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A Cell-Based High-Throughput Screening for Inducers of Myeloid Differentiation

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Abstract

Recent progress of genetic studies has dramatically unveiled pathogenesis of acute myeloid leukemia (AML). However, overall survival of AML still remains unsatisfactory and development of novel therapeutics is required. CCAAT/Enhancer Binding Protein α (C/EBPα) is one of crucial transcription factors that induce granulocytic differentiation and its activity is perturbed in human myeloid leukemias. As its re-expression can induce differentiation and subsequent apoptosis of leukemic cells *in vitro*, we hypothesized that chemical compounds that restore C/EBPα expression and/or activity would lead to myeloid differentiation of leukemic cells. Using a cell-based high-throughput screening, we identified 2-[(*E*)-2-(3,4-dihydroxyphenyl)vinyl]-3-(2-methoxyphenyl)-4(3H)-quinazolinone as a potent inducer of C/EBPα and myeloid differentiation. Leukemia cell lines and primary blast cells isolated from human AML patients treated with ICCB280 demonstrated evidence of morphological and functional differentiation, as well as massive apoptosis. We performed conformational analyses of the high-throughput screening hit compounds to postulate the spatial requirements for high potency. Our results warrant a development of novel differentiation therapies and significantly impact care of AML patients with unfavorable prognosis in the near future.

Keywords

acute myeloid leukemia; CCAAT/Enhancer Binding Protein α (C/EBPα); differentiation; 2-[(*E*)-2-(3, 4-dihydroxyphenyl)vinyl]-3-(2-methoxyphenyl)-4(3H)-quinazolinone

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Introduction

Recent advances in genetic studies led to a dramatic progress in understanding the pathogenesis of acute myeloid leukemia (AML). However, long-term survival of AML still remains unsatisfactory. In particular, AMLs with monosomal or complex karyotypes have shown the worst prognosis with 3-year rate of overall survival being only 12%.[1] Moreover, AMLs arising from myelodysplastic syndrome (MDS) or secondary to previous cytotoxic chemotherapy have a lower rate of remission than *de novo* AMLs. Patients with high-risk MDS have a 3-year survival rate of less than 10%.[2] Considering the fact that conventional chemotherapies do not have specific targets and demonstrate various levels of toxicity, there is a great demand for novel targeted therapeutics. One of the best approaches is to focus on a block of differentiation, which is a hallmark of all subtypes of AML. *All-trans* retinoic acid (ATRA) is now widely used as the first line therapy for one subtype of AML, t(15;17) positive acute promyelocytic leukemia (APL), and induces differentiation of leukemia cells and eventually leads to apoptosis. Although it has been shown that ATRA can induce remission and lead to cure in nearly 70% of patients with APL,[3] its application in other types of myeloid leukemia is limited. In addition, relapse can occur in the course of treatment. Although arsenic trioxide has a high rate (85%) of successful remission induction in patients with APL resistant to ATRA, an 18-month relapse-free survival is 60%.[4]

Expression of CCAAT/Enhancer Binding Protein α (C/EBPα) is increased and maintained during granulocytic differentiation and rapidly downregulated during the alternative monocytic pathway.[5] Conditional expression of C/EBPα in stably transfected myeloid precursor cells triggers neutrophilic differentiation, concomitant with upregulation of the granulocyte colony-stimulating factor receptor (G-CSFR) and secondary granule proteins.[5] In mice deficient in C/EBPα, there is a block in granulocytic differentiation at the myeloblast stage, while all the other blood cell types are present and intact.[6] Thus, C/EBPα is necessary and sufficient for neutrophil differentiation. Consistent with its importance in normal myeloid differentiation, expression and/or function of C/EBPα are perturbed in various types of myeloid leukemias by different mechanisms (transcriptional silencing, translational inhibition, posttranslational modification, decrease in DNA binding, or point mutations resulting in increased production of a dominant negative form).[7] Thus, restoration of C/EBPα expression and/or activity could overcome the block of differentiation and lead to growth arrest and apoptosis of leukemic cells.

In the current study, we established a stable cell line carrying luciferase gene driven by an artificial promoter composed of a tetramer of C/EBP binding sites, which responds to C/EBPα activity. By using this indicator line in a cell-based high-throughput screen, we identified one chemical compound, 2-[(*E*)-2-(3,4-dihydroxyphenyl)vinyl]-3-(2-methoxyphenyl)-4(3H)-quinazolinone (referred to as ICCB280), which induced myeloid differentiation of leukemia cell lines by increasing expression of C/EBPα and C/EBPε, and decreasing expression of c-Myc. Importantly, exposure of primary blast cells from AML patients to ICCB280 led to differentiation and apoptosis as well. Interestingly, G-CSFR, which is one of targets of C/EBPα, was upregulated by the treatment with ICCB280 and the addition of G-CSF further enhanced the differentiation induced by this compound. These results indicate that the use of ICCB280 alone, or in combination with other agents, such as

G-CSF may provide a novel means of treatment of AML. Given the fact that in addition to AML, abnormalities in C/EBPα expression and/or function have been also reported in other malignancies such as lung[8] and liver,[9] we believe that small molecule agents that selectively target C/EBPα, such as ICB280, may find broad applications in treating cancers.

Materials and Methods

Reagents

ATRA (cat. R2625) was purchased from Sigma. 2-[(*E*)-2-(3,4-dihydroxyphenyl)vinyl]-3-(2-methoxyphenyl)-4(3H)-quinazolinone (ICCB280) was purchased from ChemBridge (San Diego, CA). Stock solutions for the compounds were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. The drugs were diluted in fresh medium before each experiment, and the final DMSO concentration was <0.5%.

