Published in final edited form as:

Breast Cancer Res Treat. 2010 June ; 121(2): 355–364. doi:10.1007/s10549-009-0481-3.

A unique RNA-directed nucleoside analog is cytotoxic to breast cancer cells and depletes cyclin E levels

Christine M. Stellrecht¹, Mary Ayres¹, Rishi Arya¹, and Varsha Gandhi^{1,2}

¹Department of Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center, Houston, USA

²Department of Leukemia, University of Texas M.D. Anderson Cancer Center, Houston, USA

Abstract

In contrast to deoxyribose or arabinose containing nucleoside analogs that are currently established for cancer therapeutics, 8-chloro-adenosine (8-Cl-Ado) possesses a ribose sugar. This unique nucleoside analog is RNA-directed and is in a phase I clinical trial for hematological malignancies. RNA-directed therapies are effective for the treatment of many malignancies as their activities are primarily aimed at short-lived transcripts, which are typically encoded by genes that promote the growth and survival of tumor cells such as cyclin E in breast cancer. Based on this, we hypothesized that 8-Cl-Ado, a transcription inhibitor, will be effective for the treatment of breast cancer cells. The metabolism of 8-Cl-Ado and the effect on ATP in the breast cancer cell lines MCF-7 and BT-474 were measured using HPLC analysis. In these cells, 8-Cl-Ado was effectively taken up, converted to its cytotoxic metabolite, 8-Cl-ATP, and depleted the endogenous ATP levels. This in turn led to an inhibition of RNA synthesis. The RNA synthesis inhibition was associated with a depletion of cyclin E expression, which is indicative of a diminished tumorigenic phenotype. The final outcome of 8-Cl-Ado treatment of the breast cancer cells was growth inhibition due to an induction of apoptosis and a loss of clonogenic survival. These results indicate that 8-Cl-Ado, which is currently in clinic for hematological malignancies, may be an effective agent for the treatment of breast cancer.

Keywords

8-chloro-adenosine; Breast cancer; Nucleoside analog; RNA synthesis inhibition; Cyclin E

Introduction

Transcription is an emerging target for cancer therapeutics [1, 2]. Though RNA-directed agents globally affect RNA synthesis, their activities are most effective against transcripts with high turn-over rates. These short-lived transcripts are typically encoded by oncogenes as well as by regulators of other key processes such as cell proliferation, angiogenesis, and cell survival [3, 4]. Therefore, treatments that impede mRNA synthesis would be expected to selectively hinder their expression and, furthermore, be detrimental to neoplastic cells that are dependent on or addicted to the activity of these genes for their tumorigenicity [5, 6, 7, 8].

Though many chemotherapeutic agents currently used in the clinic have some inhibitory effects on transcription [2], it is a primary mechanism of action for the cdk7/9 inhibitors flavopiridol, Seliciclib, and SNS-032, as they prevent transcription initiation and elongation by inhibiting the phosphorylation of RNA Pol II on serine residues. In chronic lymphocytic leukemia (CLL), mantle cell lymphoma, and multiple myeloma (MM), this results in the depletion of the short-lived survival factors *MCL-1*, *XIAP*, and *MET*, which are crucial for the survival of these cells [9, 10, 11, 12, 13, 14, 15]. Furthermore, these agents have shown potent cytotoxic activity during preclinical investigations and encouraging results in clinical trials on these malignancies [10, 16].

The ribonucleoside analog, 8-chloro-adenosine (8-Cl-Ado), exhibits a strong potential for use as an effective, RNA-directed therapeutic agent and is currently in a Phase I clinical trial for the treatment of CLL. 8-Cl-Ado is cytotoxic to primary CLL cells [17], as well as to glioma [18, 19, 20], MM [21, 22, 23], myeloid leukemia [24], colon [25], and lung [26] cancer cell lines. We have shown that 8-Cl-Ado cytotoxicity is contingent on its intracellular phosphorylation to 8-Cl-AMP by adenosine kinase [21], which is followed by the formation of 8-Cl-ATP. Due to adenosine kinase's high specific activity and substantial substrate specificity for 8-Cl-Ado, in hematopoietic cells, 8-Cl-ATP accumulates to near millimolar levels [17, 21]. Parallel to 8-Cl-ATP accumulation, the endogenous ATP pool decreases, resulting in a high ratio of 8-Cl-ATP to ATP. The net effect of this is a decline in RNA synthesis. The mechanism by which the analog inhibits RNA synthesis is via incorporation of 8-Cl-ATP by RNA Pol into RNA. Of the various species of RNA, mRNA synthesis is inhibited the most due to the preferential incorporation by RNA Pol II into the body of the mRNA and the likely detrimental effects on poly(A) polymerase [27, 28]. These actions lead to premature termination of transcription [27]. Furthermore, in both primary CLL cells and MM cells, we showed that 8-Cl-Ado induces apoptosis by diminishing the expression of genes consequential for their survival [17, 29].

