Original Article

Yonsei Med J 2020 Sep;61(9):762-773
https://doi.org/10.3349/ymj.2020.61.9.762

Yonsei Medical Journal
YMJ
pISSN: 0513-5796 · eISSN: 1976-2437

All-Trans Retinoic Acid Synergizes with Enasidenib to Induce Differentiation of IDH2-Mutant Acute Myeloid Leukemia Cells

Yundeok Kim[1], Hoi-Kyung Jeung[2], June-Won Cheong[3], Jaewoo Song[4], Soo Han Bae[5], Jong In Lee[1], and Yoo Hong Min[3]

[1]Department of Internal Medicine, Yonsei Wonju College of Medicine, Wonju;
[2]Avison Biomedical Research Center, [3]Department of Internal Medicine, [4]Department of Laboratory Medicine,
[5]Severance Biomedical Science Institute, Yonsei Biomedical Research Institute, Yonsei University College of Medicine, Seoul, Korea.

Purpose: Pharmacological inhibition of mutant isocitrate dehydrogenase (IDH) reduces R-2-hydroxyglutarate (2-HG) levels and restores cellular differentiation in vivo and in vitro. The IDH2 inhibitor enasidenib (AG-221) has been approved by the FDA as a first-in-class inhibitor for the treatment of relapsed or refractory (R/R) IDH2-mutant acute myeloid leukemia (AML). In this study, the effects of a combination of all-trans retinoic acid (ATRA) and AG-221 on AML cell differentiation was explored, along with the mechanisms employed by IDH2-mutant cells in AML.

Materials and Methods: We treated the human AML cell line, IDH2-mutant-TF-1, and primary human AML cells carrying IDH2 mutation with 30 μM AG-221 and 100 nM ATRA, alone or in combination.

Results: Combined treatment with AG-221 and ATRA inhibited 2-HG production and resulted in synergistic effects on differentiation among IDH2-mutant AML cells and primary AML cells expressing IDH2 mutation. Combined treatment with AG-221 and ATRA altered autophagic activity. AG-221 and ATRA treatment-induced differentiation of IDH2-mutant AML cells was associated with autophagy induction, without suppressing autophagy flux at maturation and degradation stages. A RAF-1/MEK/ERK pathway was founded to be associated with AG-221 and ATRA-induced differentiation in IDH2-mutant AML cells. IDH-associated changes in histone methylation markers decreased after AG-221 and ATRA combination treatment.

Conclusion: Our preliminary evidence indicates that the addition of ATRA to treatments with IDH2 inhibitor may lead to further improvements or increases in response rates in IDH2-mutant AML patients who do not appear to benefit from treatments with IDH2 inhibitor alone.

Key Words: Isocitrate dehydrogenase 2-mutant acute myeloid leukemia, isocitrate dehydrogenase 2 inhibitor, all-trans retinoic acid, combination treatment, differentiation

INTRODUCTION

Acute myeloid leukemia (AML) represents a group of hematological malignancies characterized by uncontrolled hematopoietic cell proliferation, decreased apoptosis, blocked differentiation hematopoiesis, and an accumulation of clonal myeloid progenitor cells that do not differentiate normally.[1,2] While 5-year overall survival (OS) rates range from 40% to 50% in younger (<50 years) AML patients, the 5-year OS rate for older patients (>70 years) is only 5 to 10%.[3,4] Although 60–80% of adult patients treated first with intensive induction chemotherapy achieve complete remission, more than 50% of patients will

Received: March 19, 2020 **Revised:** July 10, 2020
Accepted: July 22, 2020
Corresponding author: Yoo Hong Min, MD, PhD, Department of Internal Medicine, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
Tel: 82-2-2228-1956, Fax: 82-2-393-6884, E-mail: minbrmmd@yuhs.ac

•The authors have no potential conflicts of interest to disclose.

show primary refractory or relapsed (R/R) disease,[1] which is associated with extremely poor prognosis, and treatment of R/R or older patients with AML remains highly challenging and controversial.

It has long been argued that overcoming the differentiation block in AML would improve treatment regimens. For example, all-trans retinoic acid (ATRA), a derivative of vitamin A that affects cellular development, including hematopoiesis, especially granulocyte differentiation,[5] has been used to treat acute promyelocytic leukemia (APL), in which 99% of cases display translocation t(15;17) with the consequent chimeric protein PML-RARα.[6] ATRA causes APL cells displaying PML-RARα to differentiate into "abnormal" neutrophils and decreases the risk of relapse within 2 years by more than 50%.[7] However, for other subtypes of AML, identification of practical means of differentiation therapy has proven to be more challenging.

Aberrant differentiation is often, partly at least, handled by epigenetic deregulation.[8] Research has shown that AML patients carry mutations in genes involved in DNA methylation and modification of histone marks; for example, somatic mutations in the isocitrate dehydrogenase 2 (IDH2) gene occurring at conserved arginine residues (R140 and R172)[9,10] could affect cellular metabolism, epigenetic regulation, redox states, and DNA repair. Mutations in genes encoding enzymes important in epigenetic regulation have been reported in several types of malignancies, including >80% of low-grade gliomas and secondary glioblastomas,[11] ~60% of chondrosarcomas,[12] ~20% of intrahepatic cholangiocarcinomas,[13] and ~10% of AMLs.[9,10] These mutant proteins have neomorphic enzyme activity leading to R-2-hydroxyglutarate (2-HG) accumulation. The resulting 2-HG accumulation competitively inhibits α-ketoglutarate-dependent enzymes,[10,14,15] resulting in DNA methylation,[14] increased repressive histone methylation,[16] and damaged hematopoietic differentiation. Because the neomorphic production of 2-HG is a gain of function that is exclusive to mutant IDH1/2 enzymes, researchers noted that these genetic changes may be encouraging targets for personalized anti-cancer therapy with small-molecule inhibitors.[17] Indeed, enasidenib (AG-221, IDH2-mutant inhibitor) was approved by the FDA for treatment for R/R IDH2-mutant AML patient as a first-in-class inhibitor.[17,18] Stein, et al.[18] investigated clinical responses in patients with R/R AML (n=176), and treatment with enasidenib elicited an overall response in 40.3% of patients and complete remission in about 50%. The median OS of the patients achieving complete remission was 19.7 months. In spite of the comparatively high response rates, not all patients experience clinical benefit. Therefore, differentiation-based IDH2 inhibition may need to be combined with other treatment modalities.[19]

ATRA has been used successfully in the treatment of APL. However, non-APL patients do not show comparable sensitivity to ATRA. Recent data, however, has shown that de novo AML patients with IDH1/2 mutations may benefit from ATRA. Boutzen, et al.[20] researched the sensitivity of leukemic cells with the IDH1/R132H mutation to ATRA. They noted that mutant IDH1/R132H-specific gene features controlled by key transcription factors, especially CEBPα, were involved in myeloid differentiation and retinoic responsiveness. Additionally, their results indicated the IDH1/R132H-mutated protein and its production, 2-HG, sensitized AML cell lines and primary AML cells for ATRA-induced granulocyte differentiation and apoptosis.

Therefore, in this study, the effects of combined treatment with ATRA and AG-221 on differentiation and possible underlying mechanisms in IDH2-mutant AML were explored.

MATERIALS AND METHODS

Cells and culture

The human AML cell line IDH2-mutant-TF-1 (TF-1/R140Q cells) was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The IDH2-naïve AML cell line (TF-1 cells) was kindly provided by Dr C.H. Park (Medicinal & Pharmaceutical Chemistry, Korea University of Science and Technology, Daejeon, Korea). All cell lines were maintained in RPMI-1640 medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA), 2 ng/mL of recombinant human GM-CSF (Merck KGaA, Darmstadt, Germany), and 100 U/mL penicillin in 5% CO_2 at 37°C. To maintain exponential growth, cells were seeded at 1×10^5 cells/ml and passaged every 3 days.

Patients and isolation of AML cells

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review of Board of Severance Hospital. Study participants provided written informed consent, and all patient samples were coded and linked anonymously. Identification of samples was possible using a code, and anonymized clinical information of linked samples was provided for researchers. Human leukemia cells were obtained from diagnostic bone marrow aspiration of patients with de novo AML diagnosed at Yonsei University Severance Hospital between 2006 and 2018. Mononuclear cells were isolated by Ficoll-Hypaque (GE Healthcare Life Sciences, Seoul, Korea) density gradient centrifugation and then cryopreserved. We selected four samples from AML patients with IDH2/R140Q mutation.

