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

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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-Cl-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-Cl-Ado in vitro and in vivo and provide mechanistic insight into the mode of action of 8-Cl-Ado in AML. 8-Cl-Ado markedly induced apoptosis in LSC, with negligible effects on normal stem cells. 8-Cl-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-Cl-Ado inhibits miR-155 expression levels accompanied by induction of DNA-damage and suppression of cell proliferation,

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through regulation of miR-155/ErbB3 binding protein 1(Ebp1)/p53/PCNA signaling. Finally, we determined that combined treatment of NSG mice engrafted with FLT3-ITD$^+$ MV4−11 AML cells with 8-Cl-Ado and the FLT3 inhibitor AC220 (quizartinib) synergistically enhanced survival, compared with that of mice treated with the individual drugs, suggesting a potentially effective approach for FLT3-ITD AML patients.

KEYWORDS
acute myeloid leukemia, antileukemic, apoptosis, FLT3, nucleoside, RNA

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-Cl-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-Cl-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-Cl-Ado is dependent on intracellular conversion of the parent drug into the 8-Cl-AMP metabolite, which is subsequently phosphorylated into 8-Cl-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-Cl-Ado (Gandhi et al., 2002 and unpublished data).

As 8-Cl-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-Cl-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-Cl-Ado possesses antileukemic activity in vitro and in vivo. Moreover, we report that 8-Cl-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-Cl-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.

