8-chloro-adenosine activity in FLT3-ITD acute myeloid leukemia

Ralf Buettner1* | Le Xuan Truong Nguyen1,2* | Bijender Kumar1 | Corey Morales1 | Chao Liu3 | Lisa S. Chen4 | Tea Pemovska5† | Timothy W. Synold6 | Joycelynne Palmer7 | Ryan Thompson1 | Ling Li1 | Dinh Hoa Hoang1 | Bin Zhang1 | Lucy Ghoda1 | Claudia Kowolik8 | Mika Kontro9 | Calum Leitch10 | Krister Wennerberg5 | Xiaochun Xu3 | Ching-Cheng Chen1 | David Horne8 | Varsha Gandhi4 | Vinod Pullarkat1 | Guido Marcucci1 | Steven T. Rosen1

1Hematology Malignancies and Stem Cell Transplantation Institute, Gehr Family Center for Leukemia Research, City of Hope National Medical Center, Duarte, California
2Department of Medical Biotechnology, Biotechnology Center of Ho Chi Minh City, Ho Chi Minh City, Vietnam
3Department of Cancer Genetics and Epigenetics, City of Hope National Medical Center, Duarte, California
4Helsinki Institute of Life Science, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas
5Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki, Finland
6Department of Cancer Biology, City of Hope National Medical Center, Duarte, California
7Department of Information Sciences, City of Hope National Medical Center, Duarte, California
8Department of Molecular Medicine, City of Hope National Medical Center, Duarte, California
9Department of Hematology, Helsinki University Hospital Comprehensive Cancer Center, Helsinki, Finland
10Department of Clinical Science, Center for Cancer Biomarkers CCBIO, University of Bergen, Bergen, Norway

Correspondence
Buettner and Xuan Truong Nguyen, Gehr Family Center for Leukemia Research, City of Hope National Medical Center, Kaplan CRB, 1026, 1500 East Duarte Road, Duarte, CA 91010. Email: rbuettner@coh.org (RB); lenguyen@coh.org (LXTN)

Present address:
†Tea Pemovska, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria.

Funding information
National Cancer Institute, Grant/Award Numbers: P30CA033572, R01CA201496; Rising Tide Foundation for Clinical Cancer Research

Abstract
Nucleoside analogs represent the backbone of several distinct chemotherapy regimens for acute myeloid leukemia (AML) and combination with tyrosine kinase inhibitors has improved survival of AML patients, including those harboring the poor-risk FLT3-ITD mutation. Although these compounds are effective in killing proliferating blasts, they lack activity against quiescent leukemia stem cells (LSCs), which contributes to initial treatment refractoriness or subsequent disease relapse. The reagent 8-chloro-adenosine (8-CI-Ado) is a ribose-containing, RNA-directed nucleoside analog that is incorporated into newly transcribed RNA rather than in DNA, causing inhibition of RNA transcription. In this report, we demonstrate antileukemic activities of 8-CI-Ado in vitro and in vivo and provide mechanistic insight into the mode of action of 8-CI-Ado in AML. 8-CI-Ado markedly induced apoptosis in LSC, with negligible effects on normal stem cells. 8-CI-Ado was particularly effective against AML cell lines and primary AML blast cells harboring the FLT3-ITD mutation. FLT3-ITD is associated with high expression of miR-155. Furthermore, we demonstrate that 8-CI-Ado inhibits miR-155 expression levels accompanied by induction of DNA-damage and suppression of cell proliferation.

*Buettner and Xuan Truong Nguyen have contributed equally to this manuscript.
1 | INTRODUCTION

Acute myeloid leukemia (AML) patients carrying the fms-related tyrosine kinase 3 (FLT3)-internal tandem duplication (ITD) have a poor prognosis (Gilliland & Griffin, 2002; Konig & Levis, 2015; Lagunas-Rangel & Chávez-Valencia, 2017; Swords, Freeman, & Giles, 2012). Nucleoside analogs (NAs) are the backbone of several upfront and salvage chemotherapy regimens for AML, including FLT3-ITD (Jordheim, Durantel, Zoulim, & Dumontet, 2013; Mayer et al., 1994; Robak & Robak, 2013). Combination of chemotherapy with tyrosine kinase inhibitors (TKIs) has improved survival of these patients, but a large proportion of them still die of their disease. Although these agents have significant antileukemic activity, they are not effective in eradicating leukemia stem cells (LSCs), the likely reason for treatment failures in AML. Consequently, new strategies are needed to improve the outcome for this and other molecular subsets of AML patients.