Reagents

Stock solutions of the following reagents were prepared by dissolving them in dimethyl sulfoxide (Biosesang, Seongnam, Korea): AG-221 (Selleckchem, Seoul, Korea), ATRA (Sigma-Aldrich, St. Louis, MO, USA), bafilomycin C1 from *Streptomyces griseus* (Sigma-Aldrich), U0126 (Sigma-Aldrich), and Sorafenib CSF (Merck KGaA). A stock solution of 3-methyladenine (Sigma-Aldrich) was prepared by dissolving it in distilled water.

Control cells were treated with equal volumes of the solvent.

Differentiation assay

Leukemic cell differentiation was evaluated according to morphology with Wright's staining and the expression of differentiation antigens CD14, CD15, and CD11b. CD14, CD15, and CD11b were measured using fluorescein isothiocyanate (FITC)-label anti-CD14 (BD Biosciences, Franklin Lakes, NH, USA), FITC-label anti-CD15 (BD Biosciences), and phycoerythrin (PE)-label anti-CD11b (BD Biosciences) with isotype controls by flow cytometry (BD FACSCalibur, Franklin Lakes, NH, USA).

Western blot analysis

Total cell lysates were prepared and analyzed by Western blotting as described previously.[21] Rabbit polyclonal anti-body against LC3 was obtained from Novus Biologicals (Centennial, CO, USA). P62/SQSTM1 was purchased from Abnova (Taiwan). Beclin-1 and rabbit polyclonal antibodies against phospho-c-RAF (Ser259), phospho-MEK1/2 (Ser221), and phospho-p44/42 MAPK (ERK1/2)(Thr202/Tyr204) were purchased from Cell Signaling Technology (Danvers, MA, USA). Bi-methyl H3K9, bi-methyl H3K27, and tri-methyl H3K9 antibodies were obtained from Abcam (Cambridge, UK). Mouse anti-α-tubulin monoclonal antibodies were obtained from Merck Millipore (Burlington, MA, USA). Secondary antibodies were coupled to horseradish peroxidase and visualized by enhanced chemiluminescence (GE Healthcare Life Sciences, Seoul, Korea).

2-HG measurement

A cell suspension of 1×10^7 leukemia cells was prepared, and 2-HG levels were measured by 2-HG Assay Kits (Colorimetric) (Abcam, Cambridge, UK). We set up plates in duplicate for external standard (50 μL of 20 nM 2-HG), internal standard (50 μL of 5 nM 2-HG+sample), sample (50 μL), sample background (50 μL), and reagent background (50 μL of buffer). Plates were incubated at 37°C for 60 minutes protected from light. The amount of 2-HG was measured on a microplate reader at optical density (OD) 450 nm.

Statistical analysis

Results are expressed as the mean±standard deviation of at least three independent experiments. Comparison of two groups was performed using two-tailed Student's t-test. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences with p values of less than 0.05 were considered statistically significant.

RESULTS

AG-221 induces differentiation of TF-1/R140Q cells

We initially examined the effects of AG-221 on differentiation in the IDH2-mutant AML cell line TF-1/R140Q and the IDH2-naïve AML cell line TF-1. The TF-1/R140Q cells were derived from parental TF-1 cells. TF-1/R140Q and TF-1 cells were treated for 3, 5, and 7 days with increasing concentrations of AG-221. As shown in Fig. 1A, AG-221 treatment for 7 days increased CD14 and CD15 expression in TF-1/R140Q cells in a dose-dependent manner. Treatment with more than 50 μM AG-221 for 5 days also increased CD14 and CD15 expression in TF-1/R140Q cells. However, the CD14 and CD15 expression rates were minimal when TF-1 cells were treated with up to 50 μM AG-221 for 7 days. Treatment of TF-1/R140Q cells with 10 μM AG-221 for 5 and 7 days exhibited mature-related morphological alterations, such as condensed chromatin and decreased nuclei/cytoplasm ratio with smaller nuclei (Fig. 1B).

ATRA induces differentiation of TF-1/R140Q cells

Next, we examined whether ATRA induces differentiation of TF-1/R140Q cells. Treatment of IDH2/R140Q cells with ATRA for 7 days produced significant differentiation, as determined by increases in the percentages of $CD14^+$ and $CD15^+$ cells, in a dose-dependent manner (Fig. 2A). Treatment of TF-1/R140Q cells with 100 nM ATRA for 5 and 7 days showed alterations of cell morphology with mature characters (Fig. 2B). In contrast, treatment of ATRA in TF-1 cells did not result in differentiation induction, indicated by the lack of surface antigens associated with myeloid differentiation, including CD14 and CD15, and mature-related morphologic changes (Fig. 2).

Combined treatment with AG-221 and ATRA inhibits the production of 2-HG in TF-1/R140Q cells

As reported previously[22] and consistent with observations in myeloblasts from patients with IDH1/2-mutant AML,[15] TF-1/R140Q cells induced intracellular 2-HG production at concentrations of 48.5 to 59.7 nmol/mg. However, TF-1 cells did not produce intracellular 2-HG. We treated the TF-1/R140Q cells with 30 μM AG-221 and 100 nM ATRA, alone or in combination (Fig. 3A). AG-221 induced suppression of intracellular 2-HG levels in TF-1/R140Q cells. When cultured in the presence of 30 μM AG-221, TF-1/R140Q cells showed an approximately 81% decrease in intracellular 2-HG. ATRA treatment also suppressed intracellular 2-HG levels in TF-1/R140Q cells. TF-1/R140Q cells were more sensitive to the inhibitory action of AG-221 than ATRA. Decreases in intracellular 2-HG levels were more marked when the TF-1/R140Q cells were treated with a combination of AG-221 and ATRA rather than when the cells were treated with either AG-221 or ATRA.

Combined treatment with AG-221 and ATRA elicits synergistic benefits in differentiation of IDH2/R140Q cells and primary AML samples

In order to assess the effect of the combination therapy, we treated IDH2/R140Q cells with 30 μM AG-221 and 100 nM ATRA, alone or in combination. We treated the cells continuously for 7 days. We determined the percentage of differentiated

