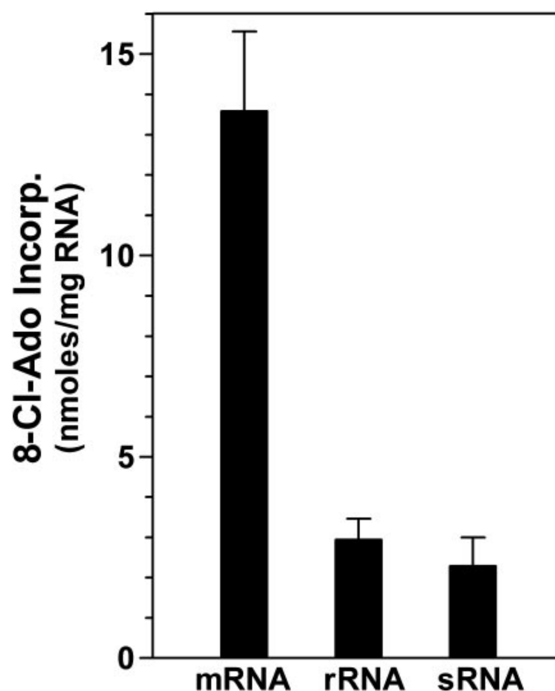

Fig. 4. Species-specific 8-Cl-Ado incorporation into RNA. Cells were treated with 10 μM [^{3}H]8-Cl-Ado for 20 h. Cells were lysed, the various RNA species were isolated, and the levels of tritium incorporation were quantitated.

S, and the 28 S rRNA products (Fig. 5*A*). Transcription of the 45 S molecule is completed in ~8 min with initial splice processing occurring before transcription is completed. The total processing takes ~1 h to complete in HeLa cells (29) and over 2 h in resting lymphocytes (30). Typically, equimolar amounts of the 28 S and 18 S rRNA are present in a cell, although, at the mass level, the amount of 28 S rRNA is twice that of 18 S rRNA. Because the 18 S rRNA is more 5′ of the rRNA gene, if transcription is inhibited by premature termination, then more 18 S than 28 S would be generated during this inhibition. In contrast, if the transcription inhibition were due to a slowing of the polymerase, then upon processing of the RNA, equimolar amounts of 18 and 28 S rRNA would be generated but would be less than that in the control cells.

With this in mind, we examined rRNA synthesis in MM.1S cells treated with 8-Cl-Ado. To visualize RNA that is synthesized during treatment (as the majority of the rRNA present in the cells would have been synthesized before drug treatment) [^{14}C]uridine was added during the final hour of treatment. The radiolabeling was followed with a 1 h chase with 5 μg/ml actinomycin D chase to allow processing of the freshly generated ^{14}C-labeled RNA while blocking any additional RNA labeling. On the ethidium bromide-stained gel (Fig. 5*B*), the ratio of 28 S to 18 S rRNA in the untreated cells was 1.8:1, which did not change after 8-Cl-Ado treatment (Fig. 5*C*). In contrast, the ratio of ^{14}C-labeled 28 S to 18 S rRNA did decrease after 8-Cl-Ado treatment. In the untreated cells, the ratio was 0.6:1 and decreased ~30% to 0.4:1 after 4-h 8-Cl-Ado treatment (Fig. 5*C*). These results demonstrate that although the ratio of 28 S to 18 S in total RNA does not significantly change after 8-Cl-Ado treatment, in the newly synthesized rRNA it does. The change in the ratio is due to a larger decrease in the generation of the 28 S rRNA as compared with 18 S, indicating that transcription is prematurely terminating. Furthermore, over time this decrease intensifies, suggesting that as the levels of 8-Cl-ATP increases in the cells, the amount of termination increases.

[4] C. M. Stellrecht, unpublished observations.