Determination of the purity and molecular weight of ICCB280

HPLC-MS analysis was performed with a Waters Alliance-Micromass ZQ instrument, an ESI source, and Waters 2489 UV/visible detector at 254 nm. Analysis was performed using an analytical Waters Symmetry C18 column (3.5 mm, 2.1×100 mm) operating at 0.5 mL/min with a linear gradient of 0–100% of acetonitrile in water (containing 0.1% formic acid, v/v) over 10 minuites.

Cells

HL-60 (ATCC; CCL-240), K562 (ATCC; CCL-243), and U937 (ATCC; CRL-1593.2) cells were maintained in RPMI 1640 with 10% fetal bovine serum at 37°C with 5% CO_2. CV-1 (ATCC; CCL-70) and 293T (ATCC; CRL-3216) were maintained in DMEM with 10% fetal bovine serum. Primary AML patient blasts were collected from peripheral blood after obtaining patients' informed consents, which were approved by the Institutional Review Boards at Beth Israel Deaconess Medical Center. Patient #0502 was diagnosed as acute myelomonocytic leukemia with abnormal eosinophils (AML M4Eo) and carried inv(16)(p13;q22). Patient #0505 was diagnosed as acute monocytic leukemia (M5) with normal karyotype. Mutations in the *CEBPA* gene were reported in neither patient. Primary AML blast cells were isolated using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ), as previously described[10] and maintained in culture in RPMI 1640 with 10% fetal bovine serum in the presence of G-CSF (60 ng/ml) at 37°C with 5% CO_2.

Plasmid constructs

Firefly luciferase gene controlled by a minimal thymidine kinase (TK) promoter and a tetramer of C/EBP-binding sites from the human G-CSFR promoter (4xCEBP-luc) was previously described.[11] To make 4x mutCEBP-luc, oligonucleotides containing mutations abolishing C/EBP binding (AAGGTGTTGCAATCCCCAGC → AAGGTGTTcaccaaCCCAGC; wild type C/EBP site underlined; mutated nucleotides in small letters) were tetramerized and inserted into SalI site of pTK min-luc [12] and pRL-TK (Promega, Madison, WI). The pGhU6 lentiviral shRNA vectors against *CEBPA* and the nonsilencing control were previously described.[13]

Generation of the C/EBP activity indicator cell line

U937 cells were co-transfected with the ScaI-linearized 4xCEBP-luc construct together with the linearized plasmid containing neomycin-resistant gene (pSV40-neo) by electroporation using 250 V and 960 μF in Gene Pulser II (BioRad, Hercules, CA), followed by selection in 1 mg/ml G418. Single clones were isolated by limiting dilution in 96-well plates.

Generation of HL-60 cells stably expressing shRNAs against CEBPA

293T cells were cotransfected with C/EBPα shRNA in pGhU6 vector or the shRNA control and lentiviral constructs Gag-Pol and Env. HL-60 cells were then infected with virus that was harvested and concentrated using a Centricon Plus-70 100000 MWCO column (Millipore, Billerica, MA). Infected cells were detected by EGFP flow cytometry analysis.

High-throughput screening of chemical libraries

Stable U937-C/EBP clones were maintained in the RPMI 1640 phenol red-free medium, 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 1 mg/ml G418 for selective propagation. Thirty μl per well (2,400 cells) of U937-C/EBP cells were plated in 384-well, flat bottom, white polystyrene plates (Nalgene, Rochester, NY). All plates were prepared in duplicates. Assay plates were then incubated in 5% CO_2 and 37°C for 24 hours. Next, 100 nano-litter of each compound dissolved in DMSO was added by a pin-transfer robot to give concentrations of 10–30 μM. ATRA and DMSO were added in every plate as positive and negative controls, respectively. Plates were incubated in 5% CO_2 and 37°C for another 24 hours, followed by the addition of 30 μl of Bright Glo (Promega, Madison, WI) to each well. Plates were incubated at room temperature for at least 3 minutes and the luciferase activity was read in sequential order using the LJL Analyst Reader (Molecular Devices, Sunnyvale CA) in Luminescence mode. The plates we screened are as follows: ICCB Bioactives 1; NINDS Custom Collection; SpecPlus Collection; ChemBridge Microformat; Commercial Diversity Set 1; Philippines Plant Extracts 1&2; Starr Foundation Extracts 1; ICCB Discretes 3&4; and DOS Collection (http://iccb.med.harvard.edu/retired-compound-libraries/). All equipment and robotic instrumentation used for screening was provided by the Institute of Chemistry and Cell Biology (ICCB) at Harvard Medical School (http://iccb.med.harvard.edu/).

Nitroblue tetrazolium (NBT) reduction assay

Reduction of NBT by respiratory burst products was performed as previously described.[5]

Growth Inhibition Assay

Cells were plated at 10,000 cells/well with specified concentrations of compounds and incubated for 48 hours. Growth inhibition was assessed by MTS assay using CellTiter 96 AQueous One solution proliferation kit (Promega). IC_{50} was calculated using Prism software (GraphPad Software, La Jolla, CA).

RNA isolation, Northern blot analysis, and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated by RNAeasy kit (Qiagen, Valencia, CA) according to manufacturer instruction. In each lane, 20 μg of RNA denatured in formamide was fractionated on 1%

agarose-2.2 M formaldehyde gels. RNA was transferred to Nylon membranes and probed as described previously.[5] Membranes were hybridized with probes for human *G-CSFR*[5] and *c-Myc*.[14] To ensure uniform levels and integrity of RNA samples loaded in each lane, the blot was stripped and rehybridized to probes specific for *GAPDH*.[15] Quantitative RT-PCR analysis was performed as previously described.[13]

Protein sample preparation and Western blotting

Whole cell lysates were prepared in cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitor cocktail set III (Millipore), and 1 mM PMSF). Lysates were cleared by centrifugation (14,000 g for 5 minutes in a pre-cooled centrifuge) and boiled with Laemmli sample buffer for 3 minutes. Protein lysates (10–30 μg) were loaded on SDS/polyacrylamide gels and blotted onto PVDF membranes (Millipore). Antibodies against C/EBPα (sc-61), C/EBPβ (sc-150), C/EBPε (sc-158), Sp-1 (sc-59), and c-Myc (sc-40) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin antibody was from Sigma-Aldrich (A5316). All antibodies were used at 1:1000 dilution.