https://doi.org/10.3349/ymj.2020.61.9.762

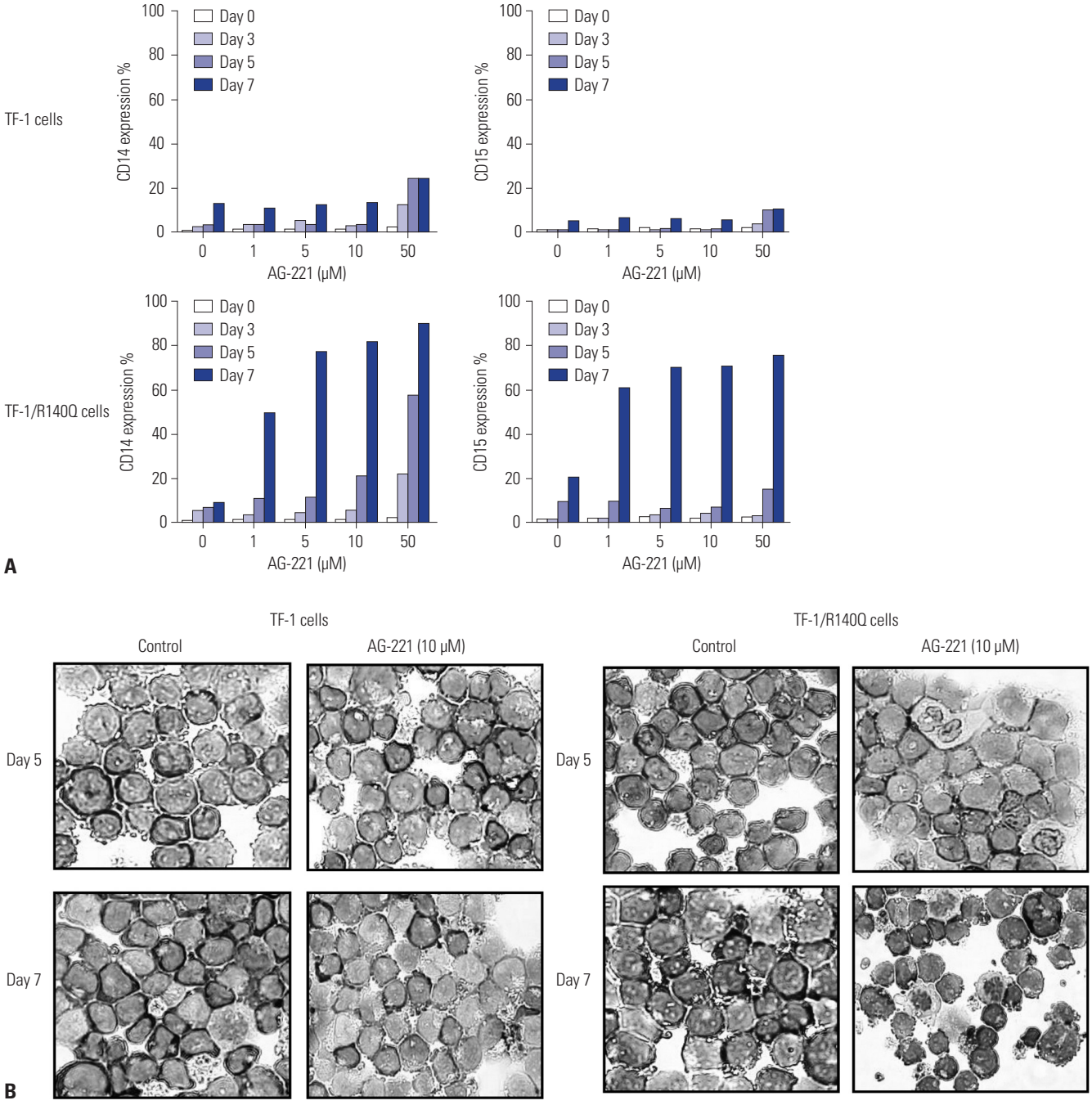


Fig. 1. AG-221 induces differentiation of TF-1/R140Q cells. (A) TF-1 and TF-1/R140Q cells were exposed to 1, 5, 10, and 50 μM AG-221 for the indicated time periods. After treatment, differentiation was assessed according to CD14 and CD15 expression by flow cytometry. (B) After treatment of TF-1 and TF-1/R140Q cells with 10 μM AG-221 for 5 and 7 days, cell morphology was observed under a microscope following Wright's staining of cells that were collected onto slides by cytospin.

cells (surface expression of myeloid marker CD14 and CD15) under the various treatment conditions by flow cytometry. The combination of 30 μM AG-221 and 100 nM ATRA induced a larger population of differentiated $CD14^+$ and $CD15^+$ cells in comparison to single ATRA and AG-221 treated cells after 7 days (Fig. 3B). Cytology revealed that by day 7 of AG-221 and ATRA combination treatment, the blasts or myeloblasts had decreased, and early signs of maturation were observed by increases in the number of myelocytes and metamyelocytes (Fig. 3C). We applied the same treatment regimens to TF-1 cell lines. As expected, cell differentiation by the AG-221 alone was reduced to 27.4±3.0%, and the cells showed minimal differentiation in the presence of AG-221 and ATRA combination treatment.

To investigate the effect of AG-221 and ATRA individually or in combination on primary human AML cells, we treated pa-

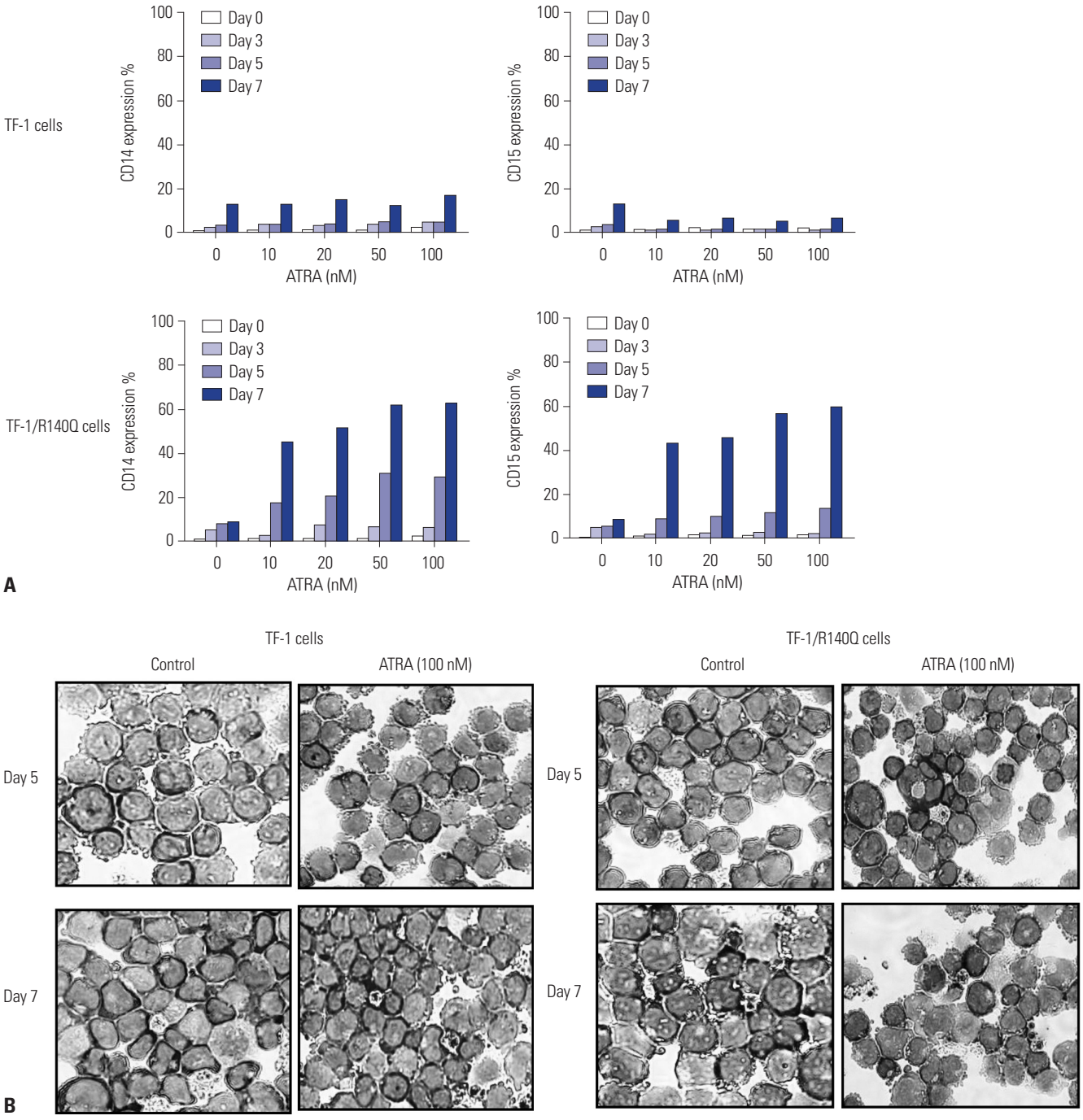


Fig. 2. ATRA induces differentiation of TF-1/R140Q cells. (A) TF-1 and TF-1/R140Q cells were exposed to 10, 20, 50, and 100 nM ATRA for the indicated time periods. After treatment, differentiation was assessed according to CD14 and CD15 expression by flow cytometry. (B) After treatment of TF-1 and TF-1/R140Q cells with 100 nM ATRA for 5 and 7 days, cell morphology was observed under a microscope following Wright's staining of cells that were collected onto slides by cytospin. ATRA, all-trans retinoic acid.

tient samples containing IDH2/R140Q mutation (n=4) (Table 1). Patient bone marrow samples were sorted and cultured in conditioned medium either in the presence or absence of 30 μM AG-221 and 100 nM ATRA, alone or in combination, and the samples of each culture were collected at 7 days. Differentiation of AML blasts was evaluated by flow cytometry analysis for changes in cell surface markers associated granulocytic differentiation (CD11b, CD14, and CD15). Treatment with AG-221 or ATRA induced an increase in the number of differentiated cells in IDH2/R140Q patient samples. In all IDH2-mutant samples, the increases in percentages for all cell surface markers were greater for AG-221 and ATRA combination therapy than for AG-221 or ATRA treatment alone (Fig. 3D).