In breast cancer, numerous studies have established a critical role that cyclin E plays in tumorigenesis, progression, and chemotherapeutic resistance, in particular, resistance to hormone-directed therapies. First, while in normal cells cyclin E in conjunction with CDK2 regulates the G₁ to S phase transition of the cell cycle, deregulation of cyclin E by various alterations such as the generation of low-molecular-weight (LMW) isoforms, gene amplification [30, 31] and mutations of its ubiquitin ligase, *CDC4* [32], has been directly implicated in breast cancer tumorigenesis. Second, deregulation of this cyclin has been shown to cause aberrant execution of mitosis [33], leads to premature degradation of cyclin B, shortens the G₂/M phase of the cell cycle, interferes with prereplication complex assembly, increases centrosome numbers, and induces chromosome instability, generating aneuploidy and multinucleated cells [34, 35, 36]. Third, high expression levels of cyclin E isoforms have not only been associated with a poorer prognosis [37, 38], but it has also been suggested to be the single most significant predictor of survival in node-negative breast tumors [39]. Fourth, tumor-specific LMW isoforms-CDK2 complexes exhibit higher kinase activity when compared to full-length cyclin E-CDK2 [40]. Fifth, due to their strong binding ability and resistance to the actions of the CDK inhibitors, p21 and p27, the LMW isoforms may sequester these inhibitors away from full-length cyclin E [34, 41]. Finally, overexpression of cyclin E in the mammary epithelial cells of transgenic mice induces tumor

formation and metastasis, both of which are markedly increased in mice specifically expressing the LMW isoforms [42]. Collectively, these data demonstrate the strong role of cyclin E in breast cancer tumorigenicity and underscore the desperate need for agents that are able to target cyclin E expression for the treatment of this disease.

Cyclin E mRNA and protein are known to have a very short half-life ($t_{1/2}$ 30 min) [30, 43]; thus, is a potential target of transcription inhibitors. In the present study, we have examined the metabolism and mechanism of action of 8-Cl-Ado in breast cancer cell lines and assessed its ability to inhibit the expression of the cyclin E gene, *CCNE1*.

Materials and methods

Materials

8-Cl-Ado was obtained from Dr. V. Rao at the Drug Development Branch of the National Cancer Institute. [5, 6-³H]uridine (~40 Ci/mM, 1 mCi/ml) was purchased from Moravek Biochemicals (Brea, CA). All of the other chemicals were of reagent grade. Primary antibodies used were rabbit polyclonal antibodies to BCL-2, MCL-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and survivin (R&D Systems Inc., Minneapolis, MN) and mouse monoclonal antibodies to poly-ADP-ribose polymerase (PARP) clone C2-10, cleaved PARP clone F21-852, BCL-X_L, XIAP, (BD Bioscience), cyclin E (Santa Cruz), and GAPDH clone 6C6 (Abcam, Inc, Cambridge, MA).

Cell lines and culture

The MCF-7 and BT-474 cell lines were obtained from the ATCC (Manassas, VA) and maintained in DMEM:F12 (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum in the presence of 5% CO₂ at 37°C. Cells were routinely tested for *Mycoplasma* infection using a commercially available kit (Invitrogen, Carlsbad, CA).

Measurement of intracellular nucleoside triphosphate by HPLC

Perchloric acid was used to extract nucleotides from MCF-7 and BT-474 cells treated with 10 μM 8-Cl-Ado, and neutralized extracts were analyzed using HPLC (Waters 600E System Controller; Waters Corp., Milford, MA, USA) as described previously [17]. The column eluate was monitored using UV absorption at 256 nm, and the nucleoside triphosphates were quantitated using 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.

Inhibition of RNA synthesis

MCF-7 and BT-474 cells were incubated with 10 μM 8-Cl-Ado for up to 12 h with the addition of 2 μCi/ml [³H]uridine 30 min prior to harvesting. Acid-insoluble cellular material was retained on multiscreen-GV filters (Millipore Corp., Bedford, MA) as described [27]. The radioactivity on the filters was measured by scintillation counting and expressed as the percentage of control (untreated) value of cells. All statistical and regression analyses were determined using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Growth inhibition analysis

For the MTS assay, 600 MCF-7 cells or 2,000 BT-474 cells were plated in 100- μ l FBS supplemented media per well of a 96-well plate. Cells were grown for 5 days, and 8-ClAdo was added sequentially allowing all time points to be assayed simultaneously using AQueous OneSolution (Promega, Madison, WI) as per the manufacturer's instructions. Each time point in an individual experiment was assayed in triplicate and the experiment was performed in triplicate. The results are presented as a percentage of the untreated cells.

Colony formation assay

One thousand exponentially growing MCF-7 cells were plated in triplicate into 6-well dishes. The cells were allowed to attach for 24 h and were then exposed to 8-Cl-Ado for 3 days before being washed with PBS (37°C) and cultured in fresh medium for 14 days. Colonies of 50 cells were counted under a dissecting microscope. The experiments were performed in triplicates.

Flow cytometry

MCF-7 and BT-474 cells were incubated with 10 μ M 8-Cl-Ado for the indicated times. The cell cycle distribution and the annexin V and propidium iodide labeling of the cells were determined, respectively, as described in [21] and [9]. All flow cytometry analysis was performed using a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA, USA).

Expression analysis

Exponentially growing cells were treated with 10- μ M 8-Cl-Ado for various amounts of times, and the RNA was isolated with the RNeasy Mini kit (Qiagen S.A., Courtabeuf, France) in duplicate. The gene expression levels were measured on an ABI prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using one-step realtime TaqMan RT-PCR as described [29]. The *CCNE1* Taqman primers and probes (Sigma GenoSys, Woodlands, TX) were designed with PrimerExpress software (Applied Biosystems) and are as follows: Forward and reverse primers 5'-TCGCTGATGAAGATGCACACA-3' and 5'-TTCTTTGCTCGGGCTTTGTC-3', Taqman probe 5'-6FAM-AGCAAATCCAAGCTGTCTCTGTGGGTCTGTAT-BHQ1-3'. Each RNA sample was assayed in triplicate, and the results are presented as a percentage of the *CCNE1* expression level in untreated cells. Western blot analysis using an Odyssey Infrared Imaging System (LI-COR Biosciences) was performed as described [29].