Apoptosis Analysis

Apoptosis was assessed using an Annexin-V-FLUOS staining kit (Roche, Penzberg, Germany) according to the manufacturer's instructions.

Conformational analysis

Energy-minimized conformers for the three isomeric molecules (ICCB280 and its *meta*- or *para*-methoxy isomer) were generated *in silico*. Structures were first drawn using Marvinsketch (Marvin 6.2.2, 2014, ChemAxon, Budapest, Hungary) and then converted into protein data bank format using the Open Babel molecular toolkit.[16] To reduce the necessary processing power, an initial steepest descent geometry optimization was performed using a MMFF94 forcefield (250 steps). Following the initial optimization, a weighted rotor conformer search was performed (200 iterations), followed by a conjugate gradient geometry optimization (250 steps) to generate the final 3D conformational structure of each isomer. Each structure was visualized and angles were calculated using the PyMOL molecular viewing program (Version 1.5.0.4, Schrödinger, LLC, New York, NY).

Statistical Analysis

Differences between the experimental groups were compared using Student's t-test. P-values of less than 0.05 were considered statistically significant.

Results

Establishment of the cell-based high-throughput drug screen for inducers of C/EBPα activity

In order to detect C/EBPα activity, we designed a luciferase construct (4xC/EBP-luc), in which a firefly luciferase gene was placed under control of a tetramer of C/EBP binding sites from the human *G-CSFR* gene promoter upstream from the minimal thymidine kinase

(TK) promoter (Fig. 1A). The response of this reporter was tested in transient co-transfection assays in CV-1cells. As shown in Figure 1B, addition of C/EBPα expression vector increased the luciferase activity 10-fold. We then transfected U937 human AML cells with the 4xC/EBP-luc construct to generate a stable cell line (U937-C/EBP). Multiple individual clones were isolated and subcloned at limiting dilution. It has been published before that treatment of U937 cells with ATRA increases levels and DNA-binding activity of C/EBPα.[5, 17, 18] Therefore, several stable clones were tested for the response of luciferase activity to treatment with ATRA (Fig. 1C) and clone #10 was chosen for the high-throughput screening. Of note is that clones that harbor the mutated C/EBP binding sites showed no increase in luciferase activity upon ATRA exposure (Fig. 1C), indicating that an increase in luciferase activity in ATRA-treated U937-C/EBP cells is specific for C/EBP proteins.

A detailed timeline of the high throughput screen is shown in Supplemental Figure S1. To optimize the assay, we plated various concentrations of U937-C/EBP cells on 384-well plates and treated them with DMSO (negative control), or ATRA (positive control). As illustrated in Figure 2A, ATRA treatment of 24,000 or 30,000 cells per well led to a 6-fold increase in luciferase activity, while no loss of cell viability was achieved by the treatment of 24,000 cells per well compared to 22% decrease in 30,000 cells per well. We therefore chose to use 24,000 cells per well for the actual drug screen. Chemical compounds were added by a pin-transfer robot to yield a final concentration of 10–30 μM. A compound was considered positive if its z-score was 3 or higher. For example, ATRA used as a positive control had a z-score of 5. A library of 67,847 compounds was screened and 320 compounds (0.47%) were found to significantly increase luciferase activity. Among them, 2-[(*E*)-2-(3,4-dihydroxyphenyl)vinyl]-3-(2-methoxyphenyl)-4(3H)-quinazolinone (Screen number 280, from now on referred to as ICCB280) displayed the strongest luciferase-inducing activity (Fig. 2B). We repurchased the compound from the supplier (ChemBridge) and confirmed its molecular mass and the purity by liquid chromatographic mass spectroscopy (LCMS) (Suppl. Fig. S2).

ICCB280 induces granulocytic differentiation, growth arrest, and subsequent apoptosis of leukemia cells

Out of the 320 initial hits, we proceeded to a second screen to identify compounds that have the ability to induce granulocytic differentiation. We used HL-60 leukemic cell line as it has been extensively used as a model for myeloid differentiation for over 30 years.[19] We treated HL-60 cells with individual compounds at 10 μM each and examined Wright-Giemsa stained cytospins for morphological changes. We also performed nitroblue tetrazolium reduction (NBT) assay detecting production of superoxide anion, which indicates functional maturation of leukemic cells to granulocytes. The strongest differentiation-promoting activity was demonstrated for ICCB280 (Fig. 3A). Seven-day treatment with ICCB280 induced granulocyte-like morphological changes such as decrease in nucleus-to-cytoplasm (N/C) ratio and nuclear lobulation in 83% of the cells, whereas no cells showed granulocyte-like morphology in cells treated with DMSO (Fig. 3B, left panels). Approximately 46% of the cells were also positive in NBT assay, compared to 1% of DMSO-treated control cells (Fig. 3B, lower right panel, black arrows). In addition, ICCB280 induced an increase in

surface CD11b expression, which is one of characteristics of neutrophilic differentiation (Fig. 5B). Furthermore, treatment of HL-60 cells with ICCB280 suppressed the cell growth in 48 hours, as assessed by MTS assay (Fig. 3C).