https://doi.org/10.3349/ymj.2020.61.9.762

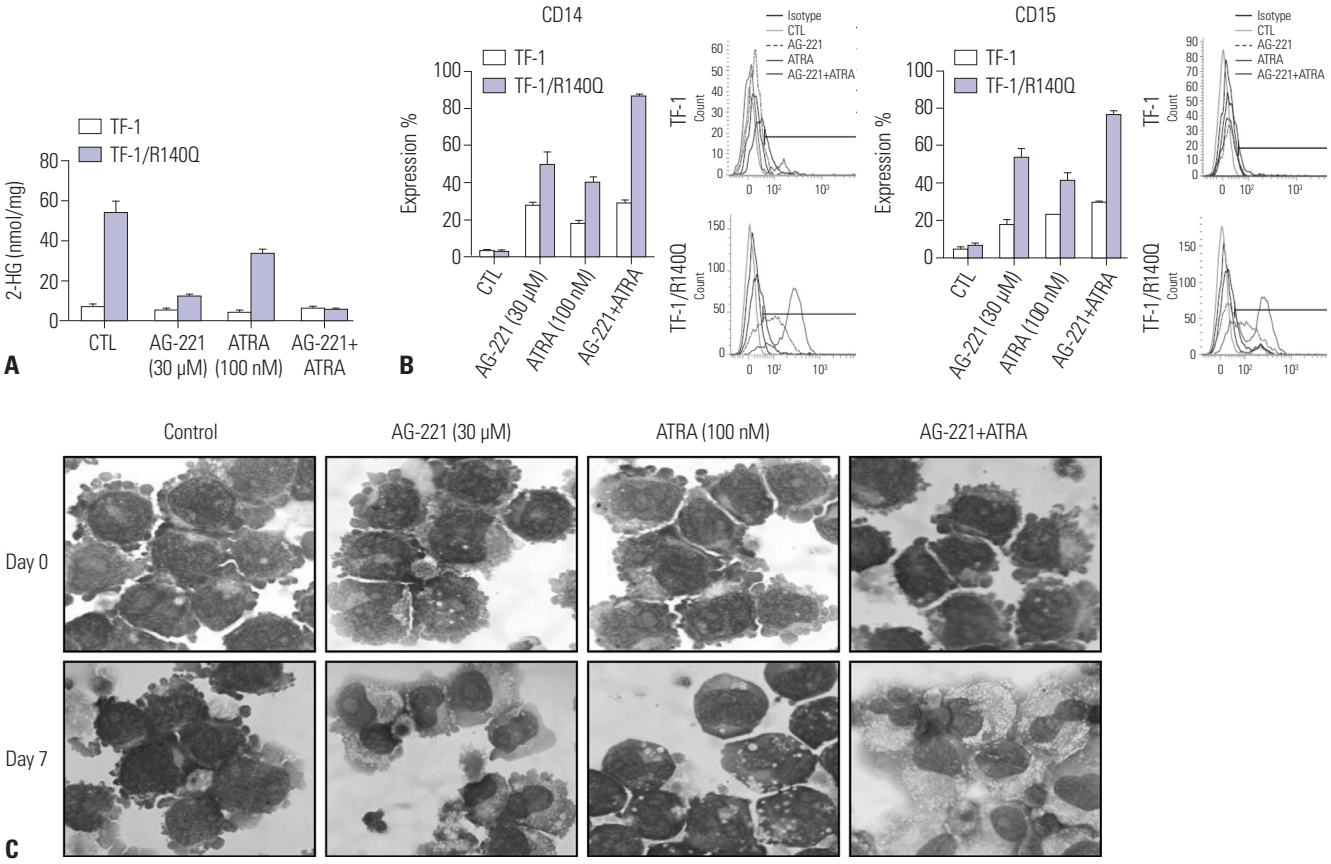


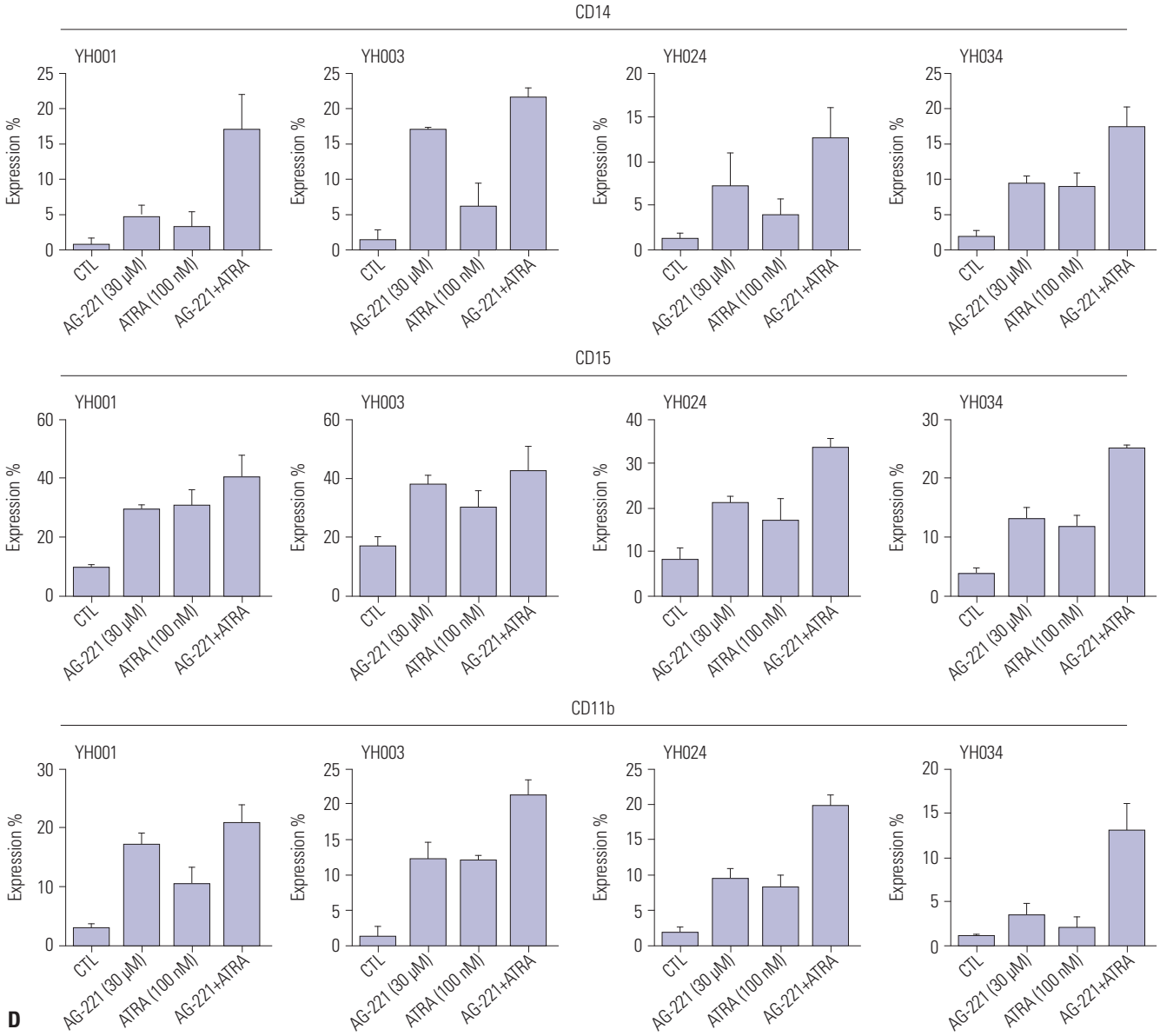
Fig. 3. AG-221 and ATRA combined effects on differentiation of TF-1/R140Q cells and primary AML samples. (A and B), TF-1 and TF-1/R140Q cells were exposed to 30 μM AG-221 and 100 nM ATRA, alone or in combination, for 7 days. (A) Intracellular amounts of 2-HG determined by D-2-HG Assay Kit (Colorimetric). (B) After treatment, differentiation was assessed according to CD14 and CD15 expression by flow cytometry. (C) After treatment of TF-1 and TF-1/R140Q cells with 30 μM AG-221 and 100 nM ATRA, alone or in combination, for 7 days, cell morphology was observed under a microscope following Wright's staining of cells that were collected onto slides by cytospin. Differentiation of AML blasts was evaluated by flow cytometry analysis for changes in CD11b, CD14, and CD15 expression. $p \leq 0.05$ indicates statistical significance of the observed differences. Columns, mean values of three independent experiments for cell lines and data obtained from four primary samples; bars, standard deviations. ATRA, all-trans retinoic acid; AML, acute myeloid leukemia; IDH2, isocitrate dehydrogenase 2.