Results

Time-dependent metabolism of 8-Cl-Ado in breast cancer cells

Typically, DNA-directed nucleoside analogs are not as effective in the treatment of solid tumors when compared to hematological malignancies. This is primarily due to the dependency of these analogs on deoxycytidine kinase for their conversion to an active metabolite, which is expressed at low levels in solid tumors [44, 45]. Previously, we have shown that 8-Cl-Ado (Fig. 1a) is cytotoxic to primary chronic lymphocytic leukemia cells [17] and multiple myeloma cell lines [23] and that 8-Cl-Ado cytotoxicity is dependent on its

metabolism to its triphosphate by adenosine kinase [21]. Unlike deoxycytidine kinase, the expression of adenosine kinase is more ubiquitous and is relatively abundant in breast cancer cells [46]. This suggests that in these cells, 8-Cl-Ado would be readily metabolized to its active triphosphate form. To address this question, MCF-7 and BT-474 cells were treated with 10 μ M 8-Cl-Ado for various times and perchloric acid extracts were analyzed using HPLC. In both the MCF-7 and the BT-474 cells, there was a rapid time-dependent increase in the intracellular level of the active metabolite, 8-Cl-ATP, (Fig. 1b, c) with the majority of the accumulation occurring in the first 4 h of treatment and by 6 h reached its plateau of ~100 and ~300 μ M, respectively. Concomitantly, there was a steady decline in the level of endogenous ATP, which reached ~40 and ~60% of the initial control levels (MCF-7 = 340 μ M and BT-474 = 1,575 μ M) by 12 h (Fig. 1b, c).

Effect of 8-Cl-ATP on RNA synthesis

Since 8-Cl-ATP is an analog of ATP, we investigated whether it had any effect on RNA synthesis. To determine the relative amount of RNA synthesis, cells were incubated with 10 μ M 8-Cl-Ado for the indicated times; however, during the last 30 min of incubation, radiolabeled uridine was added to the medium. The level of radiolabeled uridine incorporated into RNA was then compared at each time point to untreated control cells. The results indicate that there was a time-dependent decline in RNA synthesis resulting in a 50% decrease by 24 h (Fig. 2a).

Previously we showed that 8-Cl-ATP is able to incorporate into RNA and terminate transcription [27], while the decrease in ATP should also affect the cells' ability to synthesize RNA. If the RNA synthesis inhibition is dependent on one or both of these, then there should be a relationship with the accumulation of 8-Cl-ATP and/or the cellular level of ATP. To determine this, a comparison of the percent inhibition of RNA synthesis to 8-Cl-ATP concentration was made (Fig. 2b). There was a strong linear relationship between inhibition of RNA synthesis and 8-Cl-ATP accumulation in MCF-7 ($r^2 = 0.75$; $p < 0.0001$) and BT-474 cells ($r^2 = 0.80$; $p < 0.0001$). This indicates that the inhibition of RNA synthesis is dependent on the accumulation of 8-Cl-ATP. Additionally, because 8-Cl-ATP may act competitively against ATP for use during transcription, we examined the ratio of 8-Cl-ATP to ATP over time (Fig. 2c) as well as the relationship of this ratio to RNA synthesis inhibition (Fig. 2d). The results showed that inhibition attained a maximum as the ratio of 8-Cl-ATP:ATP reached ~0.5. Moreover, there was an exponential association between this ratio and RNA synthesis inhibition in MCF-7 ($r^2 = 0.83$) and BT-474 ($r^2 = 0.79$) cells, resembling a pseudo first-order enzyme inhibition reaction. This suggests that the ratio of 8-Cl-ATP:ATP plays an important role in RNA synthesis inhibition.

8-Cl-Ado inhibits cell growth and/or survival

The growth inhibitory effects of 8-Cl-Ado have been reported in lung [26], colon [25, 47], glioma [18, 20], and malignant hematopoietic cell lines [17, 21, 23, 24, 48]. To investigate the growth inhibitory effects of 8-Cl-Ado on breast cancer cells, MCF-7 and BT-474 cells were incubated with various concentrations of 8-Cl-Ado (0.1–100 μ M) for 5 days. At the end of the incubation time, growth inhibition was measured using the MTS cell titer assay. We found that growth was inhibited by as little as 0.1 μ M 8-Cl-Ado in both cell lines (Fig.

3a) with an IC_{50} for growth inhibition of 0.3 and 0.55 μM in the BT-474 and MCF-7 cells, respectively. To examine the kinetics of the growth inhibitory activities of 8-Cl-Ado, the cells were treated with 10 μM 8-Cl-Ado for various times and again measured using the MTS assay. Within 1 day of treatment, the growth of both breast cancer cells was inhibited. The kinetics of the inhibition were virtually identical between the MCF-7 and BT-474 cells and reached ~90% inhibition by day 5. These results indicate that the effects of 8-Cl-Ado on the growth of MCF-7 and BT-474 cells are both dose- and time-dependent.