The reagent 8-chloro-adenosine (8-CI-Ado) is a ribose-containing, RNA-directed NA (Gandhi et al., 2001; Stellrecht, Ayres, Arya, & Gandhi, 2010; Stellrecht, Rodriguez, Ayres, & Gandhi, 2003) which, different from other NAs, is incorporated into newly transcribed RNA rather than in DNA, as is the case for many other NAs. Treatment with 8-CI-Ado in a variety of human cancer cells results in inhibition of RNA transcription and malignant cell growth and survival (Gandhi et al., 2001; Krett et al., 1997; Stellrecht et al., 2003; 2010; 2017; Stellrecht, Vangapandu, Le, Mao, & Shentu, 2014; Zhu, Zhang, Zhao, Cui, & Strada, 2006). The anticancer activity of 8-CI-Ado is dependent on intracellular conversion of the parent drug into the 8-CI-AMP metabolite, which is subsequently phosphorylated into 8-CI-ATP (Gandhi et al., 2001). Preliminary in vivo studies (in animals and in patients with chronic lymphocytic leukemia [CLL]) showed favorable pharmacokinetic (PK) and pharmacodynamic (PD) profiles and limited toxicity for 8-CI-Ado (Gandhi et al., 2002 and unpublished data).

As 8-CI-Ado is incorporated in RNA rather than DNA, it may have cell cycle-independent antileukemic activity and be effective in AML blasts, including subpopulations enriched for LSCs. microRNA-155 (miR-155) is the most overexpressed microRNA (miRNA) in FLT3-ITD and reportedly plays a key role in FLT3-ITD blast hyper-proliferation (Khalife et al., 2015; Whitman et al., 2010). Thus, silencing of miR-155 has been proposed as a novel therapeutic approach for FLT3-ITD AML (Khalife et al., 2015). As 8-CI-Ado is incorporated mainly into RNA, we reasoned that it could also be incorporated into miR-155 (and other miRNAs). In this report, we show that 8-CI-Ado possesses antileukemic activity in vitro and in vivo. Moreover, we report that 8-CI-Ado interacts with and downregulates miR-155 expression, associated with deregulation of the miR-155/ErbB3 binding protein 1(Ebp1)/p53/PCNA signaling pathway. Finally, we show that 8-CI-Ado synergizes with the FLT3 inhibitor AC220 (quizartinib) in growth inhibition of FLT3-ITD+ AML cells in vitro and in vivo, thus suggesting a potentially effective approach for the treatment of FLT3-ITD+ AML patients.

2 | MATERIALS AND METHODS

An extensive description of the methods can be found in the Supporting Information Methods.

2.1 | Patient samples

Bone marrow (BM) and peripheral blood samples from AML patients or healthy donors were obtained under a specimen banking protocol approved by the Institutional Review Board of City of Hope National Medical Center (COH IRB #14269), in accordance with assurances filed with and approved by the Department of Health and Human Services and meeting all requirements of the Declaration of Helsinki. For drug sensitivity scoring (DSS), AML patient or healthy donor samples (mononuclear cells from BM or peripheral blood) were collected after written informed consent and ethical approval of the Institutional Review Board at Helsinki University Central Hospital, Department of Hematology, as previously described (see Pemovska et al., 2013 in Supporting Information references).

2.2 | Animal experiments

Animal care and experimental procedures were performed in accordance with approved protocols from the Institutional Animal Care and Use Committee at the City of Hope National Medical Center. All animal studies were performed in accordance with the guidelines of the City of Hope Research Animal Care Committee.
2.3 Statistical analysis

Where indicated, to compare the means of two groups, results were generally compared by using unpaired, two-tailed Student’s t test, with values from at least two independent experiments with triplicate determination. Data are presented as mean±standard error (SE), as indicated. The log-rank test was used to compare the survival distributions of the different treatment groups, from the time of cancer cell injection to the death of the animals. N = 5–8 per group for the animal experiments. p < 0.05 was considered statistically significant; ns indicates not significant. All statistical analyses were conducted using SigmaPlot 12.5 (Systat Software, Chicago, IL). All statistical tests were two-sided.