Combined treatment with AG-221 and ATRA alters autophagic activity during differentiation of IDH2/R140Q cells

To examine alterations in autophagy-related molecules by AG-221 and ATRA, Western blot analyses were performed on whole cell lysates from TF-1/R140Q and TF-1 cells collected 7 days after treatment with 30 μM AG-221 and 100 nM ATRA, alone or in combination. In TF-1/R140Q cells treated with AG-221 and ATRA, we observed an increase in the conversion of LC3-I to LC3-II, which is a widely accepted marker for autophagosome formation.[23] The increase in beclin-1, another indicator for autophagy initiation that participates in autophagosome formation by interacting with hVps34,[24] paralleled the increased LC3-II levels in TF-1/R140Q cells after treatment with AG-221 and ATRA. When 30 μM AG-221 and 100 nM ATRA was used in combination, the accumulation of LC3-II and beclin-1 proteins were further enhanced, compared to treatment with AG-221 or ATRA alone. In addition, p62/SQSTM1 protein interacts with both LC3-II and ubiquitin protein and is degraded in autophagosomes. Therefore, an increase in p62/SQSTM1 would indicate inhibited autophagic degradation, whereas a reduction in p62/SQSTM1 would indicate increased autophagic degradation. As shown in Fig. 4A, AG-221 and ATRA treatment failed to decrease the expression levels of p62/SQSTM1 protein. Collectively, these results suggested that, during AG-221 and ATRA-induced leukemic cell differentiation, autophagosome formation increases, while the degradation of autophagic vacuoles decreases.

Next, we examined whether autophagy regulation contributes to the effects of combined treatment with AG-221 and ATRA on the differentiation of TF-1/R140Q cells by autophagy inhibition using pharmacological inhibitors. The addition of 3-MA to AG-221 and ATRA combination treatment resulted in a significant decrease in CD14 and CD15 expression (Fig. 4B) (p=0.007; p=0.013), compared to treatment with AG-221 or ATRA. However, the addition of bafilomycin A1 to AG-221 and ATRA combination treatment failed to change the expression levels of CD14 and CD15 (Fig. 4B) (p=0.669; p=0.943), compared to

that treatment with AG-221 or ATRA. Bafilomycin A1 is a known inhibitor of the late phase of autophagy, preventing the maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes.[25] Thus, bafilomycin A1 was introduced to test whether autophagy deficiency affects (especially the defect of late step in autophagy) AG-221 and ATRA-



D

Fig. 3. AG-221 and ATRA combined effects on differentiation of TF-1/R140Q cells and primary AML samples. (D) Primary AML cells with IDH2-mutant (n=4) were cultured in conditioned medium either in the presence or absence of 30 μM AG-221 and 100 nM ATRA, alone or in combination, and samples for each culture were collected on day 7. Differentiation of AML blasts was evaluated by flow cytometry analysis for changes in CD11b, CD14, and CD15 expression. $p \leq 0.05$ indicates statistical significance of the observed differences. Columns, mean values of three independent experiments for cell lines and data obtained from four primary samples; bars, standard deviations. ATRA, all-trans retinoic acid; AML, acute myeloid leukemia; IDH2, isocitrate dehydrogenase 2.

Table 1. Overview of Primary Human AML Cells

Patient no.	Sex	IDH2	NGS	Chromosome	Source	CD34 %
YH001	Male	R140		46, XY [20]	BM	96.40
YH003	Male	R140	IDH2, NRAS, KMT2C, BCORL1, PHF6	47, XY, +14 [13]	BM	78.73
YH024	Male	R140	NPM1, IDH2, PTPN11	46, XY	BM	0.39
YH034	Male	R140	IDH2, NRAS, BCOR, BCORL1	46, XY	BM	1.30

AML, acute myeloid leukemia; BM, bone marrow; NGS, next generation sequencing; IDH2, isocitrate dehydrogenase 2.

https://doi.org/10.3349/ymj.2020.61.9.762

induced differentiation. The results indicated that AG-221 and ATRA treatment-induced differentiation of TF-1/R140Q cells is associated with autophagy, without suppressing autophagy flux at maturation and degradation stages.

Activation of the RAF-1/MEK/ERK pathway upon combined treatment with AG-221 and ATRA in IDH2/R140Q cells

With mounting evidence indicating that ATRA-induced differentiation in a variety of cell lines is dependent on MEK/ERK activation and with more comprehensive information of MEK/ERK signaling in some models,[26,27] we examined alterations in MEK/ERK signaling in AG-221 and ATRA-induced differentiation in TF-1/R140Q cells. We investigated whether MEK/ERK was activated with AG-221 and ATRA treatment by Western blot analysis of phosphorylated MEK1/2 (Ser221) and phosphorylated ERK1/2 (Thr202/Try204). MEK was phosphorylated even in TF-1/R140Q cells without treatment. Moreover, following treatment with 30 μM AG-221 and 100 nM ATRA alone or in combination for 7 days, the amounts of phosphorylated MEK were increased. Meanwhile, the activation of ERK1/2 occurred after 7 days by AG-221 and ATRA treatment (Fig. 5A). To further study whether MEK/ERK activation was necessary for AG-221 and ATRA-induced differentiation in TF-1/R140Q cells, the cells were incubated with 2 μM U0126, a specific inhibitor of MEK, with AG-221 and ATRA treatment. The effectiveness of U0126 was evaluated by ERK1/2 phosphorylation with the indicated treatment for 7 days. When the activation of ERK 1/2 peaked after AG-221 and ATRA combination treatment, U0126 did attenuate ERK1/2 activation (Fig. 5B). Co-