8-Cl-Ado does not alter cell cycle progression

To determine whether the growth inhibition of 8-Cl-Ado is due to cytostatic effects, exponentially growing MCF-7 and BT-474 cells were treated with 10 μM 8-Cl-Ado for various times, and their cell cycle profile was assessed using flow cytometry. In untreated MCF-7 cells, the average percentage of cells in G_1 , S, or G_2/M phase was 62, 14, and 24%, respectively (Fig. 4a). During the 3-day incubation, there were no statistically significant changes in any phase of the cell cycle. In untreated BT-474 cells, the average percentages in G_1 , S, and G_2/M phase were 71, 11, and 18%, respectively (Fig. 4b). Similar to the MCF-7 cells, there were no significant changes in G_1 or S phase of the cell cycle during the 3-day incubation. There was a statistically significant decrease in the percentage of cells in G_2/M phase during this time period, 17.0–10.4% ($p < 0.05$), which can be attributed to an increase in the sub G_1 fraction of cells. Because the 8-Cl-Ado treatment of the breast cancer cells did not result in an accumulation of cells in any particular phase of the cell cycle, this suggests that the growth inhibition seen in Fig. 3 was not attributable to cytostatic growth; but more likely to loss of cell viability.

8-Cl-Ado is cytotoxic to breast cancer cells

To determine whether the effects of 8-Cl-Ado on breast cancer cells are associated with a decrease in cellular reproductive viability, clonogenicity assays were performed in MCF-7 cells. The results showed that 8-Cl-Ado significantly decreased clonogenic viability in a dose-dependent manner ($IC_{50} = 0.75 \mu M$) (Fig. 5a). At concentrations of 10 μM 8-Cl-Ado, clonogenic viability was inhibited by >85% and reached ~95% inhibition with 100 μM . To ascertain that the loss in viability was due to an apoptotic response to 8-Cl-Ado treatment, the cells were examined for evidence of PARP cleavage using western blot analysis. Within 2 days of 10 μM 8-Cl-Ado treatment, PARP cleavage was readily detected in both cell lines (Fig. 5b). Likewise, flow cytometry analysis of both cell lines treated with 10 μM 8-Cl-Ado showed that there was an induction of annexin V and/or propidium iodide-positive cells within 1 day of treatment (Fig. 5c). These experiments establish that the growth inhibition associated with 8-Cl-Ado is due to a loss in cell viability due at least in part with an induction of apoptosis.

8-Cl-Ado inhibits cyclin E expression

Since therapeutic agents targeting cyclin E would be advantageous for the treatment of breast cancer and because *CCNE1* mRNA is rapidly turned over (half-life as short as 30 min) [30], we hypothesized that 8-Cl-Ado would inhibit the expression of cyclin E. To examine the effects of 8-Cl-Ado on cyclin E expression and to quantitate the inhibition kinetics, MCF-7 and BT-474 cells were treated with 10 μM 8-Cl-Ado for 48 h and *CCNE1*

mRNA levels were measured using real-time RT-PCR after various times of treatment. The results demonstrated that the *CCNE1* transcript levels decline in both cell lines and that this inhibition begins within the first 4 h of treatment (Fig. 6a) and continued to decrease up to ~60% by 24 h.

Additionally, a block in *CCNE1* mRNA synthesis would be expected to instigate a decrease in cyclin E protein levels as it also has a short half-life [43]. To determine this, BT-474 and MCF-7 cells were treated with 10 μ M 8-Cl-Ado and the levels of cyclin E were measured using immunoblot analysis. The analysis revealed that both the full-length 50-kDa cyclin E and the ~40-kDa LMW cyclin E isoform levels were diminished in these cells after 8-Cl-Ado treatment (Fig. 6a). Overall, these results demonstrate that 8-Cl-Ado treatment diminishes the expression levels of *CCNE1* mRNA and protein in breast cancer cells.

Effects of 8-Cl-Ado on the expression of survival factors

To determine whether 8-Cl-Ado-induced cytotoxicity was due to the depletion of an anti-apoptotic factor, the levels of MCL-1, BCL-2, BCL-X_L, survivin, and XIAP were assessed in MCF-7 and BT-474 cells using western blot analysis after 24-h treatment with increasing dosage of 8-Cl-Ado. Interestingly, the expression of these anti-apoptotic genes did not significantly change after 8-Cl-Ado treatment (Fig. 7a, b). Thus, 8-Cl-Ado-induced cytotoxicity does not appear to involve the depletion of any of these five survival factors but is likely through the loss of another factor(s) required for breast cancer cell survival.

Discussion

We show that 8-Cl-Ado has significant *in vitro* activity against breast cancer cells as it inhibits the growth and survival of MCF-7 and BT-474 cells when measured using MTS (Fig. 3) and clonogenic survival (Fig. 5a) assays. This is due, at least in part, to an induction of apoptosis as determined using PARP cleavage analysis and annexin V staining (Fig. 5b, c) but not to any blocks in the progression of the cell cycle (Fig. 4). Therefore, these results indicate that in breast cancer cells 8-Cl-Ado is primarily cytotoxic as opposed to cytostatic.