3 RESULTS

3.1 Antileukemic effects of 8-Cl-Ado in AML cells

To test the antileukemic activity of 8-Cl-Ado in AML, we first evaluated two representative AML cell lines, FLT3-ITD+ MV4-11 and FLT3 WT KG-1a, by treating with 10 µM 8-Cl-Ado for 12 hr. We measured intracellular concentrations of the 8-Cl-ATP triphosphate metabolite, which reached 680–870 µM, and found a concurrent 20–22% decrease in endogenous levels of ATP, in contrast to vehicle-treated controls (p < 0.05; Figure 1a). Accumulation of 8-Cl-Ado triphosphate and 8-Cl-Ado-dependent depletion of endogenous cellular ATP pools were also observed in lymphocytes from CLL patients treated with 8-Cl-Ado (Stellrecht et al., 2017). Exposure to increasing concentrations of 8-Cl-Ado (0.1–100 µM) for 72 hr resulted in significant dose-dependent inhibition of cell growth, with IC50 values in the sub- to low-micromolar range (Figure 1b). 8-Cl-Ado increased the apoptosis rate only in FLT3-ITD+ cells (MV4-11; Figure 1c) but not in FLT3 WT cells (KG-1a, THP1, HL-60; Figure 1d). Cell cycle analysis demonstrated that 8-Cl-Ado-induced G0/G1 arrest and decreased proteins involved in cell cycle regulation, including cyclins A2 and E2, CDC25a, and CDK2 (Figures 1e and S1). Of note, 8-Cl-Ado increased the sub-G0/G1 peak and the apoptosis rate only in FLT3-ITD+ cells (Figure 1e).

3.2 FLT3-ITD+ AML sensitivity to 8-Cl-Ado

Among AML primary blasts from patients with distinct cytogenetic and molecular subtypes of AML (n = 11) and hematopoietic stem cells (HSCs) from healthy donors, those harboring activating FLT3 mutations (both FLT3-ITD and FLT3-TKD; Figure 2a, top) had the
highest sensitivity to 8-Cl-Ado, as quantified by DSS, an algorithm based on continuous modeling and integration of multiple dose-response relationships (Figure 2a, lower panel; Yadav et al., 2014). To test the antileukemic activity of 8-Cl-Ado in vivo, we injected luciferase-expressing FLT3-ITD+ MOLM-14 AML cells into immunodeficient NOD scid γ (NSG) mice. Mice were treated with 50 mg/kg/day 8-Cl-Ado for 16 days, starting 3 days after AML cell injection. Tumor growth was monitored by bioluminescence imaging. We observed significant antileukemic activity by 8-Cl-Ado 3 days before subcutaneous surgical implantation of an osmotic infusion pump releasing control vehicle or 8-Cl-Ado at 50 mg·kg⁻¹·day⁻¹. Leukemia growth was determined 16 days after the start of treatment using bioluminescence imaging (*p < 0.01). N = 6 mice per group, data are presented as mean±SE. (c and d) 8-Cl-Ado treatment inhibits leukemia stem cells (LSC)-enriched subpopulations of AML blasts. CD34⁺/CD38⁻ and CD34⁺/CD38⁺ subpopulations were isolated from peripheral blood mononuclear cells of a healthy donor or an AML patient and treated with 5 µM 8-Cl-Ado or control for 24 hr (2 × 10⁵ cells/ml). Apoptosis was measured using Annexin V and flow cytometry, N = 2, data are presented as mean±SE, with triplicate determination. (d) LSC subpopulations (2 × 10⁵ cells/ml) from an AML patient were treated with 5 µM 8-Cl-Ado 24 hr before plating on methylcellulose. After 14 days, colonies were counted, and half of the cells were replated for an additional 14 days. N = 2, data are presented as mean±SE, with triplicate determination. (e) 8-Cl-Ado treated (5 µM), or untreated primary FLT3-ITD+ AML blasts (10⁶) were transplanted into sublethally irradiated 4-day-old Rag2/γC double knockout mice, and survival was determined. N = 5–7 mice per group. The log-rank test was used to compare the survival distributions of the different treatment groups. Asterisk indicates significantly different based on unpaired t test analysis. 8-Cl-Ado: 8-chloro-adenosine; AML: acute myeloid leukemia; DMSO: dimethyl sulfoxide; ns: not significant; SE: standard error [Color figure can be viewed at wileyonlinelibrary.com]
levels, as well as markers of DNA fragmentation and apoptosis (Figure 3a–c).