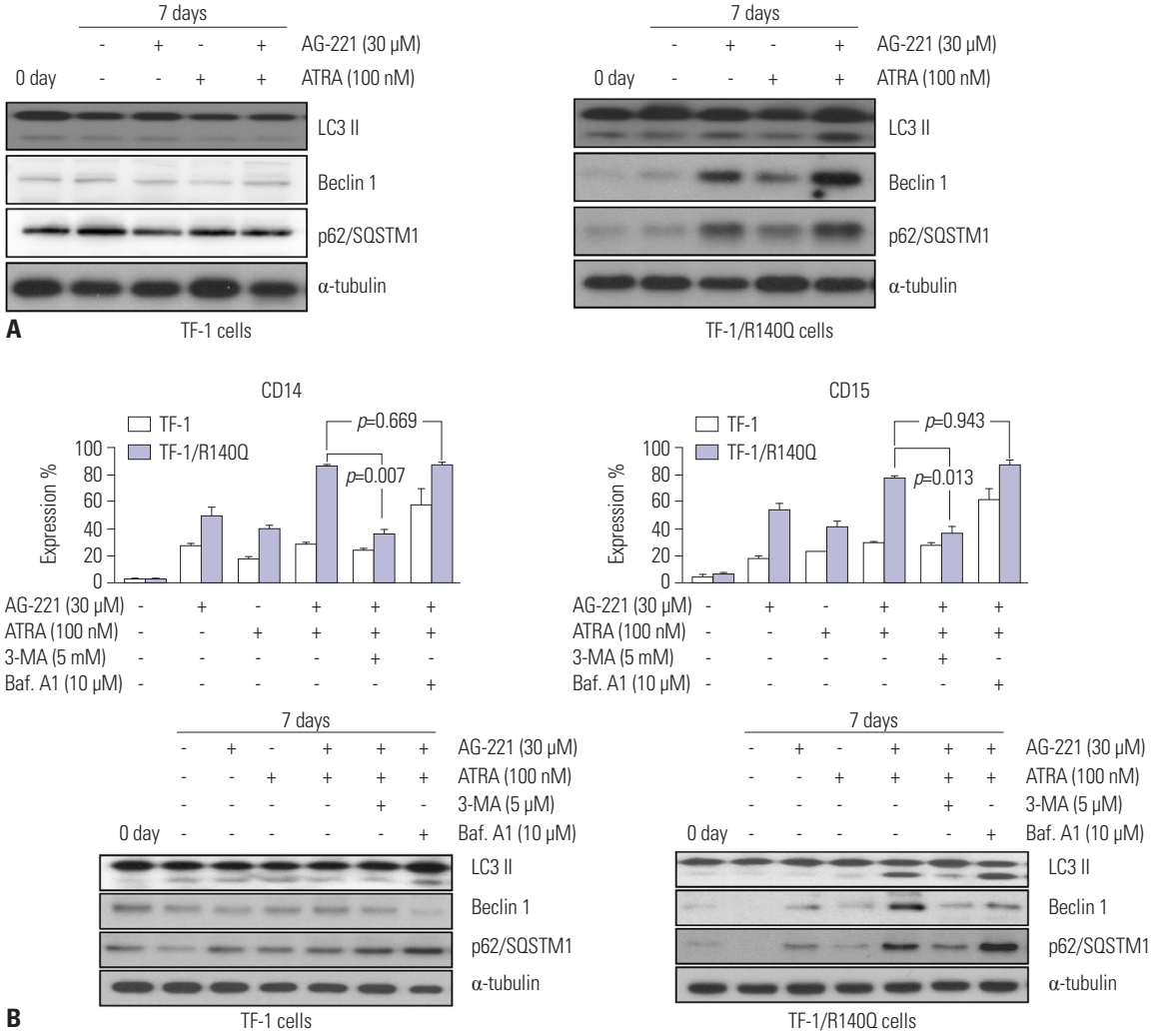


Fig. 4. Effects of autophagy inhibition on AG-221 and ATRA-induced differentiation of TF-1/R140Q cells. TF-1 and TF-1/R14-Q cells were treated with 30 μM AG-221 and 100 nM ATRA alone or in combination for 7 days. (A) Autophagy was measured in both cells by evaluating protein levels of LC3-I/II, beclin-1, and p62/SQSTM1 in whole cell lysates. α-tubulin was used as a protein-loading control. (B) TF-1 and TF-1/R140Q cells were treated for 7 days with 30 μM AG-221 and 100 nM ATRA combination treatment or in addition of autophagy inhibitor, either 3-methyladenine (3-MA, 5 μM) or bafilomycin A1 (Baf. A1, 10 μM). Inhibition of AG-221 and ATRA-induced autophagy by 3-MA or Baf. A1 was measured by Western blotting for LC3-I/II, beclin-1, and p62/SQSTM1 expressions. CD14 and CD15 expression by flow cytometry on day 7 was used as a marker of differentiation. $p \leq 0.05$ indicates statistical significance of the observed differences. ATRA, all-trans retinoic acid.

treatment with 2 μM U0126 significantly suppressed CD14 and CD15 expression (Fig. 5B) (p=0.008; p=0.006). These results highlighted the role of the MEK/ERK signal pathway in the differentiation-inducing effects of AG-221 and ATRA in TF-1/R140Q cells. To investigate the upstream molecules in the MEK/ERK signal pathway involved in AG-221 and ATRA-induced differentiation of TF-1/R140Q cells, we assessed RAF-1, a classical upstream regulator of the MEK/ERK signal pathway. The activation of RAF-1 by AG-221 and ATRA was analyzed by Western blot of Phospho-c-Raf (Ser259). RAF-1 was phosphorylated even in TF-1/R140Q cells without treatment. The phosphorylation of RAF-1 at Ser338 was not increased with AG-221 and ATRA treatment (Fig. 5A). To further investigate whether RAF-1 activation was required for MEK/ERK signaling, cells were co-treated with 2.5 μM sorafenib tosylate, a specific inhibitor of RAF-1. The inhibitory effect of sorafenib tosylate on RAF-1 activation was evaluated by phosphorylation of MEK1/2, as well as ERK1/2, with the indicated treatments for 7 days. The phosphorylation of MEK1/2 ERK1/2 upon combined treatment with AG-221 and ATRA for 7 days was decreased by sorafenib tosylate co-treatment (Fig. 5B). Thus, the activation of MEK/ERK by AG-221 and ATRA was deemed to be dependent on RAF-1. Moreover, 2 μM sorafenib tosylate also attenuated AG-221 and ATRA-induced differentiation, as assessed by CD14 and CD15 expression (p=0.012; p=0.013) (Fig. 5B). These results demonstrated the involvement of RAF-1/MEK/ERK in AG-221 and ATRA-induced differentiation in TF-1/R140Q cells.

Combined treatment with AG-221 and ATRA alters isocitrate dehydrogenase-associated changes in histone methylation

Studies have demonstrated that 2-HG-producing IDH mutants can prevent histone demethylation, which is required for lineage-specific progenitor cells to differentiate into terminally differentiated cells.[16] To test whether histone lysine methyla-

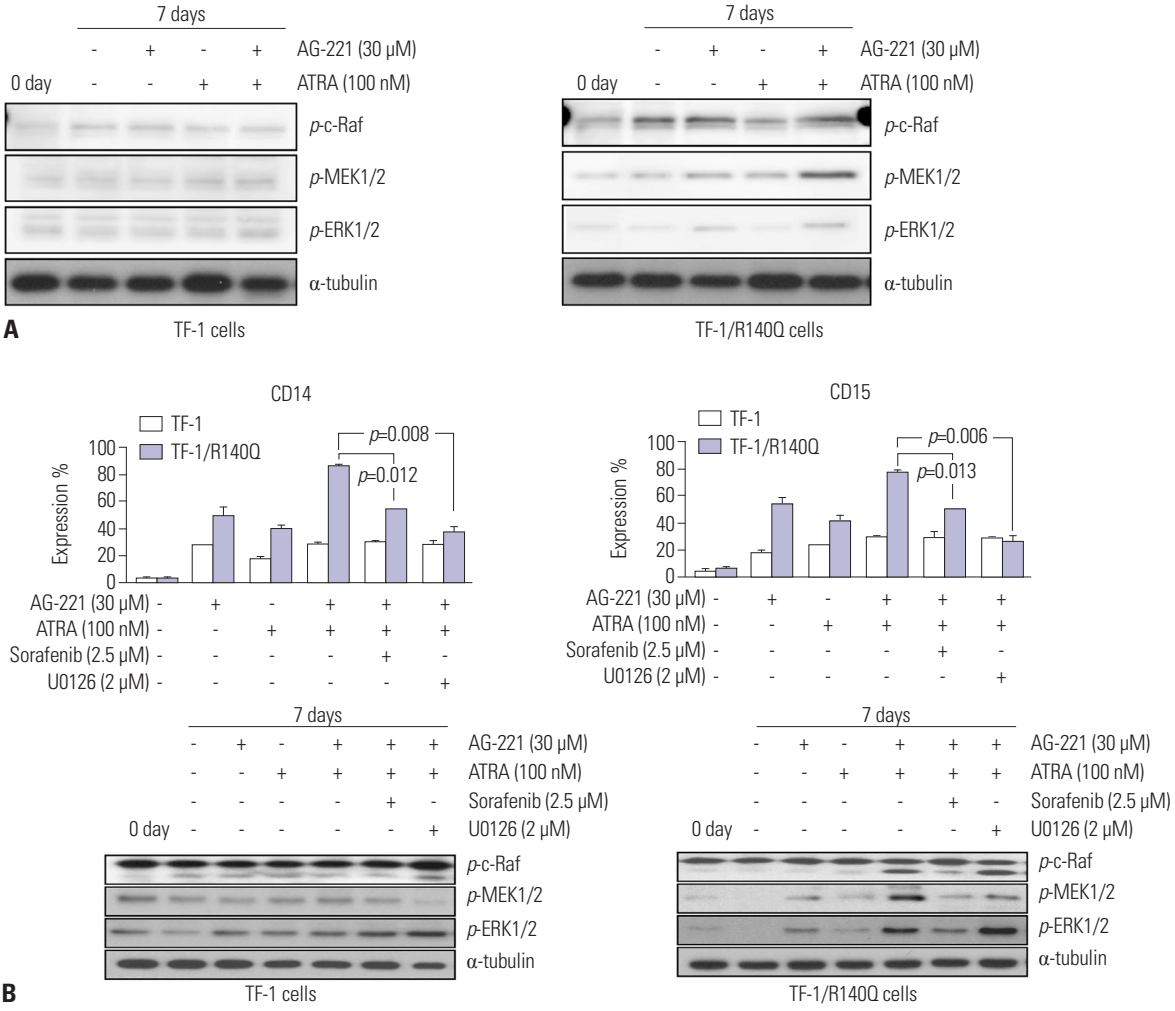


Fig. 5. Activation of RAF-1/MEK/ERK on AG-221 and ATRA-induced differentiation of TF-1/R140Q cells. (A) The activation of RAF-1/MEK/ERK signaling was evaluated by Western blot analysis of the phosphorylation status of MEK1/2, ERK1/2, and c-Raf in TF-1 and TF-1/R140Q cells treated with 30 μM AG-221 and 100 nM ATRA for 7 days. α-tubulin was used as a protein-loading control. (B) Cells were co-treated with either 2 μM U0126 or 2.5 μM sorafenib, and AG-221 and ATRA-induced differentiation for 7 days was evaluated by flow cytometry using CD14 and CD15 expressions. $p \leq 0.05$ indicates statistical significance of the observed differences. ATRA, all-trans retinoic acid.

https://doi.org/10.3349/ymj.2020.61.9.762

tion is dysregulated in IDH2-mutant AML, Western blot analysis of TF-1/R140Q and TF-1 cells was performed for well-characterized histone markers. Compared to IDH2-naïve AML cells, we noted an increase in the repressive trimethylation of H3K9 (H3K9me3) and no significant difference in biomethylation of H3K27 (H3K27me2) and H3K9 (H3K9me2) in IDH2-mutant AML cells (Fig. 6). To further study whether the induction of differentiation by AG-221 and ATRA treatment alters histone methylation markers, Western blot analysis of histone methylation markers was performed on whole cell lysates from TF-1/R140Q collected 7 days after treatment with 30 μM AG-221 and 100 nM ATRA alone or in combination. H3K9me3, H3K9me2, and H3K27me2 proteins were decreased significantly only after combined treatment with AG-221 and ATRA (Fig. 6).