During treatment, 8-Cl-Ado is taken up by the cells and is phosphorylated to its cytotoxic moiety, 8-Cl-ATP (Fig. 1b) while concomitantly diminishing the endogenous ATP pool (Fig. 1c). Although the exact mechanism of 8-Cl-Ado-mediated cell death in these cells is not yet elucidated, the depletion of cellular ATP pool is expected to be a contributing factor as depletion of greater than 70% of the ATP levels in MCF-7 cells has been shown to inhibit clonogenic survival [49] and induce apoptosis [50]. A key contributor to the drug's cytotoxic activity likely involves 8-Cl-Ado-mediated inhibition of RNA synthesis as this may lead to depletion of specific transcript(s) required for tumor cell survival. Both the accumulation of 8-Cl-ATP and the decrease in the cellular ATP pool may play a role in the inhibition of RNA synthesis. ATP is not only a substrate for RNA polymerases, but it also promotes activation of these enzymes. We have found that 8-Cl-ATP is a substrate for all three human RNA polymerases, in particular RNA polymerase II, which leads to incorporation of the analog into the RNA chain, prematurely terminating transcription [27]. Furthermore, 8-Cl-ATP incorporation inhibits polyadenylation, a step required for synthesis and stability of the

majority of mRNA transcripts [28]. These actions lead to a greater inhibition of mRNA transcription when compared to other RNA species. In breast cancer cells, we have found a strong linear relationship between inhibition of RNA synthesis and 8-Cl-ATP accumulation, while there was an exponential association with the 8-Cl-ATP:ATP ratio (Fig. 2d). These results suggest that the 8-Cl-ATP:ATP ratio may be a key inhibitor of RNA synthesis in these cells.

The development of agents capable of targeting cyclin E is highly desirable for the treatment of breast cancer [51] as well as for other types of tumors with dysregulated cyclin E [52, 53, 54]. Deregulation of cyclin E and its LMW isoforms [30, 31] induces chromosome instability [34, 35, 36] and promotes breast cancer tumorigenesis and progression. Additionally, high expression levels of cyclin E isoforms have been associated with a poorer prognosis [37, 38] and resistance to hormone-directed therapies [34]. We have shown that 8-Cl-Ado treatment diminishes cyclin E levels in breast cancer cells (Fig. 6); thus, is an effective agent for targeting cyclin E. In contrast, the expression of several small survival factors was not inhibited by 8-Cl-Ado in these cells (Fig. 7). Similar results were also seen in 8-Cl-Ado treatment of MM cells [29]. The reason for this is not known but possibly indicates that in addition to the turn-over rate, transcript size may also play an important role in 8-Cl-Ado-mediated transcription repression.

In conclusion, our data demonstrate that 8-Cl-Ado was effectively taken up, converted to its cytotoxic metabolite, 8-Cl-ATP, and depleted the endogenous ATP levels in breast cancer cells. This in turn leads to an inhibition of both RNA synthesis and cyclin E expression with the final outcome being loss of the tumor cell survival. This cytotoxic effect indicates that the analog may be an effective agent for the treatment of breast cancer. Additionally, because RNA-directed therapeutic agents exploit tumor cells' dependency or addiction [5, 7, 8] on short-lived oncogenes, growth regulators, and survival factors, 8-Cl-Ado is likely to alter the expression of a yet to be determined gene required for the survival of these cells.

Acknowledgments

This study was supported by Grants KG080366 from the Susan G. Komen for the Cure and CA85915 from the National Cancer Institute.

References

1. Stellrecht, CM., Chen, LS., Gandhi, V. Inhibition of oncogene expression by RNA-directed agents. In: Gandhi, V., editor. Transcription as a target for cancer therapeutics AACR educational book. American Association for Cancer Research; Philadelphia: 2005. p. 338-343.
2. Derheimer FA, Chang CW, Ljungman M. Transcription inhibition: a potential strategy for cancer therapeutics. *Eur J Cancer*. 2005; 41:2569–2576. [PubMed: 16213135]
3. Bakheet T, Williams BR, Khabar KS. ARED 2.0: an update of AU-rich element mRNA database. *Nucleic Acids Res*. 2003; 31:421–423. [PubMed: 12520039]
4. Lam LT, Pickeral OK, Peng AC, et al. Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. *Genome Biol*. 2001; 2 research0041.0041-research0041.0011.
5. Weinstein IB. Addiction to oncogenes—the Achilles heel of cancer. *Science*. 2002; 297:63–64. [PubMed: 12098689]
6. Weinstein IB, Joe A. Oncogene addiction. *Cancer Res*. 2008; 68:3077–3080. [PubMed: 18451130]