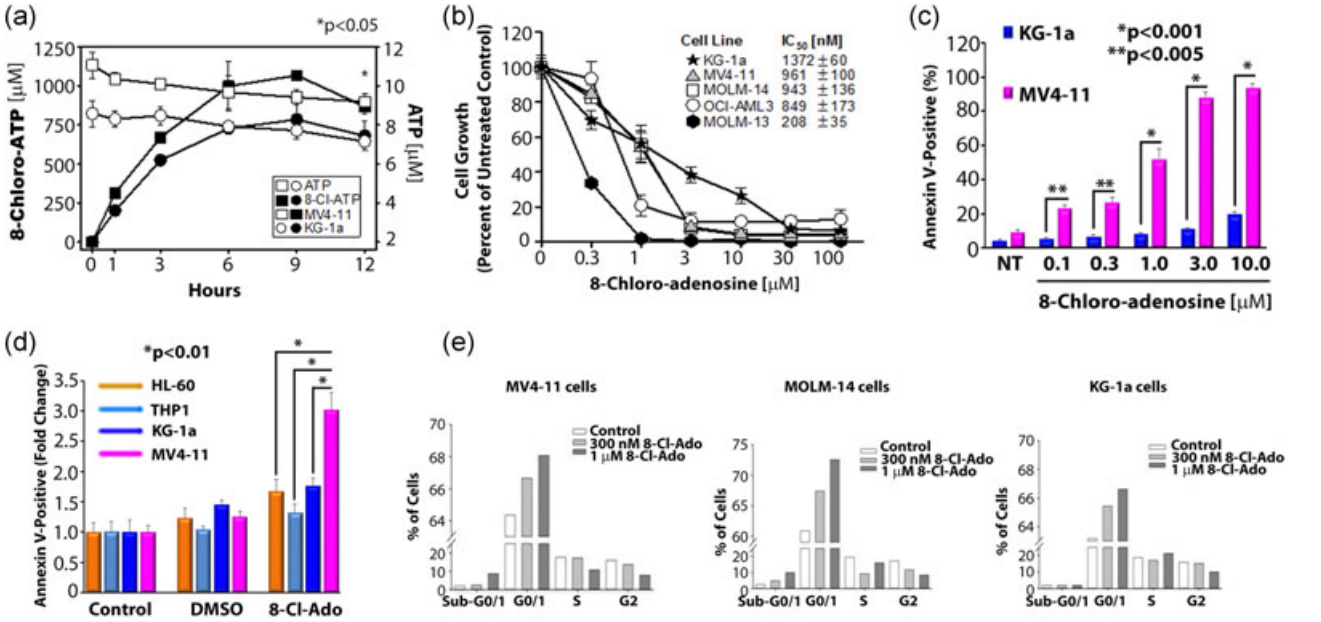

FIGURE 1 Antileukemic activities of 8-Cl-Ado in AML cell lines. (a) Intracellular concentrations of 8-Cl-ATP and ATP in MV4–11 (FLT3-ITD$^+$) and KG-1a (FLT3 WT) cells (2×10^5 cells/ml) treated with 10 μM 8-Cl-Ado for up to 12 hr. *N* = 2, data are presented as mean ±*SE*, with triplicate determination. (b) Effect of 8-Cl-Ado on the proliferation of (2×10^5 cells/ml) leukemic cells (KG-1a: FLT3 WT; MV4–11, MOLM-14, OCI-AML3, MOLM-13: FLT3-ITD$^+$) treated with increasing concentrations of 8-Cl-Ado for 72 hr. *N* = 2, data are presented as mean±*SE*, with quadruplicate determination. (c and d) Effect of 8-Cl-Ado on apoptosis of leukemic cells. (c) MV4–11 and KG-1a cells (2×10^5 cells/ml) were treated with up to 10 μM 8-Cl-Ado for 24 hr. (d) FLT3 WT cells (2×10^5 cells/ml; HL-60, THP1, KG-1a), and FLT3-ITD$^+$ cells (MV4–11) were treated with 500 nM 8-Cl-Ado for 24 hr. Apoptosis was determined by Annexin V staining and flow cytometry. *N* = 2, data are presented as mean±*SE*, with triplicate determination. (e) Effect of 8-Cl-Ado on cell cycle distribution in MV4–11, MOLM-14, and KG-1a cells. Cells (2×10^5 cells/ml) were treated with vehicle control, 300 nM or 1 μM of 8-Cl-Ado for the indicated times. Flow cytometric cell cycle analysis (propidium iodide staining) was performed 24 hr after treatment and presented as bar graphs. *N* = 2 (representative results from one out of two independent experiments shown). Asterisk indicates significantly different based on unpaired *t* test analysis. 8-Cl-Ado: 8-chloro-adenosine; AML: acute myeloid leukemia; DMSO: dimethyl sulfoxide; *SE*: standard error; WT: wild-type [Color figure can be viewed at wileyonlinelibrary.com]

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 IC_{50} 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