3.4 | 8-CI-Ado inhibits miR-155 which is associated with induction of SHIP1, downregulation of AKT, activation of Ebp1, and enhanced p53/PCNA promoter binding, followed by growth inhibition and apoptosis, in FLT3-ITD+ AML

As 8-CI-Ado is incorporated into RNA, we reasoned that it should also target short noncoding RNA species such as miRNA. We and others have reported the correlation of FLT3-ITD with high expression of miR-155, which contributes to the leukemogenic activity of the mutant protein (Khalife et al., 2015). Consistent with our hypothesis, we demonstrated a significant decrease in miR-155 levels in 8-CI-Ado-treated FLT3-ITD+ blast cells (Figure 4a and S2A). This effect appeared to be specific to miR-155, as no changes were observed in the levels of miR-125b, which is also upregulated in FLT3-ITD+ blast cells (Figure S2B). Colocalization of 8-CI-Ado and miR-155, shown by fluorescence-labeled 8-CI-Ado (8-CI-Ado-FAM) and miR-155 staining with SmartFlare probes (Figure 4b), supported a direct interaction of 8-CI-Ado with miR-155 and in turn drug-dependent miR-155 degradation. The use of 8-CI-Ado-FAM led to induction of apoptosis similarly to the unlabeled compound (Figure S2C). Overexpression of miR-155 reversed 8-CI-Ado-induced apoptosis in MV4–11 cells (Figures 4c and S2D), supporting miR-155 as a relevant target for 8-CI-Ado antileukemic activity. Suppression of miR-155 by 8-CI-Ado was associated with upregulation of SHIP1, a phosphatase that negatively regulates AKT phosphorylation and signaling, and decreased p-AKT (Ser473) levels (Figures 4a, right and S2A, right).

Of note, we also observed disruption of interaction of AKT and ErbB3 binding protein (Ebp1), a highly expressed protein in AML (Nguyen, Zhu, Lee, Ta, & Mitchell, 2016), which regulates p53 expression and is required for preventing DNA fragmentation and apoptosis (Figure 4d; Ahn et al., 2006; D. Q. Nguyen et al., 2015; L. X. T. Nguyen et al., 2018).

In contrast to Ebp1 WT mouse embryonic fibroblasts (MEF), Ebp1-null MEF (Ebp1−/−) present with elevated p53 protein levels (Figure S3A, left; Nguyen et al., 2015). The binding of p53 protein to the PCNA promoter regulates PCNA transcription through inhibition of acetyl-histone H4 (H4Ac) binding to the transcription site of the PCNA promoter (Figure S3B; Shan, Xu, Zhuo, Morris, & Morris, 2003). A significant increase in p53 binding, decrease in H4Ac binding to the PCNA promoter, and reduced PCNA transcription was detected in MEF Ebp1−/− cells using western blot analysis, q-PCR and ChIP assays (Figure S3A). Re-expression of Ebp1 in MEF Ebp1−/− cells effectively reversed the Ebp1 knockdown (KD) effects on p53-regulated PCNA transcription (Figure S3C). Consistent with these results, we demonstrated that Ebp1 KD in primary FLT3-ITD+ blasts resulted in increased p53 expression (Figure 4e, left), increased p53-PCNA promoter binding and decreased H4Ac-PCNA promoter binding (Figure S4A, bottom). Subsequently, there was also an increase in DNA fragmentation and apoptosis (Figures 4e and S4A, top) and a decrease in PCNA messenger RNA (Figure 4f, left) and
protein levels (Figure 4e, left) and decrease in colony formation activity (Figures 4f, right and S4A, middle). Although Ebp1 overexpression did not restore miR-155 levels, it rescued cells from 8-Cl-Ado-induced apoptosis and enhanced PCNA levels and colony formation activity (Figures 4g and S4B), thereby suggesting that decreased 8-Cl-Ado-dependent p-AKT levels are an important step in the antileukemic activity of this compound. Indeed, overexpression of a constitutive active form of Akt (Akt-myr) rescued cells from 8-Cl-Ado-induced apoptosis (Figure S4C).
3.5 | 8-Cl-Ado synergizes with the FLT3 inhibitor AC220 (quizartinib) in FLT3-ITD* AML

We next examined the effects of the combination of 8-Cl-Ado with AC220 (quizartinib), a TKI, in FLT3-ITD* AML cells. Compared to either drug alone, combination treatment was more effective in decreasing miR-155 and p-AKT expression, in increasing p53 protein levels and in inhibiting PCNA transcription by p53, as well as in inhibiting colony formation and proliferation and increasing induction of apoptosis in FLT3-ITD* AML cells (Figures 5a–c and 5A–D). Finally, NSG mice engrafted with FLT3-ITD* MV4–11 cells survived significantly longer when treated with the combination of 8-Cl-Ado (75 mg·kg⁻¹·day⁻¹; osmotic pump) and AC220 (1 mg·kg⁻¹·day⁻¹, daily oral), as compared with single-agent or vehicle control treatment (Figure 5d).