7. Weinstein B. Relevance of the concept of oncogene addiction to hormonal carcinogenesis and molecular targeting in cancer prevention and therapy. *Adv Exp Med Biol.* 2008; 617:3–13. [PubMed: 18497026]
8. Certo M, del Moore VG, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell.* 2006; 9:351–365. [PubMed: 16697956]
9. Chen R, Wierda WG, Chubb S, et al. Mechanism of action of SNS-032, a novel cyclin-dependent kinase inhibitor, in chronic lymphocytic leukemia. *Blood.* 2009; 113:4637–4645. (<https://doi.org/10.1182/blood-2008-12-190256>). DOI: 10.1182/blood-2008-12-190256 [PubMed: 19234140]
10. Chen R, Keating MJ, Gandhi V, Plunkett W. Transcription inhibition by flavopiridol: mechanism of chronic lymphocytic leukemia cell death. *Blood.* 2005; 106:2513–2519. [PubMed: 15972445]
11. Gojo I, Zhang B, Fenton RG. The cyclin-dependent kinase inhibitor flavopiridol induces apoptosis in multiple myeloma cells through transcriptional repression and down-regulation of Mcl-1. *Clin Cancer Res.* 2002; 8:3527–3538. [PubMed: 12429644]
12. Pepper C, Thomas A, Hoy T, Fegan C, Bentley P. Flavopiridol circumvents Bcl-2 family mediated inhibition of apoptosis and drug resistance in B-cell chronic lymphocytic leukaemia. *Br J Haematol.* 2001; 114:70–77. [PubMed: 11472347]
13. Phillip CJ, Stellrecht CM, Nimmanapalli R, Gandhi V. Targeting MET transcription as a therapeutic strategy in multiple myeloma. *Cancer Chemother Pharmacol.* 2009; 63:587–597. (<https://doi.org/10.1007/s00280-008-0770-2>). DOI: 10.1007/s00280-008-0770-2 [PubMed: 18509644]
14. Raje N, Kumar S, Hideshima T, et al. Seliciclib (CYC202 or R-roscovitine), a small-molecule cyclin-dependent kinase inhibitor, mediates activity via down-regulation of Mcl-1 in multiple myeloma. *Blood.* 2005; 106:1042–1047. [PubMed: 15827128]
15. MacCallum DE, Melville J, Frame S, et al. Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. *Cancer Res.* 2005; 65:5399–5407. [PubMed: 15958589]
16. Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. *J Clin Oncol.* 2006; 24:1770–1783. [PubMed: 16603719]
17. Balakrishnan K, Stellrecht CM, Genini D, et al. Cell death of bioenergetically compromised and transcriptionally challenged CLL lymphocytes by chlorinated ATP. *Blood.* 2005; 105:4455–4462. (<https://doi.org/10.1182/blood-2004-05-1699>). DOI: 10.1182/blood-2004-05-1699 [PubMed: 15718423]
18. Langeveld CH, Jongenelen CA, Theeuwes JW, et al. The antiproliferative effect of 8-chloro-adenosine, an active metabolite of 8-chloro-cyclic adenosine monophosphate, and disturbances in nucleic acid synthesis and cell cycle kinetics. *Biochem Pharmacol.* 1997; 53:141–148. S0006-2952(96)00593-X[pil] ([https://doi.org/S0006-2952\(96\)00593-X%5Bpii%5D](https://doi.org/S0006-2952(96)00593-X%5Bpii%5D)). [PubMed: 9037246]
19. Langeveld CH, Jongenelen CA, Heimans JJ, Stoof JC. 8-Chloro-cyclic adenosine monophosphate, a novel cyclic AMP analog that inhibits human glioma cell growth in concentrations that do not induce differentiation. *Exp Neurol.* 1992; 117:196–203. 0014-4886(92)90127-C[pil] ([https://doi.org/0014-4886\(92\)90127-C%5Bpii%5D](https://doi.org/0014-4886(92)90127-C%5Bpii%5D)). [PubMed: 1379937]
20. Langeveld CH, Jongenelen CA, Heimans JJ, Stoof JC. Growth inhibition of human glioma cells induced by 8-chloroadenosine, an active metabolite of 8-chloro cyclic adenosine 3':5'-monophosphate. *Cancer Res.* 1992; 52:3994–3999. [PubMed: 1617676]
21. Gandhi V, Ayres M, Halgren RG, et al. 8-chloro-cAMP and 8-chloro-adenosine act by the same mechanism in multiple myeloma cells. *Cancer Res.* 2001; 61:5474–5479. [PubMed: 11454694]
22. Krett NL, Zell JL, Halgren RG, et al. Cyclic adenosine-3', 5'-monophosphate-mediated cytotoxicity in steroid sensitive and resistant myeloma. *Clin Cancer Res.* 1997; 3:1781–1787. [PubMed: 9815564]
23. Halgren RG, Traynor AE, Pillay S, et al. 8Cl-cAMP cytotoxicity in both steroid sensitive and insensitive multiple myeloma cell lines is mediated by 8Cl-adenosine. *Blood.* 1998; 92:2893–2898. [PubMed: 9763575]