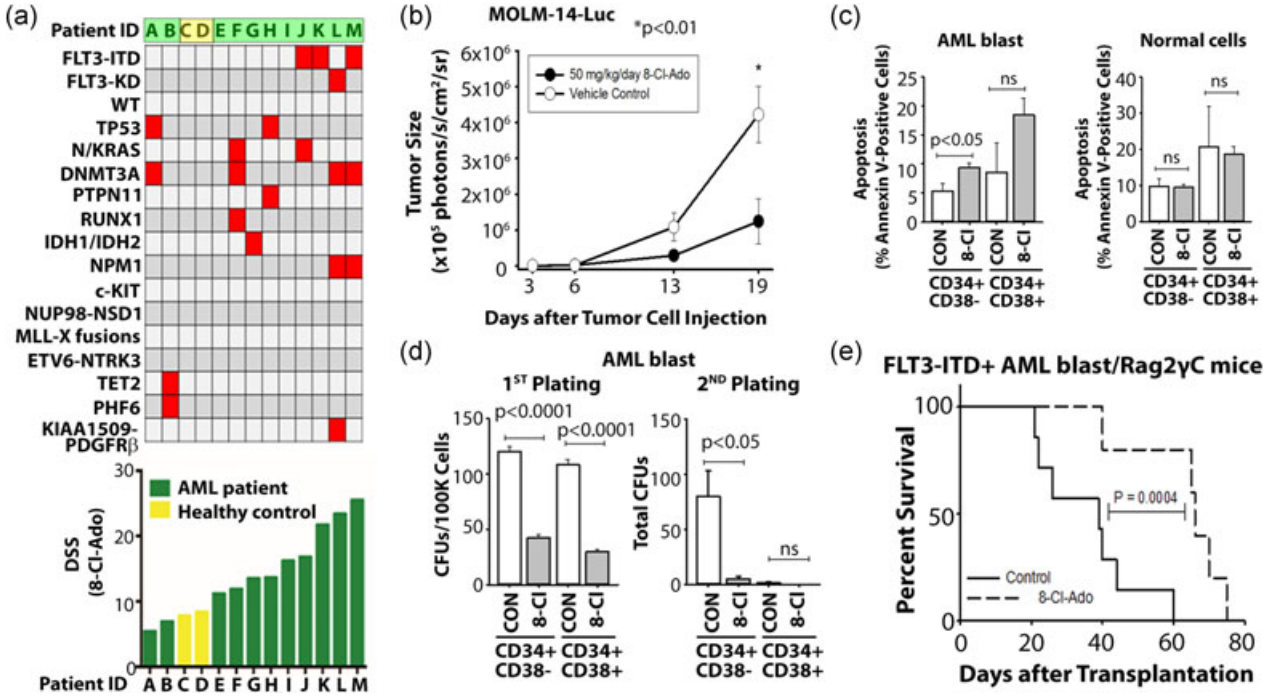

FIGURE 2 Effects of 8-Cl-Ado on primary FLT3-ITD$^+$ AML cells in vitro and in vivo. (a) Drug sensitivity scoring (DSS) responses across molecularly characterized primary AML patient samples ex vivo after 72 hr exposure to 8-Cl-Ado. DSS was determined as previously described (Yadav et al., 2014). Top panel: Significant AML mutations and recurrent gene fusions in red. Bottom panel: AML and control sample responses (5×10^5 cells/ml). $N = 1$. (b) Effects of 8-Cl-Ado on tumor formation in vivo. MOLM-14-Luc cells (2.5×10^6) were intravenous injected in NSG mice 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. (c) 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 (2×10^5 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 (2×10^5 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 pretreated (5 μM), or untreated primary FLT3-ITD$^+$ AML blasts (10^7) 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]

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 ɣ (NSG) mice. Mice were treated with 50 mg·kg^{-1}·day^{-1} 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 in treated mice, in contrast to vehicle-treated controls 16 days after the start of treatment ($p < 0.01$; Figure 2b). Interestingly, treatment of primary FLT3-ITD + AML blasts with 8-Cl-Ado as low as 5 μM for 24 hr resulted in a significantly higher level of apoptosis induction in the more primitive CD34$^+$ CD38$^-$ cells (typically LSC-enriched subpopulation) compared with CD34$^+$ CD38$^+$ cells, normal HSCs, or vehicle-treated controls ($p < 0.05$; Figure 2c). Colony forming activity was significantly reduced in both 8-Cl-Ado-treated CD34$^+$ CD38$^-$ and CD34$^+$ CD38$^+$ cells after the first plating ($p < 0.0001$) and completely eliminated after the second plating ($p < 0.05$; Figure 2d). Rag2/γC mice engrafted with FLT3-ITD$^+$ AML primary blast cells pretreated with 8-Cl-Ado (5 μM, 24 hr) also survived significantly longer than controls engrafted with vehicle-treated blasts (median survival: 66 vs. 39 days; $p = 0.0004$; Figure 2e).