Once differentiated, neutrophils have a half-life of only 6–10 hours in the circulation and are eventually cleared by constitutive apoptosis.[10] Therefore, induction of the differentiation-induced apoptosis would be a key to eliminate leukemic cells in differentiation therapies. To examine whether ICCB280 induces apoptosis, HL-60 cells were treated with ICCB280 and apoptosis was assessed by AnnexinV assay. An increase in early apoptotic cells ($AnnexinV^+/PI^-$) was followed by that of late apoptotic cells ($AnnexinV^+/PI^+$), indicating that cell death was indeed caused by apoptosis (Fig. 3D). Furthermore, 41% of cells treated with ICCB280 for 7 days underwent apoptotic cell death (late apoptotic cells) (Fig. 3D). These results indicate that differentiated cells induced by ICCB280 eventually undergo apoptosis.

In order to confirm that ICCB280 has therapeutic potential, we treated two samples of primary human acute leukemia cells with ICCB280 *in vitro*. Leukemic blasts were isolated from either bone marrow or peripheral blood from patients with acute myeloid leukemia subtype M4 or M5, in which ATRA is not effective. After a 5-day treatment with ICCB280, leukemic cells showed a decrease in N/C (Fig. 3E) in 90% the cells. Moreover, Annexin V assay showed that ICCB280 induced more cell death than DMSO vehicle control (Suppl. Fig. S3), possibly due to differentiation-induced apoptosis. Taken together, these results indicate that ICCB280 induces granulocytic differentiation, growth arrest, and subsequent apoptosis in myeloid leukemia cells.

ICCB280 induces upregulation of C/EBPα and C/EBPε, but not C/EBPβ

Since the high-throughput screen was specifically designed to induce C/EBPα expression/activity, we sought to determine whether the differentiation-promoting effect of ICCB280 leukemic cells was indeed mediated by C/EBP proteins. All C/EBP family members have the capacity to bind to the G-CSFR promoter-derived C/EBP site. Therefore, in addition to C/EBPα, we also examined two other C/EBP family members, C/EBPβ and C/EBPε. It has been show that in the absence of C/EBPα, C/EBPβ can induce emergency granulocytic differentiation by cytokine stimulation.[20] C/EBPε on the other hand is induced by C/EBPα[21] and is required for terminal neutrophilic maturation.[22] When HL60 cells were treated with ICCB280, a time-dependent increase in *CEBPA* mRNA was detected by quantitative RT-PCR (Fig. 4A). Consistent with this data, upregulation of C/EBPα protein was observed as early as on day 4, followed by increase in C/EBPε on day 6 (Fig. 4B). In contrast, no changes in C/EBPβ expression were noted through the 8 days of the experiment, as compared to control cells (Fig. 4B). T These results demonstrate that ICCB280 upregulates C/EBPα at the transcriptional level.

ICCB280 modulates expression of C/EBPα target genes

Next, we examined whether ICCB280 alters expression of downstream targets of C/EBPα. Northern blot analysis demonstrated that mRNA expression of G-CSFR (*CSF3R)*, which is a known target of C/EBPα, is rapidly upregulated in cells treated with ICCB280 (Fig. 4C). In

contrast, *both c-Myc* mRNA and its protein were strongly downregulated by ICCB280 (Fig. 4C and Suppl. Fig. S4). This result is consistent with the previous report demonstrating that *c-Myc* expression is negatively regulated by C/EBPα.[14] Next, we asked whether upregulation of C/EBPα is necessary for ICCB280-induced differentiation. To this end, we generated HL-60 cells expressing shRNA against *CEBPA*. HL-60/sh*CEBPA*#1 cells showed 64% reduction of *CEBPA* mRNA, while suppression in HL-60/sh*CEBPA*#2 cells was only 32% (Fig. 4D, upper left). When cells were treated with ICCB280 for 7 days, we observed that only 48% of HL-60/sh*CEBPA*#1 cells were morphologically differentiated upon ICCB280 treatment, compared to 90% differentiation of HL-60/sh*CEBPA*#2 and HL-60/control shRNA cells (Fig. 4D). Taken together, our results demonstrate that ICCB280 mediates granulocytic differentiation of AML cells by increasing the expression and activity of C/EBPα transcription factor.

ICCB280 and G-CSF cooperatively induce granulocytic differentiation

Upregulation of *CSF3R* by ICCB280 prompted us to hypothesize that cells treated with ICCB280 may be more susceptible to G-CSF and that a combination of ICCB280 and G-CSF would enhance neutrophilic differentiation. As shown in Figure 5A, in the absence of ICCB280 HL60 cells did not respond to the treatment with G-CSF. However, when treated with ICCB280 together with G-CSF for 7 days, HL-60 cells showed morphology consistent with neutrophilic differentiation and N/C ratio was even lower than in cells treated with ICCB280 alone (Fig. 5A). In addition, flow cytometric analysis detected more surface CD11b-positive cells treated with ICCB280/G-CSF combination (47%), than G-CSF (6%) or ICCB280 (25%) alone (Fig. 5B). These results suggest that ICCB280 induces upregulation of G-CSFR, which renders leukemic cells more susceptible to G-CSF stimulation.