DISCUSSION

Although IDH2 inhibitor, such as AG-221, has shown early success in AML,[28] not all patients experience clinical benefits. It is expected that for more powerful responses, differentiation-based IDH2 inhibition will need to be combined with other treatment modalities, such as standard chemotherapy, or other types of mechanism-based target therapy.[19] This study showed that combination therapy, specifically AG-221 and ATRA, in IDH2-mutant AML achieves therapeutic efficacy through induction of differentiation.

Previous studies have shown that AG-221 results in differentiation in IDH2-mutant TF-1 cells and primary AML cells ex vivo, as well as in IDH2/R140Q mutant AML xenograft mouse models in vivo, and have reported a survival benefit in an in vivo AML xenograft model.[29] Our findings confirm the AG-221 causes differentiation of IDH2-mutant AML cells, as demonstrated by changes in morphology and myeloid-related antigens. AG-221 is a first-in-class inhibitor of the mutant IDH2 enzyme. AG-221 binds to an allosteric site within the dimer link, stabilizing the open configuration of the enzyme and inhibiting the change of α-ketoglutarate to 2-HG. AG-221 exhibits excellent pharmaceutical characteristics, including proper solubility, low clearance, and good oral bioavailability, and powerfully inhibits 2-HG production by both the IDH2/R140Q-WT heterodimer and IDH2/R140Q homodimer.[29] Our data indicated that in IDH2-mutant AML cells, IDH2/R140Q expression in the TF-1 erythroleukemia cell line produced intracellular 2-HG and that 2-HG levels were decreased by AG-221 treatment.

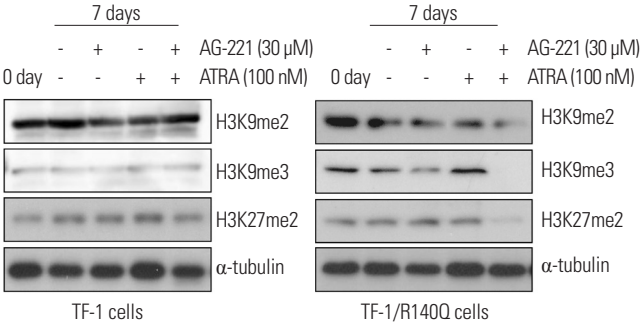


Fig. 6. Decreases in histone methylation after AG-221 and ATRA combination treatment. TF-1 and TF-1/R140Q cells were treated with 30 μM AG-221 and 100 nM ATRA alone or in combination for 7 days. TF-1 and TF-1/R140Q cells were assessed for expression levels of histone methylation by Western blot with specific antibodies. α-tubulin was used as a protein-loading control. Similar results were obtained in three independent experiments. ATRA, all-trans retinoic acid.

Although sustained efforts are being made to successfully combine IDH2 inhibitors with chemotherapy,[30] we also now have a better understanding of the molecular changes of specific pathways that frequently occur in AML. Therefore, the investigation of further targeted therapeutic agents that may synergize with IDH2 inhibition could move clinical trials toward the goal of eliminating chemotherapy altogether. Here, ATRA has been shown to act synergistically with IDH2 inhibitor-induced differentiation in IDH2 mutant AML cells and primary AML cells, compared with either drug alone. The enhanced effectiveness of AG-221 and ATRA combination treatment in inducing differentiation was most evident after 7 days of treatment in IDH2-mutant AML cells.

Autophagy plays an intrinsic role as an protein turnover in mammalian development and differentiation.[31,32] Within the hematopoietic system, autophagy plays a vital role in organelle clearance during reticulocyte differentiation,[32] lymphocyte differentiation[32] and plasma cell differentiation,[33] indicating that autophagy may also be essential for normal myeloid differentiation. Autophagy is increased during ATRA-induced granulocytic differentiation of the APL cell line, and this is linked with increased expression of LC3II and GATE-16 proteins involved in autophagosome formation. Autophagy inhibition attenuates myeloid differentiation.[34] Consistent with this finding, AG-221 and ATRA combination treatment was found to alter autophagic activity in a manner of considerable accumulation of autophagosomes without an increase in the degradation of autophagic vacuoles, as assessed by the elevation of LC3-II and beclin-1 protein levels, as well as no significant decrease of p62/SQSMT1. Autophagy inhibition using a pharmacological inhibitor resulted in a decrease of AG-221 and ATRA-induced differentiation in IDH2-mutant AML cells. However, a decrease in AG-221 and ATRA-induced differentiation following cotreatment with the autophagic degradation inhibitor bafilomycin A1 in IDH2-mutant AML cells was not observed. Taken together, the results indicated that AG-221 and ATRA treatment-induced differentiation of IDH2-mutant AML cells was associated with autophagy induction, without suppressing autophagy flux at maturation and degradation stages.

Recently, RAF-1/MEK/ERK cascade has been reported to be associated with ATRA-induced differentiation in APL cells.[35] Consistent with this finding, MEK/ERK activation after AG-221 and ATRA treatment was found to induce differentiation

through MEK1/2 (Ser221) and ERK1/2 (Thr202/Tyr204) phosphorylation in IDH2-mutant AML cells. The role of MEK/ERK activation in AG-221 and ATRA-induced differentiation was shown by targeting MEK with a pharmacologic inhibitor. MEK inhibition reduced AG-221 and ATRA-induced differentiation of IDH2-mutant AML cells. To investigate the upstream regulators of MEK/ERK, RAF-1 was selected. Similar to MEK activation, the phosphorylation of RAF-1 at Ser259 was found and continued with AG-221 and ATRA treatment. The inhibition of RAF-1 promoted MEK/ERK inhibition and decreased AG-221 and ATRA-induced differentiation in IDH2-mutant AML cells. This suggested that RAF-1 is a positive upstream regulator of the MEK/ERK signal pathway involved in AG-221 and ATRA-induced differentiation in IDH2-mutant AML cells. Indeed, research has shown that RAF-1 is an upstream molecule of MEK/ERK and that RAF-1/MEK/ERK cascade participates in ATRA-triggered differentiation in HL-60 cells.[36]

A recent study reported that IDH mutation worsens histone demethylation and blocks cell differentiation.[16] In the present study, we discovered a marked increase in the histone marker H3K9me3 in IDH2-mutant AML cells, compared with IDH2-WT AML cells. Meanwhile, biochemical studies have suggested that 2-HG is a universal inhibitor of Jumonji-C domain histone demethylase (JHDM) family members.[37] Future investigation of sensitivity to 2-HG inhibition among JHDM family members and/or cellular feedback mechanisms promoted after faulty histone demethylation will be needed.

This study showed that ATRA enhances IDH2 inhibitor-induced differentiation in IDH2-mutant AML cells. These results suggest that combining IDH2 inhibition with ATRA treatment would likely be minimal cytotoxic to normal cells and would be well tolerated by older AML patients, giving at least transient disease control in the absence of cytotoxic chemotherapy. Furthermore, we provide preliminary evidence that the addition of ATRA may lead to further improvements or increases in response rates to treatment with IDH2 inhibitor in IDH2-mutant AML patients who show no benefits from them otherwise. Our results indicate that autophagy is a vital component of the influence of AG-221 and ATRA on differentiation in IDH2 mutant AML and that pharmacologic autophagy regulation is this context may potentiate the AG-221 and ATRA-induced differentiation of IDH2-mutant AML cells. The definitive mechanisms through which AG-221 and ATRA activate autophagy remain to be explained. Further studies will address this issue so that we can understand how best to target this process for patient benefits. The present results further demonstrated that the induction of differentiation by AG-221 and ATRA requires the activation of RAF-1/MEK/ERK cascades. Considering the complex mechanisms involved in the activation of the RAF/MAK/ERK pathway, further studies will need to be performed to better understand the effect of AG-221 and ATRA-induced activation of MEK/ERK kinases, and the identification of more downstream targets of the MEK/ERK signal pathway in this cell model might be crucial to explain its translational role.