3.3 | 8-Cl-Ado induces DNA damage and apoptosis in FLT3-ITD$^+$ AML

Treatment with 8-Cl-Ado resulted in increased DNA damage pathway signaling and apoptosis induction in the FLT3-ITD$^+$ MV4–11 cell line and in FLT3-ITD$^+$ primary AML blast cells but not in FLT3 WT KG-1a cells and FLT3 WT primary AML blasts, as demonstrated by changes in levels of γ-H2AX, 53BP1, p53, p21, and proliferating cell nuclear antigen (PCNA) protein

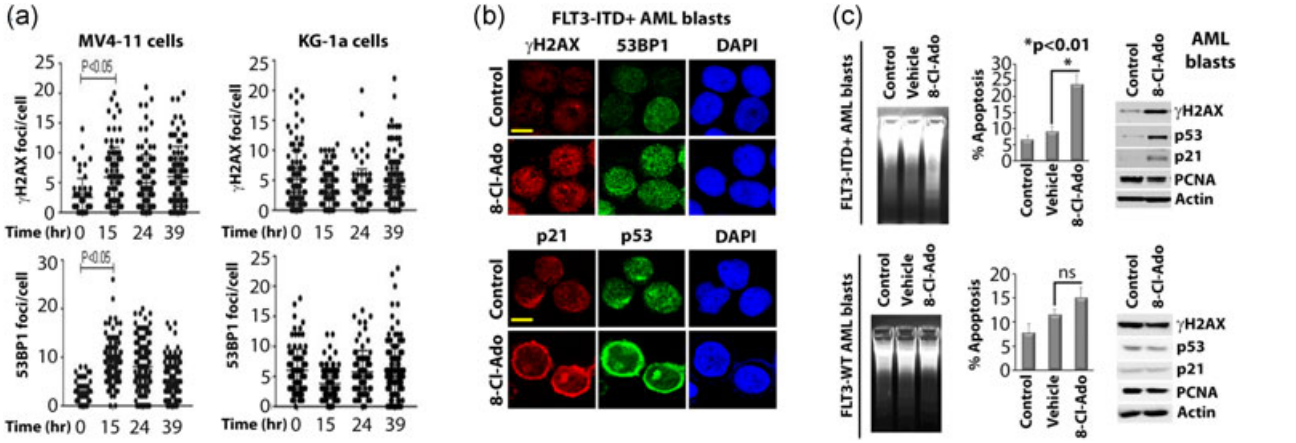

FIGURE 3 Effects of 8-Cl-Ado on DNA damage signaling in FLT3-ITD$^+$ and FLT3 WT AML cells. (a and b) Effect of 8-Cl-Ado on γH2AX, 53BP1, p53, and p21 protein expression in FLT3-ITD$^+$ and FLT3 WT AML cells. (a) MV4–11 (FLT3-ITD$^+$) and KG-1a (FLT3 WT) cells (2×10^5 cells/ml) were treated with 500 nM 8-Cl-Ado for up to 39 hr before measurement and quantification of double-strand DNA breaks as indicated by a number of γH2AX and 53BP1 foci. *N* = 3. (b) Primary FLT3-ITD$^+$ AML blasts (a mixture of 2×10^5 cells/ml from five patients) were treated with 500 nM 8-Cl-Ado or control for 24 hr, stained with indicated antibodies, and subjected to confocal microscopy. *N* = 2 (representative images from one out of two independent experiments shown). (c) Effects of 8-Cl-Ado on apoptosis of FLT3 WT and FLT3-ITD$^+$ primary AML blasts. A mixture of cells (2×10^5 cells/ml, from five patients) was treated with 500 nM 8-Cl-Ado for 24 hr, followed by genomic DNA fragmentation analysis (left panel), Annexin V apoptosis measurement by flow cytometry (middle panel) and immunoblot as indicated (right panel). *N* = 2, data are presented as mean±*SE*, with triplicate determination for apoptosis assay. Asterisk indicates significantly different based on unpaired *t* test analysis. 8-Cl-Ado: 8-chloro-adenosine; AML: acute myeloid leukemia; DAPI: 4′,6-diamidino-2-phenylindole; *SE*: standard error; WT: wild-type [Color figure can be viewed at wileyonlinelibrary.com]

levels, as well as markers of DNA fragmentation and apoptosis (Figure 3a–c).