Mechanism for 8-Cl-Ado Transcription Termination. The premature transcription termination may be occurring at the position of the analogue incorporation by chain termination, or it is possible that it allows incorporation of an additional nucleotide(s) but then causes the polymerase to disassociate, *i.e.,* steric constraints. This issue was addressed by digesting [^{3}H]8-Cl-A(RNA) with micrococcal nuclease and spleen PDE. These enzymes specifically cleave at the 5′ position, leaving the phosphate group on the 3′ position of the preceding nucleotide; thus, the internal and terminal nucleotide will be liberated as a nucleotide monophosphate and nucleoside, respectively. After digestion, the resulting free nucleotides and nucleosides were resolved and quantitated by HPLC with an online scintillation counter. Initially, an ammonium acetate/methanol buffer system, which allows for the resolution of the monophosphate nucleotides from the nucleosides, was used (25). The results show that ~65% and ~30% of the radioactivity above background resolved with a ~24 and a ~26 min retention time, similar to nucleosides, whereas none resolved as a monophosphate, ~20 min (Fig. 6, *A* and *B*). In contrast, digest of [^{3}H]uridine-labeled RNA resolved primarily as UMP and, to a lesser extent, uridine (data not shown). Thus, it appears that the 8-Cl-Ado does not incorporate at internal positions of the transcripts as observed for uridine.

Interestingly, although 30% of the radioactive materials from the [^{3}H]8-Cl-A(RNA) digests corresponded to 8-Cl-Ado, ~65% resolved with a ~24 min retention time, similar to the retention times of 8-Cl-inosine and adenosine (Fig. 6*B*). To better resolve the adenosine from the 8-Cl-inosine, a methanol buffering system was used. Under these conditions, the digested [^{3}H]8-Cl-A(RNA) resolved as 8-Cl-Ado (~31 min) and adenosine (~27 min). For additional confirmation, the reactions were repeated without the addition of any adenosine deaminase inhibitors. If the peak in question is adenosine, then during the digestion it would be deaminated to inosine by the contaminating deaminases. In the absence of adenosine deaminase inhibitors, the peak in question resolved similarly to inosine (~20 min) indicating that originally it was adenosine. To try to understand why the tritiated RNA liberates a terminal radioactive adenosine we tested the possibility that this compound was dechlorinated *in vitro* during the harsh acidic phenol RNA extraction conditions. This does not appear to be the case, as [^{3}H]8-Cl-Ado placed in RNAzol overnight followed by the same extraction procedure use to isolate the RNA did not affect the HPLC resolution of the [^{3}H]8-Cl-Ado. Similarly, the nuclease digestion of [^{3}H]8-Cl-Ado also did not affect its resolution. These results indicate 8-Cl-Ado incorporates at the terminus, and a portion is dechlorinated to adenosine.

DISCUSSION

MM is an invariably fatal plasma cell malignancy with a median 3-year survival. This poor prognosis is primarily due to resistance that ultimately arises. MM is resistant *de novo* to DNA-directed purine nucleoside analogues that are active in other indolent B-cell malignancies. Surprisingly, we have demonstrated that the ribonucleoside analogue, 8-Cl-Ado, is cytotoxic in a number of MM cell lines that are resistant to traditional chemotherapeutic agents (4, 9, 13).

Inhibition of RNA synthesis may be an important mechanism in 8-Cl-Ado-induced cytotoxicity of MM cell. We have shown that before cell death, there is a progressive accumulation of the analogue triphosphate in the cells with a concurrent reduction in the ATP levels (Fig. 1; Ref. 13). This is associated with an inhibition of RNA synthesis, whereas DNA synthesis is not affected. With data presented in the present work and based on the actions of 8-Cl-Ado, it appears that this analogue has a multifactorial route for inhibition of RNA synthesis. First, the effect may be in part due to an overall decline in