Although all of the events responsible for AG-221 and ATRA-induced differentiation in IDH2-mutant AML cells have not yet been elucidated, we discovered that combining IDH2 inhibition with ATRA treatment may be useful for inducing the differentiation of leukemia cells, thereby providing improved therapeutic responses in AML patients.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2017R1D1A1B03036419).

AUTHOR CONTRIBUTIONS

Conceptualization: Yoo Hong Min. Data curation: Yoo Hong Min. Formal analysis: Yundeok Kim. Funding acquisition: Yundeok Kim. Investigation: Hoi-Kyung Jeung. Methodology: Jaewoo Song. Project administration: June-Won Cheong. Resources: Soo Han Bae. Software: Jong In Lee. Supervision: June-Won Cheong. Validation: Yoo Hong Min. Visualization: Hoi-Kyung Jeung. Writing—original draft: Yundeok Kim. Writing—review & editing: June-Won Cheong and Yundeok Kim. Approval of final manuscript: all authors

ORCID iDs

Yundeok Kim https://orcid.org/0000-0002-5336-7936
Hoi-Kyung Jeung https://orcid.org/0000-0003-1075-8627
June-Won Cheong https://orcid.org/0000-0002-1744-0921
Jaewoo Song https://orcid.org/0000-0002-1877-5731
Soo Han Bae https://orcid.org/0000-0002-8007-2906
Jong In Lee https://orcid.org/0000-0002-3027-8762
Yoo Hong Min https://orcid.org/0000-0001-8542-9583

REFERENCES

1. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood 2017;129:424-47.
2. Estey E, Döhner H. Acute myeloid leukaemia. Lancet 2006;368:1894-907.
3. Walter RB, Kantarjian HM, Huang X, Pierce SA, Sun Z, Gundacker HM, et al. Effect of complete remission and responses less than complete remission on survival in acute myeloid leukemia: a combined Eastern Cooperative Oncology Group, Southwest Oncology Group, and M. D. Anderson Cancer Center Study. J Clin Oncol 2010;28:1766-71.
4. Juliusson G, Lazarevic V, Hörstedt AS, Hagberg O, Höglund M; Swedish Acute Leukemia Registry Group. Acute myeloid leukemia in the real world: why population-based registries are needed. Blood 2012;119:3890-9.
5. Tocci A, Parolini I, Gabbianelli M, Testa U, Luchetti L, Samoggia P, et al. Dual action of retinoic acid on human embryonic/fetal hematopoiesis: blockade of primitive progenitor proliferation and shift from multipotent/erythroid/monocytic to granulocytic differentiation program. Blood 1996;88:2878-88.

6. Fenaux P, Chomienne C, Degos L. All-trans retinoic acid and chemotherapy in the treatment of acute promyelocytic leukemia. Semin Hematol 2001;38:13-25.
7. Fenaux P, Chevret S, Guerci A, Fegueux N, Dombret H, Thomas X, et al. Long-term follow-up confirms the benefit of all-trans retinoic acid in acute promyelocytic leukemia. European APL group. Leukemia 2000;14:1371-7.
8. Cancer Genome Atlas Research Network, Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 2013;368:2059-74.
9. Gross S, Cairns RA, Minden MD, Driggers EM, Bittinger MA, Jang HG, et al. Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. J Exp Med 2010;207:339-44.
10. Janin M, Mylonas E, Saada V, Micol JB, Renneville A, Quivoron C, et al. Serum 2-hydroxyglutarate production in IDH1- and IDH2-mutated de novo acute myeloid leukemia: a study by the Acute Leukemia French Association group. J Clin Oncol 2014;32:297-305.
11. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science 2008;321:1807-12.
12. Pansuriya TC, van Eijk R, d'Adamo P, van Ruler MA, Kuijjer ML, Oosting J, et al. Somatic mosaic IDH1 and IDH2 mutations are associated with enchondroma and spindle cell hemangioma in Ollier disease and Maffucci syndrome. Nat Genet 2011;43:1256-61.
13. Borger DR, Tanabe KK, Fan KC, Lopez HU, Fantin VR, Straley KS, et al. Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. Oncologist 2012;17:72-9.
14. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 2010;18:553-67.
15. Fathi AT, Sadrzadeh H, Borger DR, Ballen KK, Amrein PC, Attar EC, et al. Prospective serial evaluation of 2-hydroxyglutarate, during treatment of newly diagnosed acute myeloid leukemia, to assess disease activity and therapeutic response. Blood 2012;120:4649-52.
16. Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. Nature 2012;483:474-8.
17. Popovici-Muller J, Saunders JO, Salituro FG, Travins JM, Yan S, Zhao F, et al. Discovery of the first potent inhibitors of mutant IDH1 that lower tumor 2-HG in vivo. ACS Med Chem Lett 2012;3:850-5.
18. Stein EM, DiNardo CD, Pollyea DA, Fathi AT, Roboz GJ, Altman JK, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. Blood 2017;130:722-31.
19. Shih AH, Meydan C, Shank K, Garrett-Bakelman FE, Ward PS, Intlekofer AM, et al. Combination targeted therapy to disrupt aberrant oncogenic signaling and reverse epigenetic dysfunction in IDH2- and TET2-mutant acute myeloid leukemia. Cancer Discov 2017;7:494-505.
20. Boutzen H, Saland E, Larrue C, de Toni F, Gales L, Castelli FA, et al. Isocitrate dehydrogenase 1 mutations prime the all-trans retinoic acid myeloid differentiation pathway in acute myeloid leukemia. J Exp Med 2016;213:483-97.
21. Kim YR, Eom JI, Kim SJ, Jeung HK, Cheong JW, Kim JS, et al. Myeloperoxidase expression as a potential determinant of parthenolide-induced apoptosis in leukemia bulk and leukemia stem cells. J Pharmacol Exp Ther 2010;335:389-400.
22. Wang F, Travins J, DeLaBarre B, Penard-Lacronique V, Schalm S, Hansen E, et al. Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. Science 2013;340:622-6.
23. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010;140:313-26.
24. Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Rep 2001;2:330-5.
25. Mauvezin C, Neufeld TP. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. Autophagy 2015;11:1437-8.
26. Battle TE, Roberson MS, Zhang T, Varvayanis S, Yen A. Retinoic acid-induced blr1 expression requires RARalpha, RXR, and MAPK activation and uses ERK2 but not JNK/SAPK to accelerate cell differentiation. Eur J Cell Biol 2001;80:59-67.
27. Piskunov A, Rochette-Egly C. A retinoic acid receptor RARα pool present in membrane lipid rafts forms complexes with G protein αQ to activate p38MAPK. Oncogene 2012;31:3333-45.
28. Amatangelo MD, Quek L, Shih A, Stein EM, Roshal M, David MD, et al. Enasidenib induces acute myeloid leukemia cell differentiation to promote clinical response. Blood 2017;130:732-41.
29. Yen K, Travins J, Wang F, David MD, Artin E, Straley K, et al. AG-221, a first-in-class therapy targeting acute myeloid leukemia harboring oncogenic IDH2 mutations. Cancer Discov 2017;7:478-93.
30. Stein EM, Tallman MS. Emerging therapeutic drugs for AML. Blood 2016;127:71-8.
31. Guan JL, Simon AK, Prescott M, Menendez JA, Liu F, Wang F, et al. Autophagy in stem cells. Autophagy 2013;9:830-49.
32. Mizushima N, Levine B. Autophagy in mammalian development and differentiation. Nat Cell Biol 2010;12:823-30.
33. Conway KL, Kuballa P, Khor B, Zhang M, Shi HN, Virgin HW, et al. ATG5 regulates plasma cell differentiation. Autophagy 2013;9:528-37.
34. Orfali N, O'Donovan TR, Nyhan MJ, Britschgi A, Tschan MP, Cahill MR, et al. Induction of autophagy is a key component of all-trans-retinoic acid-induced differentiation in leukemia cells and a potential target for pharmacologic modulation. Exp Hematol 2015;43:781-93.
35. Weng XQ, Sheng Y, Ge DZ, Wu J, Shi L, Cai X. RAF-1/MEK/ERK pathway regulates ATRA-induced differentiation in acute promyelocytic leukemia cells through C/EBPβ, C/EBPβ and PU.1. Leuk Res 2016;45:68-74.
36. Hong HY, Varvayanis S, Yen A. Retinoic acid causes MEK-dependent RAF phosphorylation through RARalpha plus RXR activation in HL-60 cells. Differentiation 2001;68:55-66.
37. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. Cancer Cell 2011;19:17-30.