3.4 | 8-Cl-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-Cl-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-Cl-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-Cl-Ado and miR-155, shown by fluorescence-labeled 8-Cl-Ado (8-Cl-Ado-FAM) and miR-155 staining with SmartFlare probes (Figure 4b), supported a direct interaction of 8-Cl-Ado with miR-155 and in turn drug-dependent miR-155 degradation. The use of 8-Cl-Ado-FAM led to induction of apoptosis similarly to the unlabeled compound (Figure S2C). Overexpression of miR-155 reversed 8-Cl-Ado-induced apoptosis in MV4–11 cells (Figures 4c and S2D), supporting miR-155 as a relevant target for 8-Cl-Ado antileukemic activity. Suppression of miR-155 by 8-Cl-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

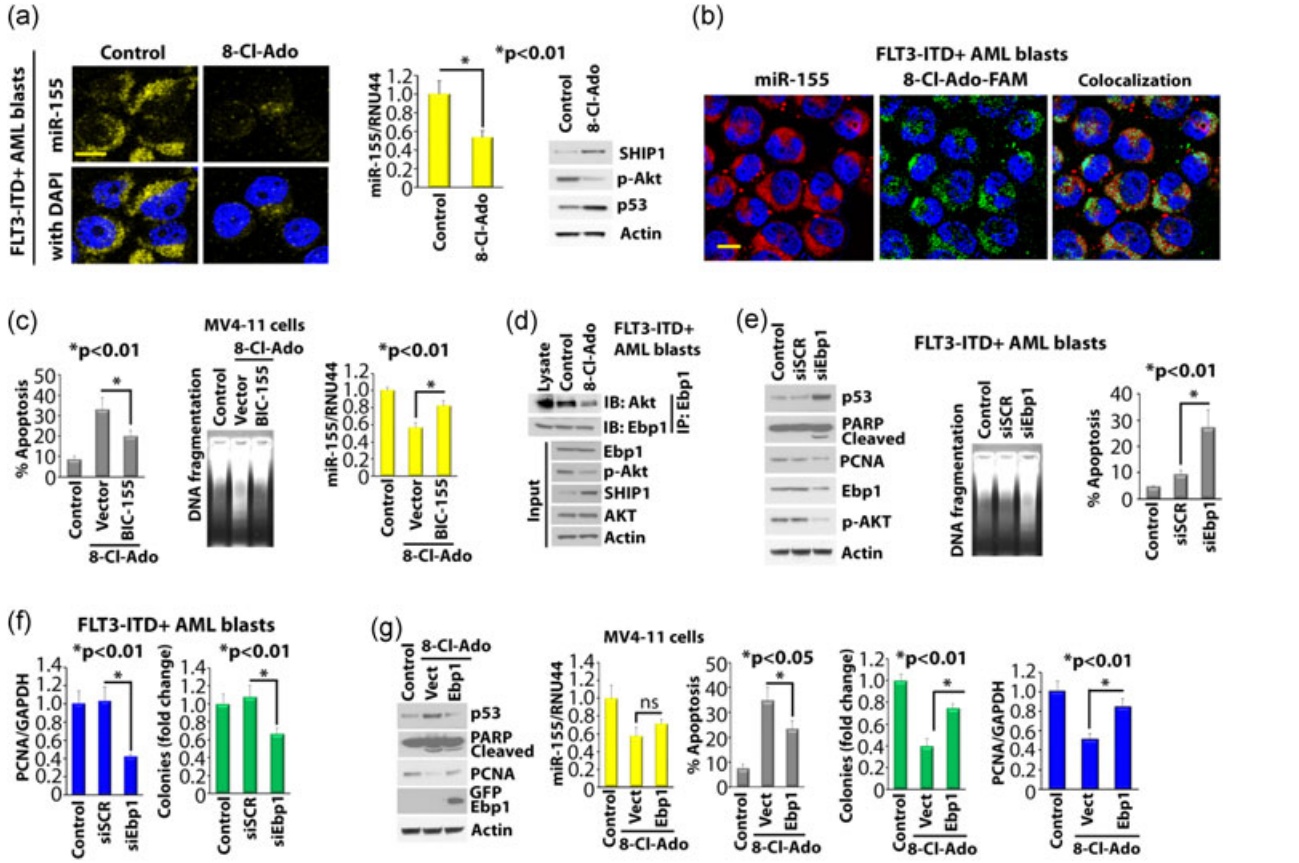

FIGURE 4 Effects of 8-Cl-Ado on apoptosis and proliferation of FLT3-ITD$^+$ AML cells through regulation of miR-155 and Ebp1 protein. (a) Effect of 8-Cl-Ado on miR-155 expression in FLT3-ITD$^+$ primary AML cells. FLT3-ITD$^+$ AML blast cells (mixture of 2×10^5 cells/ml from five patients) were treated with 500 nM 8-Cl-Ado for 24 hr, followed by miR-155 staining using SmartFlare probes (left panel, representative results from one out of two independent experiments shown), q-PCR of miR-155 (middle panel) and immunoblot as indicated (right panel). *N* = 2, data are presented as mean±*SE*, with triplicate determination for q-PCR assay. (b) Colocalization of miR-155 and 8-Cl-Ado in FLT3-ITD$^+$ primary AML blasts. Cells (a mixture of 2×10^5 cells/ml from five patients) were incubated with FAM-conjugated 8-Cl-Ado and SmartFlare probes specific for miR-155, followed by confocal microscopy and colocalization analysis (Zeiss, colocalization shown in cyan). *N* = 2, representative images from one out of two independent experiments shown. (c) Effect of miR-155 expression on 8-Cl-Ado-induced apoptosis of MV4–11 cells. Cells (2×10^5 cells/ml) were transfected with vector control or BIC-155 (long noncoding