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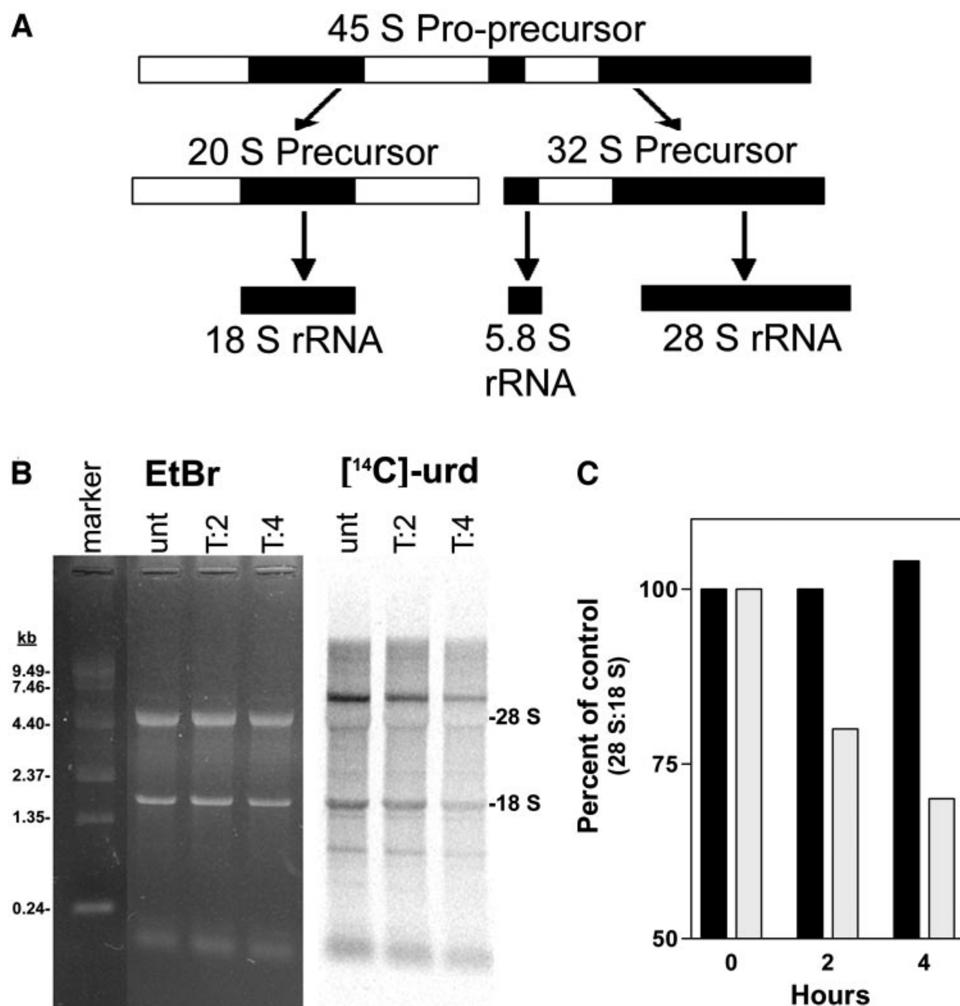

Fig. 5. Effects of 8-Cl-Ado on rRNA synthesis. *A*, schematic diagram of rRNA transcript processing. *B,* effect of 8-Cl-Ado on the production of newly synthesized rRNA. MM.1S cells were treated with 8-Cl-Ado for the indicated time with the addition of [^{14}C]uridine during the final hour. The radiolabeling was followed with a 1 h 5 mg/ml actinomycin D chase to allow processing of the ^{14}C-labeled RNA while blocking any additional RNA labeling. After electrophoresis, total RNA was visualized by ethidium bromide staining (*EtBr*), *left*. The newly synthesized and processed ^{14}C-labeled RNA was visualized by phosphorimaging, *right*. *Lanes* are as marked. *C,* quantitation of the newly synthesized 28 S rRNA as compared with 18S rRNA. The ratios of the 28 S rRNA and 18 S rRNA bands on the ethidium bromide stained gel, ■, or ^{14}C-labeled bands, □, were quantitated and expressed as a percentage of the ratio in untreated control cells.

the ATP pool, a precursor for RNA synthesis. Second, the action may be due, at least partially, to the incorporation of fraudulent nucleotide into RNA and prematurely terminating transcription. Finally, both the incorporation of the analogue and reduction in ATP pool may affect polyadenylation of mRNA transcript, which is required for full-length mRNA transcript processing. This latter action would predominantly affect mRNA synthesis.

To additionally understand the species-specific RNA synthesis inhibition, we examined the synthesis of the three main RNA forms, r, m, and sRNA, in 8-Cl-Ado-treated cells, as they would reflect the action of the analogue triphosphate on the activity of the three RNA polymerases. The incorporation of uridine revealed that compared with untreated cells, mRNA synthesis was inhibited the most in drug-treated cultures, followed by rRNA synthesis (Figs. 2 and 3). In contrast, negligible change in sRNA synthesis was seen after 8-Cl-Ado treatment. This differential and maximal effect of 8-Cl-Ado on mRNA synthesis may be due to several reasons. First, as demonstrated by our additional data (Fig. 4), there is a higher number of 8-Cl-AMP residues in mRNA species. Second, this maximal incorporation may reflect on the affinity of RNA Pol II to the analogue. Third, RNA Pol II may be more susceptible to a decline in the ATP pool. Fourth, the function of polyadenylase may be hampered due to the high ratio of 8-Cl-ATP to ATP, as reported for other analogues (31–34). Finally, in general and also in this cell line, the rate of mRNA synthesis is higher than that of rRNA (Fig. 2). Hence, this faster synthesis rate provides a favorable environment for increased analogue incorporation and maximal inhibition of the RNA species.