24. Zhu B, Zhang LH, Zhao YM, Cui JR, Strada SJ. 8-chloroadenosine induced HL-60 cell growth inhibition, differentiation, and G(0)/G(1) arrest involves attenuated cyclin D1 and telomerase and up-regulated p21(WAF1/CIP1). *J Cell Biochem.* 2006; 97:166–177. [PubMed: 16173047]
25. Carlson CC, Chinery R, Burnham LL, Dransfield DT. 8-Cl-adenosine-induced inhibition of colorectal cancer growth in vitro and in vivo. *Neoplasia.* 2000; 2:441–448. [PubMed: 11191111]
26. Zhang HY, Gu YY, Li ZG, et al. Exposure of human lung cancer cells to 8-chloro-adenosine induces G2/M arrest and mitotic catastrophe. *Neoplasia.* 2004; 6:802–812. [PubMed: 15720807]
27. Stellrecht CM, Rodriguez CO Jr, Ayres M, Gandhi V. RNA-directed actions of 8-chloro-adenosine in multiple myeloma cells. *Cancer Res.* 2003; 63:7968–7974. [PubMed: 14633728]
28. Chen LS, Sheppard TL. Chain termination and inhibition of *Saccharomyces cerevisiae* poly(A) polymerase by C-8-modified ATP analogs. *J Biol Chem.* 2004; 279:40405–40411. [PubMed: 15265873]
29. Stellrecht CM, Phillip CJ, Cervantes-Gomez F, Gandhi V. Multiple myeloma cell killing by depletion of the MET receptor tyrosine kinase. *Cancer Res.* 2007; 67:9913–9920. (<https://doi.org/10.1158/0008-5472.CAN-07-0770>). DOI: 10.1158/0008-5472.CAN-07-0770 [PubMed: 17942923]
30. Keyomarsi K, Pardee AB. Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc Natl Acad Sci U S A.* 1993; 90:1112–1116. [PubMed: 8430082]
31. Keyomarsi K, O'Leary N, Molnar G, et al. Cyclin E, a potential prognostic marker for breast cancer. *Cancer Res.* 1994; 54:380–385. [PubMed: 7903908]
32. Reed, SI. Deregulation of cyclin E in cancer. In: Reed, SI., editor. *The ubiquitin proteasome system: roles and targets in cancer AACR educational book.* American Association for Cancer Research; Philadelphia: 2005. p. 53-56.
33. Keck JM, Summers MK, Tedesco D, et al. Cyclin E overexpression impairs progression through mitosis by inhibiting APCCdh1. *J Cell Biol.* 2007; 178:371–385. [PubMed: 17664332]
34. Akli S, Zheng PJ, Multani AS, et al. Tumor-specific low molecular weight forms of cyclin E induce genomic instability and resistance to p21, p27, and antiestrogens in breast cancer. *Cancer Res.* 2004; 64:3198–3208. [PubMed: 15126360]
35. Ekholm-Reed S, Mendez J, Tedesco D, et al. Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *J Cell Biol.* 2004; 165:789–800. [PubMed: 15197178]
36. Bagheri-Yarmand R, Keyomarsi K. Overexpression of full length or low molecular weight of cyclin E leads to differential G2/M transition and mitotic defects through deregulation of cdc25c. *AACR Meet Abstr.* 2009; 2009:2473.
37. Keyomarsi K, Tucker SL, Buchholz TA, et al. Cyclin E and survival in patients with breast cancer. *N Engl J Med.* 2002; 347:1566–1575. [PubMed: 12432043]
38. Sieuwerts AM, Look MP, Meijer-van Gelder ME, et al. Which cyclin E prevails as prognostic marker for breast cancer? Results from a retrospective study involving 635 lymph node-negative breast cancer patients. *Clin Cancer Res.* 2006; 12:3319–3328. [PubMed: 16740753]
39. Keyomarsi K, Tucker SL, Bedrosian I. Cyclin E is a more powerful predictor of breast cancer outcome than proliferation. *Nat Med.* 2003; 9:152. [PubMed: 12563321]
40. Wingate H, Bedrosian I, Akli S, Keyomarsi K. The low molecular weight (LMW) isoforms of cyclin E deregulate the cell cycle of mammary epithelial cells. *Cell cycle.* 2003; 2:461–466. [PubMed: 12963845]
41. Wingate H, Zhang N, McGarhen MJ, et al. The tumor-specific hyperactive forms of cyclin E are resistant to inhibition by p21 and p27. *J Biol Chem.* 2005; 280:15148–15157. [PubMed: 15708847]
42. Akli S, Van Pelt CS, Bui T, et al. Overexpression of the low molecular weight cyclin E in transgenic mice induces metastatic mammary carcinomas through the disruption of the ARF-p53 pathway. *Cancer Res.* 2007; 67:7212–7222. [PubMed: 17671189]
43. Clurman BE, Sheaff RJ, Thress K, Groudine M, Roberts JM. Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev.* 1996; 10:1979–1990. [PubMed: 8769642]
44. Hapke DM, Stegmann AP, Mitchell BS. Retroviral transfer of deoxycytidine kinase into tumor cell lines enhances nucleoside toxicity. *Cancer Res.* 1996; 56:2343–2347. [PubMed: 8625309]

45. Arner ES, Eriksson S. Mammalian deoxyribonucleoside kinases. *Pharmacol Ther.* 1995; 67:155–186. [PubMed: 7494863]
46. Shedden K, Townsend LB, Drach JC, Rosania GR. A rational approach to personalized anticancer therapy: chemoinformatic analysis reveals mechanistic gene-drug associations. *Pharm Res.* 2003; 20:843–847. [PubMed: 12817886]
47. Carlson CC, Burnham LL, Shanks RA, Dransfield DT. 8-Cl-adenosine induces differentiation in LS174T cells. *Dig Dis Sci.* 2001; 46:757–764. [PubMed: 11330409]
48. Van Lookeren Campagne MM, Villalba Diaz F, Jastorff B, Kessin RH. 8-Chloroadenosine 3', 5'-monophosphate inhibits the growth of Chinese hamster ovary and Molt-4 cells through its adenosine metabolite. *Cancer Res.* 1991; 51:1600–1605. [PubMed: 1998950]
49. Lu X, Errington J, Chen VJ, et al. Cellular ATP depletion by LY309887 as a predictor of growth inhibition in human tumor cell lines. *Clin Cancer Res.* 2001; 6:271–277.
50. Jang JY, Choi Y, Jeon YK, Kim CW. Suppression of adenine nucleotide translocase-2 by vector-based siRNA in human breast cancer cells induces apoptosis and inhibits tumor growth in vitro and in vivo. *Breast Cancer Res.* 2008; 10:R11. [PubMed: 18267033]
51. Akli S, Keyomarsi K. Cyclin E and its low molecular weight forms in human cancer and as targets for cancer therapy. *Cancer Biol Ther.* 2003; 2:S38–S47. [PubMed: 14508079]
52. Spruck CH, Won KA, Reed SI. Deregulated cyclin E induces chromosome instability. *Nature.* 1999; 401:297–300. [PubMed: 10499591]
53. Ma Y, Fiering S, Black C, et al. Transgenic cyclin E triggers dysplasia and multiple pulmonary adenocarcinomas. *Proc Natl Acad Sci U S A.* 2007; 104:4089–4094. [PubMed: 17360482]
54. Sgambato A, Camerini A, Pani G, et al. Increased expression of cyclin E is associated with an increased resistance to doxorubicin in rat fibroblasts. *Br J Cancer.* 2003; 88:1956–1962. [PubMed: 12799643]