ICCB280 adopts a unique 3-dimentional conformation that affects its potency

In order to identify key structural attributes that are critical to the activity of ICCB280, we searched for its analogs. We identified 61 2-(2-arylvinyl)-3-aryl-4(3H)-quinazolinone analogs available at the Institute of Chemistry and Cell Biology (Harvard Medical School) and these compounds were screened at 10 μM each for induction of morphological changes and NBT positivity (Table S1). HL-60 cells were treated with each compound and stained with Wright Giemsa and NBT as in Figure 3C. Among all 61 analogs, 9 showed differentiation activity, but ICCB280 was the most potent (Fig. 6A). Structure comparisons indicate that the catechol moiety and the ortho-methoxy (MeO) group on the 3-phenyl ring are important for the high activity of ICCB280. In contrast, the meta-MeO (on the 3-phenyl ring) regioisomer of ICCB280 is far less active, while the para-isomer is completely inactive. We conducted conformational analyses of the three isomeric compounds (Fig. 6B). Although the position of the MeO group has only marginal effects on the torsional angle between the 3-phenyl ring and the quinazolinone ring (57.5° for ICCB280 vs. 52.1° and 50.5° for the meta- and para-isomers, respectively), we observed a profound effect on the conformational change of the catechol moiety. While the catechol ring of the meta- and para-isomers remains essentially coplanar with the vinyl-quinazolinone, the catechol ring in ICCB280 has rotated nearly 60° away from a conjugated planar geometry. This significant

change is likely the cause of the othro-MeO group that is propelled toward the catechol electrostatically by the carbonyl group.

Discussion

Introduction of *all-trans* retinoic acid (ATRA) as the first-line therapy for t(15;17) positive acute promyelocytic leukemia has dramatically improved prognosis with a cure rate being nearly 70%.[23] However, despite tremendous efforts made even prior to great success of ATRA, a differentiation therapy for other types of AML has been unavailable so far. Among the promising targets for differentiation therapy were two nuclear receptors, vitamin D receptor (VDR) and peroxisome proliferator activated receptor gamma (PPARγ), which were described to play crucial roles in induction of differentiation of leukemic cells.[24] However, neither vitamin D compounds, nor PPARγ agoinsts have shown clinical impacts.[24] Thus, identification of a new strategy for differentiation therapy is still warranted.

Given that C/EBPα transcription factor plays a central role in myeloid differentiation program, here we describe a successful establishment of a cell-based high-throughput screening to identify novel chemical compounds that enhance C/EBPα expression and/or activity and thus induce granulocytic differentiation. We identified ICCB280 as a lead compound, which led to myeloid differentiation of leukemia cells morphologically and functionally, accompanied by increased expression/activity of C/EBPα and its downstream target genes. Importantly, we showed that ICCB280 indeed induced differentiation-like morphological changes and apoptosis in primary human acute leukemia cells from two patients with AML in this proof-of-principle study. In addition to its role in myeloid leukemia, we have shown that C/EBPα is necessary for lung alveolar cell development and downregulation of C/EBPα is detected in approximately half of primary human lung cancers.[8, 25, 26] Thus, our C/EBPα-inducing strategy may find applications in treatment of solid tumors as well. Further studies are necessary to demonstrate clinical effectiveness and safety of this class of chemical compounds.

We demonstrated that ICCB280 exhibits anti-leukemic properties including terminal differentiation, proliferation arrest, and apoptosis through activation of C/EBPα and affecting its downstream targets. One such target is C/EBPε, which is transcriptionally and directly regulated by C/EBPα and responsible for progression of later stages of granulocytic differentiation of myeloid cells.[21] In addition to transcriptional transactivation of neutrophil-specific genes, such as CD11b and G-CSFR, C/EBPα also hinders cell cycle progression by inhibiting cyclin-dependent kinase-2 (CDK2) through physical interaction with p21.[27] ICCB280-mediated inhibition of cell proliferation can be also explained by a dramatic downregulation of *c-Myc* by ICCB280, consistent with our previous report demonstrating C/EBPα-mediated transcriptional downregulation of *c-Myc* gene expression.[14] As Myc proteins contribute to cell proliferation by activating cell cycle and inducing DNA replication,[28] our results indicate that downregulation of c-Myc contributes to ICCB280-induced proliferation arrest. Furthermore, ICCB280 caused 80% reduction in cell growth in 48 hours, whereas differentiation and apoptosis were observed at much later time (5–7 days). These results indicate that growth arrest precedes differentiation and apoptosis induced by ICCB280.

Although hematopoietic cytokines are essential for differentiation of normal hematopoietic stem and progenitor cells into mature cells, only modest effects are observed in leukemia cell lines or primary human AML cells, both *in vitro* and *in vivo*.[24] One of the reasons may be lack of signals from G-CSF due to low expression of its receptor, G-CSFR. Indeed, G-CSFR is restricted in a subset of myelomonocytic cell lines and shows considerable variability among primary AML blasts.[29] We showed that ICCB280 induced up-regulation of G-CSFR in HL-60 cells, which do not express appreciable amounts of G-CSFR on the surface under normal conditions.[29] It is of interest to note that G-CSF significantly enhanced ICCB280-induced differentiation of leukemia cells. Therefore, a combination of chemical compounds that induce C/EBPα activity and hematopoietic cytokines such as G-CSF makes it a plausible strategy to induce differentiation of leukemic blasts more effectively.

The biological activity of an organic compound depends on its interactions with its biological target, and this interplay is profoundly affected by stable 3 dimensional structures presented by the organic molecule. Our conformational analyses imply that ICCB280 adopts a unique spatial geometry that might be preferential for its high activity. We envision that the torsional angle and associated conformational change may prove to be critical parameters to guide future structure-activity relationship (SAR) study, which may result in novel compounds with improved activity.