RNA containing miR-155 sequence) and treated with 500 nM 8-Cl-Ado for 24 hr. Left, the percentage of apoptosis. Middle, genomic DNA fragmentation. Right, levels of miR-155 RNA expression. *N* = 2, data are presented as mean±*SE*, q-PCR and apoptosis in triplicate. (d) Effects of 8-Cl-Ado on AKT/Ebp1 interaction in FLT3-ITD$^+$ primary AML blasts. A mixture of 2×10^5 cells/ml from five patients was treated with or without 500 nM 8-Cl-Ado for 24 hr before immunoprecipitation with anti-Ebp1 and immunoblotting with anti-AKT antibodies. *N* = 1. (e and f) Effects of Ebp1 knockdown on apoptosis and proliferation of FLT3-ITD$^+$ primary AML blasts. A mixture of 2×10^5 cells/ml from five patients was transfected with 50 nM of scrambled control (siSCR) or siEbp1 for 36 hr. (e) Left, immunoblot with antibodies as indicated. Middle, genomic DNA fragmentation. Right, the percentage of apoptosis. *N* = 1, data are presented as mean±*SE*, apoptosis assay with triplicate determination. (f) Left, PCNA messenger RNA expression. Right, colony formation. *N* = 1, data are presented as mean ±*SE*, with triplicate determination. (g) Effects of Ebp1 overexpression on 8-Cl-Ado-induced apoptosis and proliferation in MV4–11 cells. Cells (2×10^5 cells/ml) were transfected with vector control or GFP-Ebp1 for 12 hr followed by 24 hr-treatment with 500 nM 8-Cl-Ado. Left to right, immunoblot with antibodies as indicated; miR-155 RNA expression levels; percentage of apoptosis; colony formation; q-PCR of PCNA. *N* = 1, data are presented as mean±*SE*, with triplicate determination. Asterisk indicates significantly different based on unpaired *t* test analysis. 8-Cl-Ado: 8-Chloro-adenosine; AML: acute myeloid leukemia; DAPI: 4′,6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; GADPH: glyceraldehyde-3-phosphate dehydrogenase; q-PCR: quantitative polymerase chain reaction; *SE*: standard error; WT: wild-type [Color figure can be viewed at wileyonlinelibrary.com]

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).