Fig. 1.

Accumulation of 8-Cl-ATP and effects on ATP levels **a** Structure of 8-Cl-Ado. Time-dependent accumulation of 8-Cl-ATP (*filled symbols*) and depletion of ATP pool (*open symbols*) in **b** MCF-7 and **c** BT-474 cells. Cells were treated with 10 μ M 8-Cl-Ado for the indicated times, and acid extracts were analyzed using HPLC to measure nucleotide levels

Fig. 2.

Effect of 8-Cl-Ado on RNA synthesis **a** MCF-7, ●, and BT-474 cells, ○, were treated with 10 μM 8-Cl-Ado for the indicated times and pulsed with [^3H]-uridine for the final 30 min of treatment. Uridine incorporation into RNA was measured and expressed as percentage of control (untreated) cells. **b** Data from Figs. 1b, c and 2a were plotted to determine the relationship between intracellular level of 8-Cl-ATP and percent inhibition of RNA synthesis in MCF-7, ●, and BT-474 cells, ○. **c** The ratio of the intracellular 8-Cl-ATP levels to ATP levels in MCF-7, ●, and BT-474 cells, ○ was assessed over time. **d** Data from Fig. 2a and c were plotted to determine the relationship between the ratio of 8-Cl-ATP to ATP and the percent inhibition of RNA synthesis in MCF-7, ●, and BT-474 cells, ○

Fig. 3. Effect of 8-Cl-Ado on the growth of breast cancer cells **a** MTS assay was used to measure the dose- and **b** time-dependent effects of 8-Cl-Ado on the growth of MCF-7, ●, and BT-474 cells, ○. An 8-Cl-Ado dose-dependent inhibition of cell growth was apparent in both cell lines after a 5-day treatment, $\text{IC}_{50} = 0.55 \mu\text{M}$ and $0.30 \mu\text{M}$ in MCF-7 and BT-474 cells, respectively. A time-dependent growth inhibition was seen in both cell lines treated with $10 \mu\text{M}$ 8-Cl-Ado

Fig. 4. Effect of 8-Cl-Ado on cell cycle **a** MCF-7 and **b** BT-474 cells were treated with 10 μM 8-Cl-Ado for the indicated times, stained with propidium iodide, and analyzed using flow cytometry. The percentage of G₁, ▲, G₂/M, ▼, and S, ◆ populations of cells were plotted

Fig. 5.

Effect of 8-Cl-Ado on the survival of breast cancer cells **a** The effects of 8-ClAdo on the clonogenic growth of MCF-7 cells, $\bullet$. Cells were treated with 8-Cl-Ado for 3 days, washed with PBS and cultured in fresh medium for 14 days. Colonies of >50 cells were counted under a dissecting microscope. **b** Immunoblot analysis of PARP cleavage in MCF-7 and BT-474 cells treated with 10 μM 8-Cl-Ado for the indicated times. Immunoblot of GAPDH was used to as a gel loading control. **c** Flow cytometry analysis of annexin V and propidium iodine staining in BT-474 cells, *hatched bars*, and MCF-7, *black bars*, treated with 10 μM 8-Cl-Ado for the indicated times

Fig. 6. Effect of 8-Cl-Ado on cyclin E expression **a** Real-time RT-PCR analysis of *CCNE1* mRNA levels in MCF-7, ●, and BT-474 cells, ○, treated with 10 μ M 8-Cl-Ado for the times indicated and graphed as a percentage of the untreated cells. **b** Immunoblot analysis of cyclin E from MCF-7 and BT-474 cells treated with 10 μ M 8-Cl-Ado for the indicated times. Immunoblot of GAPDH was used as a gel loading control

Fig. 7. Effects of 8-Cl-Ado on the expression of anti-apoptotic factors in breast cancer cells **a** Immunoblot analysis of MCL-1, BCL-2, BCL-X_L, and survivin levels in MCF-7 cells treated with 0, 1, 3, 10, 30, and 100 μM of 8-Cl-Ado for 24 h. **b** Immunoblot analysis of MCL-1, BCL-2, BCL-X_L, survivin, and XIAP levels in BT-474 cells treated with 0, 1, 3, 10, 30, and 100 μM of 8-Cl-Ado for 24 h. Immunoblot of GAPDH was used as a gel loading control