In summary, we successfully established a cell-based high throughput screening to identify chemical compounds capable of inducing myeloid differentiation. Our data showing that ICCB280 was capable of inducing differentiation and apoptosis of ATRA-resistant patient blasts strongly signify that the activity of this compound can overcome resistance to other current therapies for AML with unfavorable prognosis. It will be interesting to test the potential synergistic effect of ICCB280 and other agents affecting granulocytic differentiation, such as ERK1/2 or cdc2 inhibitors.[13, 30] Finally, our data indicate that similar target-oriented drug screening approaches may be applied to other malignancies affecting different specific pathways. It is expected that identification of novel differentiation therapies will benefit patients with AML, who are resistant to current treatment in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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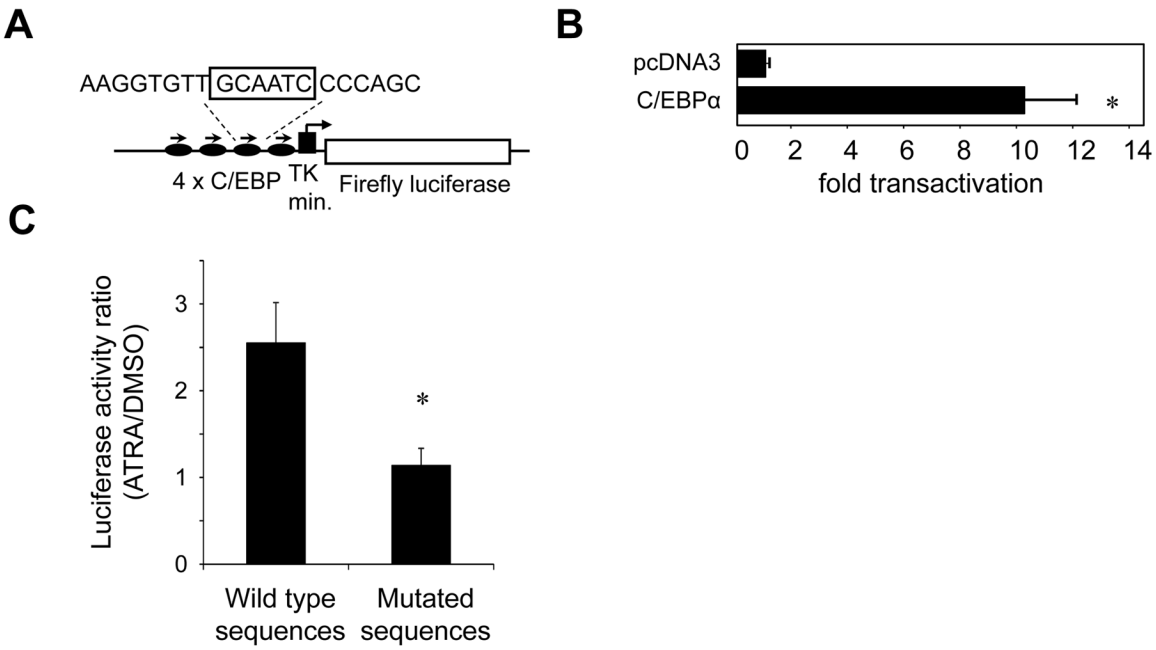

Figure 1. Establishment of the cell line which detect C/EBPa activity

(A) A firefly luciferase gene was placed under the control of tetramer of C/EBP binding sites from the human G-CSF receptor gene. U937 cell line was stably transfected with this construct, together with a neo-resistant gene. (B) CV-1 cells were transiently transfected with C/EBPα expression vector and the reporter shown above and luciferase activity was analyzed. Data represents mean ± standard deviation from three independent experiments. * indicates $p \leq 0.05$. (C) Stable U937-C/EBP cells were treated with either 0.1% DMSO or 1 μM ATRA for 24 hours. Luciferase activity was measured using a benchtop luminometer. Data represents mean ± standard deviation from three independent experiments. * indicates $p \leq 0.05$.

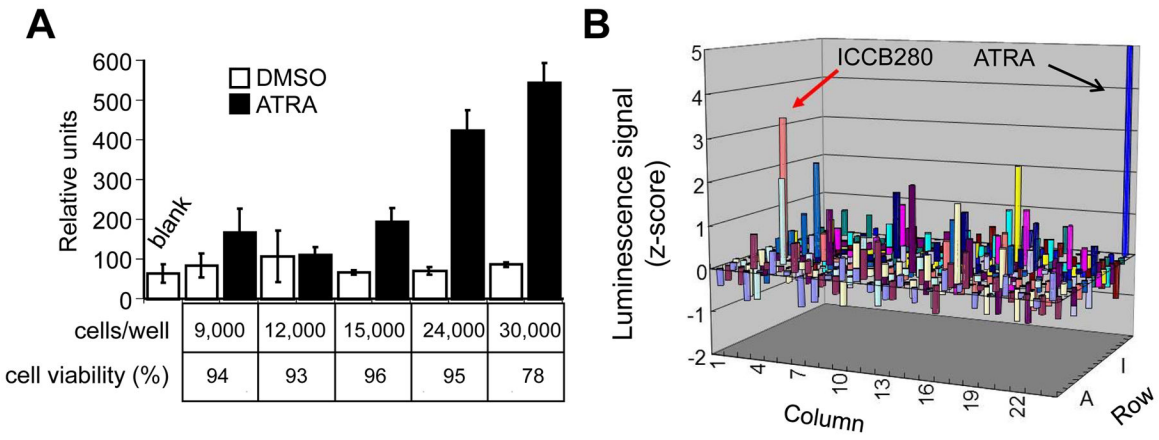

Figure 2. High-thourghput screening of chemical compounds that increase C/EBP expression and/or activity

(A) Stable U937-C/EBP cells were plated in a 384-well plate and treated with either 0.1% DMSO or 10 μM ATRA. Luciferase activity was measured using multiplate luminometer. Data represents mean ± standard deviation from three independent experiments. (B) The plate which contains a luminescence signal of ICCB280 (red arrow). A black arrow shows positive control.