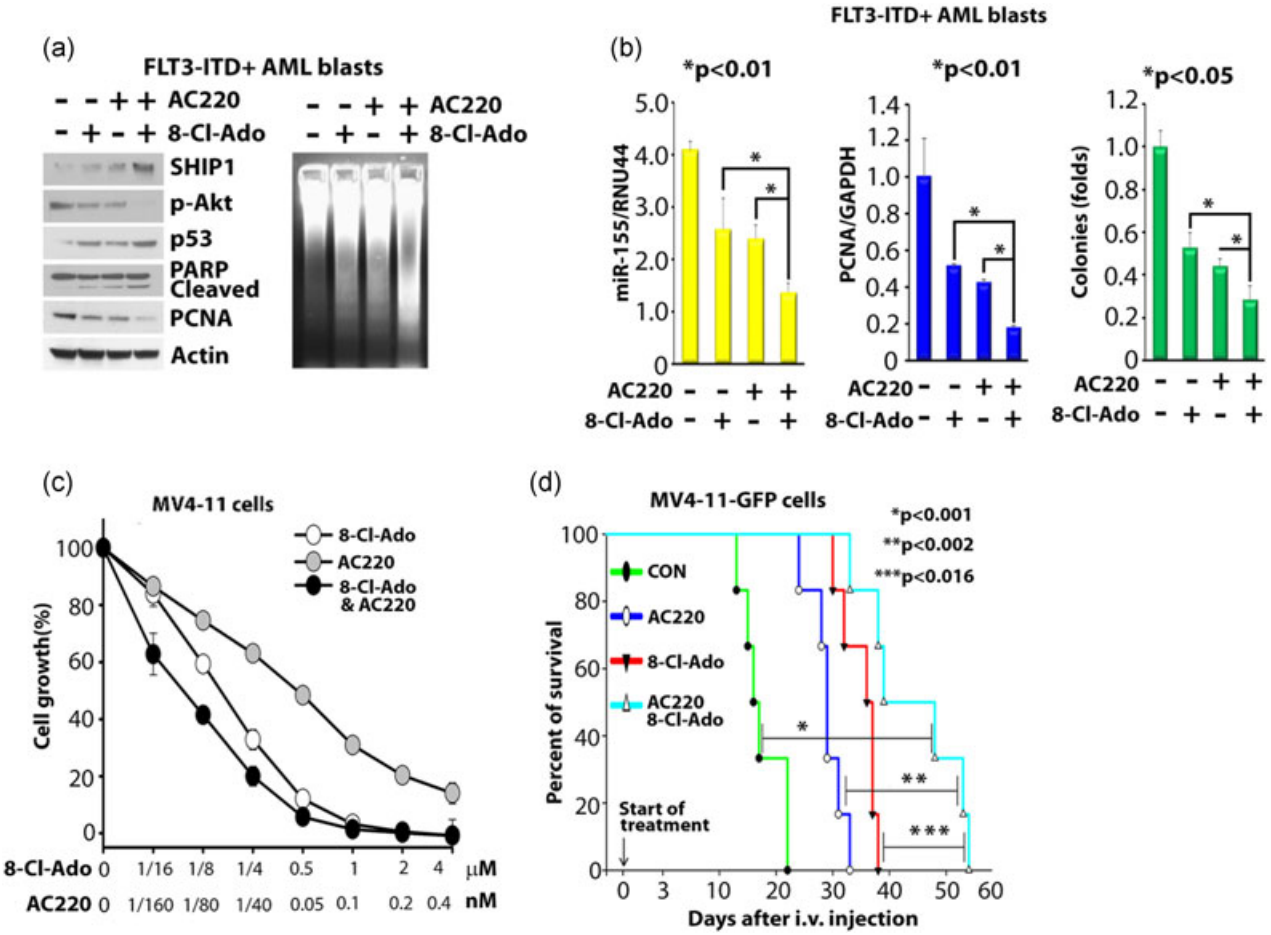

FIGURE 5 Effects of 8-Cl-Ado plus AC220 combination treatment on FLT3-ITD$^+$ AML cells in vitro and in vivo. (a and b) Combination treatment with AC220 and 8-Cl-Ado and its effects on apoptosis and proliferation of FLT3-ITD$^+$ primary AML blasts. A mixture of FLT3-ITD$^+$ cells (2×10^5 cells/ml from five patients) was treated for 24 hr with 0.5 nM AC220, 500 nM 8-Cl-Ado, or both. (a) Left, immunoblot with antibodies as indicated. Right, DNA fragmentation. $N = 1$. (b) miR-155 RNA expression (left), PCNA messenger RNA expression (middle), colony formation (right). $N = 2$, data are presented as mean±*SE*, with triplicate determination. (c) Synergistic effect of AC220 and 8-Cl-Ado on the growth of FLT3-ITD$^+$ MV4–11 AML cells. Cells (2×10^5 cells/ml) were cultured for 72 hr with constant ratios of AC220 and 8-Cl-Ado, based on previously established IC_{50} values, as indicated, followed by MTS assay. The synergy between AC220 and 8-Cl-Ado was analyzed using CalcuSyn program. Combination indices (CI) for effective doses (ED) were: CI = 0.81 (ED_{50}), CI = 0.77 (ED_{75}), CI = 0.78 (ED90), and CI = 0.79 (ED95). $N = 2$, data are presented as mean±*SE*, with quadruplicate determination. (d) MV4–11-Luc cells (5×10^6) were intravenously injected in NSG mice 3 days before subcutaneous implantation of an osmotic infusion pump releasing 8-Cl-Ado at 