Additional examination of the mechanism for RNA synthesis inhibition indicates that incorporation of analogue results in premature termination of transcription. This was evidenced by the analysis of rRNA synthesis; 8-Cl-Ado treatment resulted in a greater loss of the rRNA 3′ splice product, 28 S, as compared with the 5′ 18 S splice product (Fig. 5). Several groups have shown both 28 S and 18 S rRNA have a long half-life in proliferating cells but are reduced under senescence (30, 35, 36). This effect would not pertain to the MM cells, because 8-Cl-Ado does not affect the cell cycle progression or distribution (13). During apoptosis after caspase 3 activation, a selective cleavage of the 28 S molecule occurs at a time coinciding with internucleosomal DNA fragmentation. This cleavage results in primarily the generation of ~3.5- and 1.2-kb fragments, which are readily discernable by ethidium bromide staining (37, 38). Similarly, double-stranded RNA induces RNase L-selective cleavage of 28 S rRNA into ~4- and 0.7-kb fragments (39). These types of degradation do not appear to be the reason for the phenomenon we observed. In 8-Cl-Ado-treated cells, we do not see a selective decrease in total 28 S rRNA as compared with 18 S rRNA by ethidium bromide staining, rather a decrease only in newly synthesized [^{14}C]uridine-labeled 28 S rRNA. If 28 S rRNA cleavage was occurring, the RNA that had been synthesized before the addition of [^{14}C]uridine would be equally affected. Moreover, the appearance of cleavage fragments was not detected either by ethidium bromide staining or by [^{14}C]uridine labeling. Additionally, internucleosomal DNA fragmentation of MM.1S cells does not appear until at least 16 h of 8-Cl-Ado treatment (9), yet we see a reduction of *de novo* 28 S production as early as 2 h of

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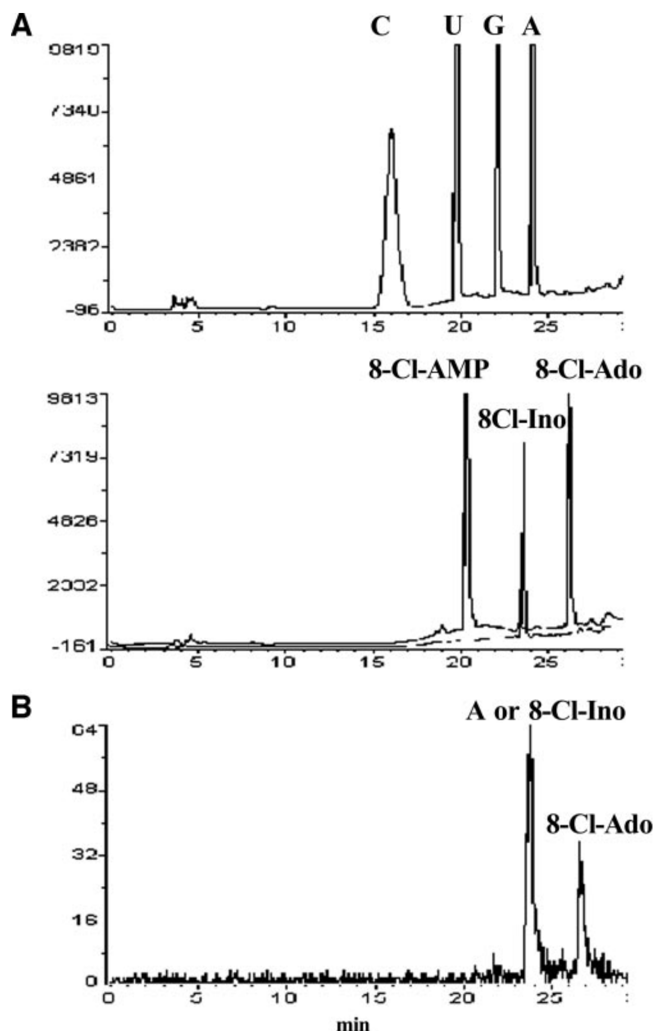