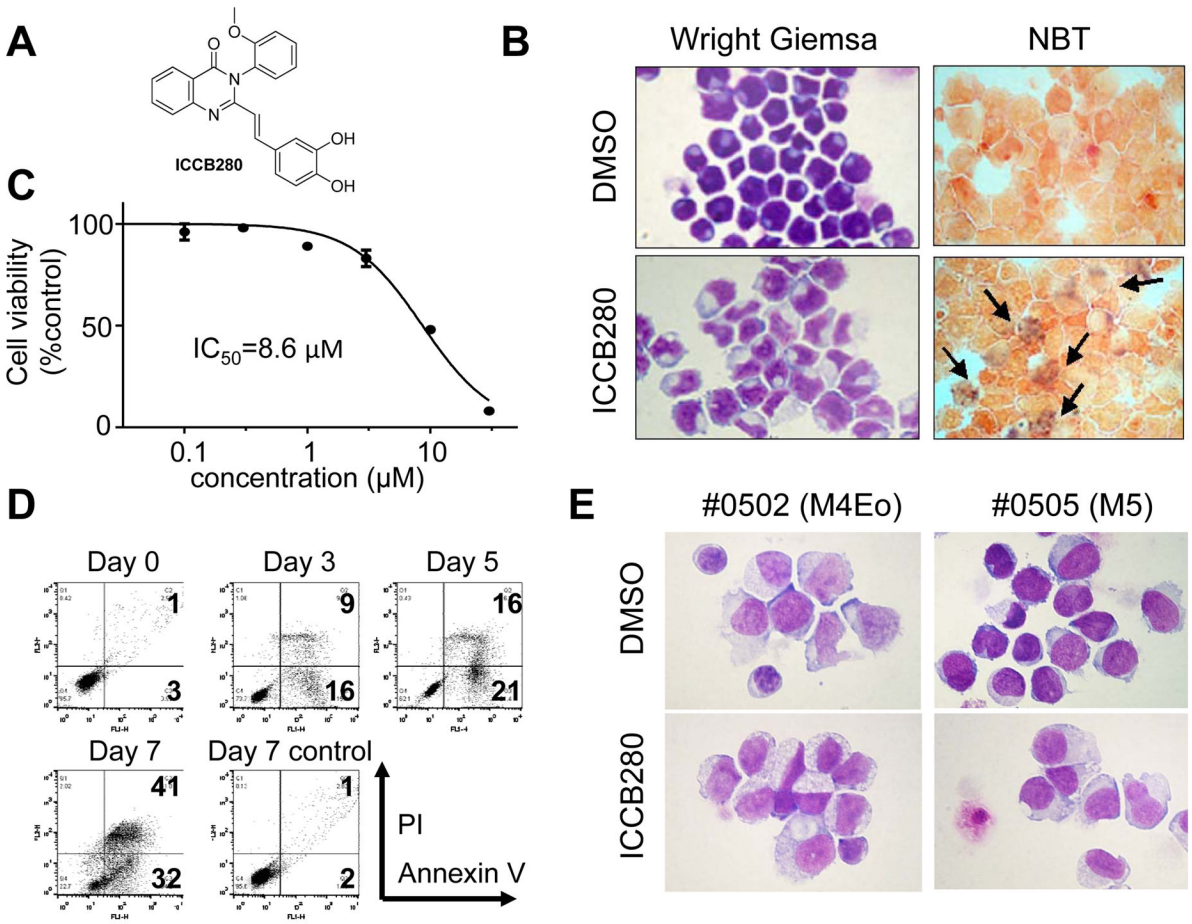

Figure 3. Identification of ICCB280

(A) Structure of ICCB280 (2-[2-(3,4-dihydroxyphenyl)vinyl]-3-(2-methoxyphenyl)-4(3H)-quinazolinone). (B) HL-60 cells were treated with 10 μM ICCB280 or 0.1% DMSO for 7 days and stained with Wright Giemsa (left panels) and NBT (right panels) (x40). (C) HL-60 cells were plated in 96 plates and treated with 10μM ICCB280. After 48 hours, MTS assay was performed. Data represents mean ± standard deviation from four independent experiments. (D) HL-60 cells were treated with 10 μM ICCB280 or 0.1% DMSO for indicated period and stained with Annexin V and PI. Cells were then subjected to flow cytometric analysis. (E) Mononuclear cells were isolated from two patients with acute myeloid leukemia (#0502 and #0505) and treated with either 10 μM ICCB280 or 0.01% DMSO for 5 days. Cells were stained with Wright Giemsa (x40).