4 | DISCUSSION

We here report the in vitro and in vivo antileukemic activity of the ribose-containing, RNA-directed nucleoside 8-Cl-Ado, which appears to spare normal HSCs. Among AML molecular subtypes, the more pronounced impact of 8-Cl-Ado in FLT3-ITD* blasts may
Our data support a mechanism where 8-Cl-Ado decreases miR-155 expression, followed by induction of SHIP1, dephosphorylation of AKT, and dissociation of the AKT/Ebp1 protein complex. Dissociation of AKT/Ebp1 then leads to increased p53 binding to the PCNA promoter, which is associated with inhibition of H4Ac binding to the transcription site of the PCNA promoter and inhibition of cell growth and induction of apoptosis.

AC220 (quizartinib) is a selective and potent next-generation FLT3 kinase inhibitor with clinical antileukemic activity as single agent in patients with relapsed/refractory AML, and is particularly effective in those patients that harbor the FLT3-ITD mutation (J. Cortes et al., 2018; J. Cortes et al., 2013). It has also been shown that AC220 can safely be combined with conventional chemotherapy in patients with AML and that such drug combinations may be beneficial as evidenced by recently presented data (Bowen et al., 2013). Importantly, our results support a potential synergistic activity of 8-Cl-Ado with TKIs, such as the FLT3 inhibitor AC220, on FLT3-ITD+ AML cells, in vitro and in vivo. The combined treatment of AML cells with 8-Cl-Ado and AC220 further augmented the effect of the individual drugs on activation of the proapoptotic miR-155/Ebp1/p53/PCNA signaling pathway.

On the basis of these encouraging results, and given the fact that 8-Cl-Ado as a ribose-containing, RNA-directed NA—possesses a unique mechanism of action, a combination of TKI (i.e., AC220) with 8-Cl-Ado may represent a potentially novel clinical approach for FLT3 kinase inhibitor with clinical antileukemic activity as single agent in patients with relapsed/refractory AML, and is particularly effective in those patients that harbor the FLT3-ITD mutation (J. Cortes et al., 2018; J. Cortes et al., 2013). It has also been shown that AC220 can safely be combined with conventional chemotherapy in patients with AML and that such drug combinations may be beneficial as evidenced by recently presented data (Bowen et al., 2013). Importantly, our results support a potential synergistic activity of 8-Cl-Ado with TKIs, such as the FLT3 inhibitor AC220, on FLT3-ITD+ AML cells, in vitro and in vivo. The combined treatment of AML cells with 8-Cl-Ado and AC220 further augmented the effect of the individual drugs on activation of the proapoptotic miR-155/Ebp1/p53/PCNA signaling pathway.

On the basis of these encouraging results, and given the fact that 8-Cl-Ado as a ribose-containing, RNA-directed NA—possesses a unique mechanism of action, a combination of TKI (i.e., AC220) with 8-Cl-Ado may represent a potentially novel clinical approach for FLT3-ITD AML. A single-agent Phase I/II clinical trial with 8-Cl-Ado for the treatment of patients with relapsed or refractory AML has recently been initiated (ClinicalTrials.gov identifier: NCT02509546).

ACKNOWLEDGMENTS

This project was supported by the Analytical Pharmacology (Dr. Timothy Synold) and Small Animal Imaging (Dr. Jun Wu) Core Facilities at City of Hope supported by the National Cancer Institute under award number P30CA033572; the Helsinki/Bergen AML individualized systems medicine team (Drs. Tero Aittokallio, Björn Tore Gjertsen, Caroline Heckman, Olli Kallioniemi, Kimmo Porkka, Krister Wennerberg) and the FIMM High Throughput Biomedicine, Information Technology and Sequencing units. The study was also in part supported by the National Cancer Institute grant R01CA201496 and the Rising Tide Foundation for Clinical Cancer Research (RTFCCR). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors like to thank James Sanchez (City of Hope) for editorial support.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Le Xuan Truong Nguyen http://orcid.org/0000-0001-5464-0861

REFERENCES


FIGURE 6 Schematic diagram of the proposed signaling pathway of 8-chloro-adenosine regulated PCNA transcription through miR-155 and Ebp1 protein [Color figure can be viewed at wileyonlinelibrary.com]


**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.


https://doi.org/10.1002/jcp.28294