Fig. 6. Terminal incorporation of 8-Cl-AMP into RNA. *A,* HPLC elution profiles of ribonucleoside standards using ammonium acetate and methanol buffers; *top*, ribonucleoside standards; *bottom*, 8-Cl-Ado, 8-Cl-Ino, and 8-Cl-AMP ribonucleoside standards. *B,* HPLC analysis of liberated nucleosides from digests of $[^3H]$8-Cl-Ado-labeled RNA. Total RNA was isolated from MM.1S cells treated with 10 μM HPLC-purified $[^3H]$8-Cl-Ado for 8 h. 8-Cl-RNA was digested with micrococcal nuclease and spleen PDE in the presence of deoxycoformycin. Digests were then separated by HPLC using ammonium acetate and methanol buffers.

8-Cl-Ado treatment. Hence, the temporal sequence of events indicates that 28 S apoptotic cleavage is not the cause of the decline of this rRNA subunit. Instead, the kinetics of the *de novo* 28 S reduction parallels the accumulation of 8-Cl-ATP, additionally corroborating that this is due to premature termination of transcription.

Confirmation for this apparent premature transcription termination by 8-Cl-Ado comes from the analysis of the position of analogue incorporation. Analysis of digested, 8-Cl-Ado-incorporated total RNA, as well as both mRNA and rRNA[2] demonstrated incorporation of 8-Cl-Ado in the 3′ terminus. Surprisingly, the tritiated drug was often released as adenosine instead of 8-Cl-Ado. Because the HPLC-purified $[^3H]$8-Cl-Ado was not contaminated with $[^3H]$Ado, and $[^3H]$AMP was not detected in the digested RNA, the data indicate that the dechlorination of $[^3H]$8-Cl-Ado is occurring after incorporation into RNA. Whether this dechlorination occurs *in vivo* or *in vitro* still remains to be determined. Nonetheless, absence of any $[^3H]$monophosphate peak, as seen with $[^3H]$uridine-labeled RNA clearly demonstrates terminal incorporation of 8-Cl-Ado in this cell line.

The finding of 8-Cl-Ado-induced chain termination is intriguing as to why RNA polymerases do not add additional nucleotides after insertion of the analogue. One possibility may be due to the nonconventional conformational change adopted by the analogue as seen in halogenated purines (40–42) including 8-Cl-Ado/8-Cl-AMP (43–45). This change is the result of base rotations to a syn conformation induced by the chlorine substitution and would be expected to prevent the typical hydrogen bonding between the new transcript and the DNA template at the site of adenine-thymine base pairing. The lack of this base pairing may hamper the polymerase from reading and incorporating the next nucleotide in the elongating RNA chain. Indeed, 8-Cl-AMP has been found to destabilize an RNA duplex when base-paired to UMP (46).

Needless to say, the RNA chain termination induced by 8-Cl-Ado treatment of MM cells inhibits RNA synthesis. The highest level of inhibition was seen in the mRNA population. This is likely to result in a significant loss of expression of genes with short mRNA half-lives. The loss of expression of critical MM survival genes such as *Mcl-1* (47), *Met* (48), and $Bcl\text{-}X_L$ (49) would then lead to cell death. Analyses of 8-Cl-Ado-induced changes in gene expression in MM cells are currently underway.

In addition to overcoming constitutively active survival pathways, the transcriptional inhibitory activities of 8-Cl-Ado may also be effective in overcoming cellular defenses induced during treatment with other chemotherapeutic agents, thus increasing the cytotoxicity when used in combination with 8-Cl-Ado. Evidence for this was seen in combinational studies of 8-Cl-cAMP with paclitaxel or cisplatin (50, 51). To use the transcription inhibition action of 8-Cl-Ado, additional agents need to be tested in combination with this analogue.

In conclusion, 8-Cl-Ado inhibits RNA transcription by incorporating into the transcript causing chain termination. Furthermore, the incorporation was the highest in transcripts polymerized by Pol II resulting in maximal inhibition of mRNA synthesis. This is likely to result in a significant loss of gene expression. Thus, 8-Cl-Ado cytotoxicity may be the result of diminished expression of key survival genes to below levels critical for sustaining cell viability.

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