75 mg·kg^{-1}·day^{-1} for 16 days. Daily oral treatment with 1 mg/kg AC220 or control solution was initiated at the same day the osmotic pump was implanted. Sixteen days after implantation of the 8-Cl-Ado-releasing osmotic pumps, the pumps were removed, and all treatment was stopped. Survival was quantified using the log rank test. $N = 6$ animals per group. Asterisk indicates significantly different based on unpaired *t* test analysis. 8-Cl-Ado: 8-Chloro-adenosine; AML: acute myeloid leukemia; GADPH: glyceraldehyde-3-phosphate dehydrogenase; *SE*: standard error; WT: wild-type [Color figure can be viewed at wileyonlinelibrary.com]

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 S5A–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

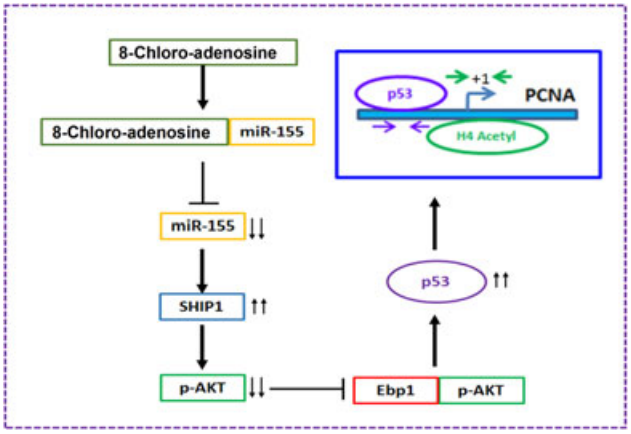

FIGURE 6 Schematic diagram of the proposed signaling pathway of 8-chloro-adenosine regulated PCNA transcription through miR-155 and Ebp1 protein [Color figure can be viewed at wileyonlinelibrary.com]

be achieved through previously unidentified targeting of anti-apoptotic mechanisms involving miR-155 and Ebp1 proteins (Figure 6). 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; Foran 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).

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

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

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