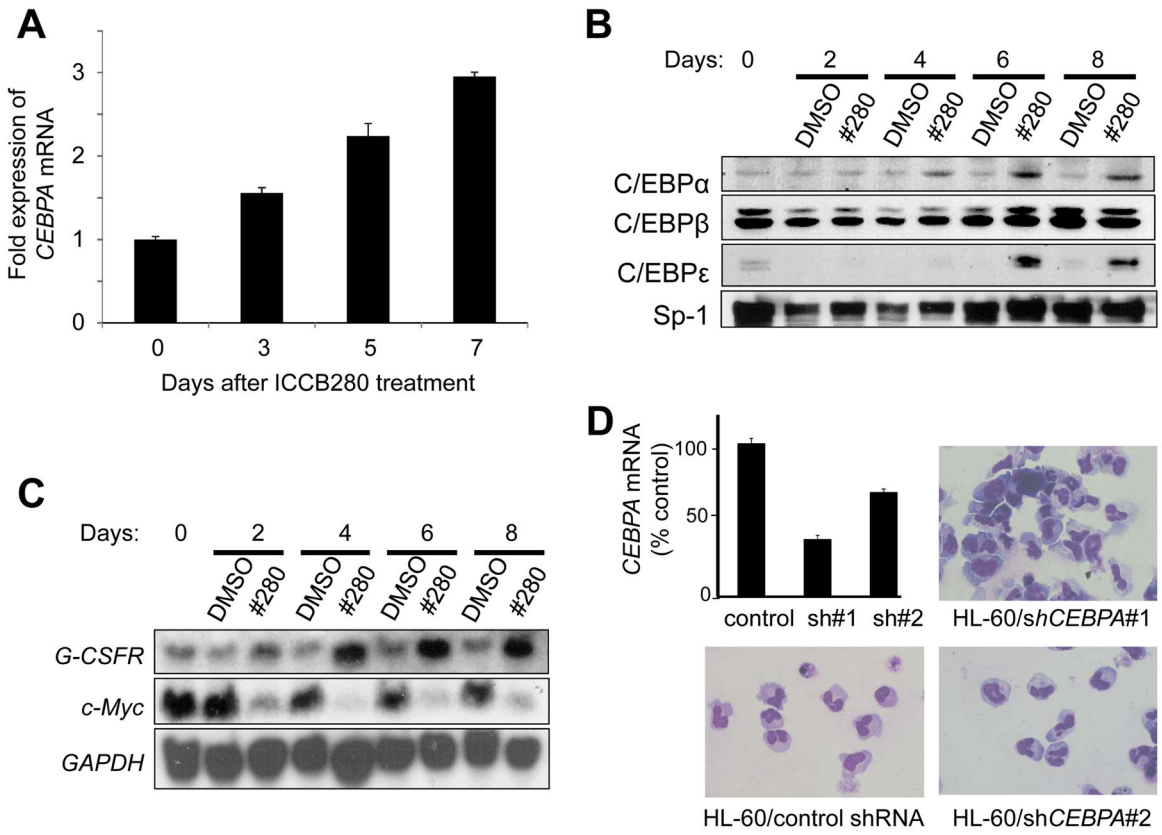

Figure 4. ICCB280 leads to an increase in C/EBPα expression and modulates its target genes

(A) HL-60 cells were treated with 10 μM ICCB280 for indicated period. cDNAs were subjected to real-time PCR. *CEBPA* mRNA expression was normalized to the housekeeping gene *GAPDH* and is shown as n-fold changes compared to non-treated HL-60. Data represents mean ± standard deviation from three independent experiments. (B) HL-60 cells were treated with 10 μM ICCB280 or 0.1% DMSO for indicated period. Cell extracts were subjected to western blot analysis. (C) Total RNAs were isolated and subjected to northern blot analysis. (D) HL-60 cells were infected with lentiviral vectors containing shRNA against *CEBPA* (#1 or #2) or the scramble control. Reduction of *CEBPA* expression is shown compared to HL-60 treated with the scramble vector. Data represents mean ± standard deviation from three independent experiments (upper left). Cells were treated with 10 μM ICCB280 for 7 days and stained with Wright Giemsa (x40).

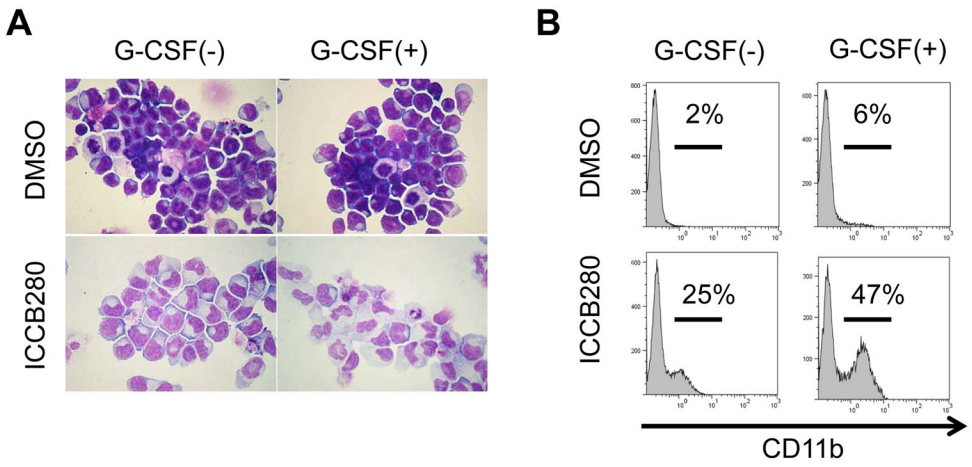

Figure 5. G-CSF enhances ICCB280 induced myeloid differentiation

HL-60 cells were treated with either 10 μM ICCB280 or 0.03% DMSO in the presence or absence of 6 μg/ml of G-CSF for 7 days. (A) Cells were stained with Wright Giemsa (x40). (B) Treated cells were stained with CD11b antibody and subjected to flow cytometric analysis.

A

Similarity	ChemBankID	Morphology	NBT activity
1	1946343	++	++
0.915	1947187	+	+
0.9	1946438	+	+
0.859	1944802	+	+
0.852	1942789	-	+
0.834	1947170	+	-
0.819	1947169	-	+
0.818	1947148	-	+
0.805	1977220	-	+

B

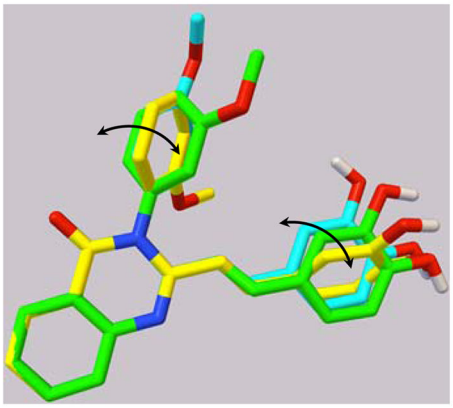

Figure 6. Conformational analyses of ICCB280

(A) ICCB280 was the most potent inducer of differentiation among 61 similar compounds. HL-60 cells were treated with each compound and stained with Wright Giemsa and NBT. (B) Overlay of the most energy-minimized conformers of ICCB280 (yellow), its *meta*-(green) and *para*-isomers (cyano). Hydrogen atoms are omitted except for the OH groups