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Identification of small compounds that inhibit multiple myeloma proliferation by targeting c-Maf transcriptional activity

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ABSTRACT

Multiple myeloma displays the clonal B cell expansion and the overproduction of monoclonal immunoglobulins. Genetic translocations at 14q32, particularly with partners like 16q23, lead to the dysregulation of oncogene expression, including the significant enhancement of c-Maf. This aberrant expression of c-Maf has prompted research into strategies for targeting this transcription factor as a potential therapeutic avenue for multiple myeloma treatment. In this study, we introduce a screening pipeline to test small compounds for their ability to inhibit c-Maf. Using a luciferase indicator driven by the *Ccl8* gene promoter, we identified two small compounds that inhibit transcriptional activity of c-Maf. These molecules impede the proliferation of c-Maf-expressing myeloma cells, and repress the expression of c-Maf target genes such as *ITGB7* and *CCR1*. Importantly, these molecules target c-Maf-expressing multiple myeloma cells, but not c-Maf-negative myeloma cells, showing potential for tailoring therapeutic intervention. In conclusion, our screening pipeline is effective to explore leads for a novel c-Maf inhibitor for multiple myeloma therapy.

1. Introduction

The *Maf* gene was originally identified in musculo-aponeurotic fibrosarcoma of chickens infected with the retrovirus AS42 [1]. Later, its cellular counterparts were cloned as c-Maf, or MAF, from vertebrates. The Maf transcription factors are divided into two subgroups: large Mafs that have transactivation domain and small Mafs that do not have one. The large Maf proteins include MAFA, MAFB, c-Maf/MAF, and neural retina-specific leucine zipper (NLR). The small Mafs comprise MafG, MafF, and MafK. The small Mafs regulate transcriptional activity of large Mafs by heterodimerizing with them although not having their own transactivation domains [2]. c-Maf is expressed in various cell types and regulates a wide range of cellular processes such as cell differentiation, tissue development, and inflammation. For example, c-Maf-deficiency impairs erythropoiesis in fetal liver [3] and the differentiation of the lens fiber cell and lens development [4] in mice. In immune cells, c-Maf is essential for Interleukin (IL)-4 production by T helper cells [5], and an anti-inflammatory cytokine interleukin IL-10 production by regulatory T cells [6] and macrophages [7]. In a previous study, we found that c-Maf also directly binds to the consensus sequence of Mafs called Maf recognition element (MARE)-half sites in *Ccl8* gene promoter and upregulates its transcription by bone marrow-derived macrophages (BMDMs) [8].

In human, the *c-Maf* gene is expressed at high levels in multiple myeloma cells carrying the chromosomal translocation t(14; 16)(q32; q23), which results in fusing immunoglobulin heavy chain gene (14q32) and *c-Maf* gene (16q23) loci [9,10] in 5–10 % of multiple myeloma patients. In those patients, an aberrant overexpression of c-Maf drives the transcription of *cyclin D2* (*CCDN2*), *Integrin β7* (*ITGB7*), and *CCR1*, leading to malignant transformation of plasma cells [11]. Upregulated cyclin D2 and Integrin β7 expression enhances proliferation of multiple myeloma cells and their adhesion to bone marrow stroma, respectively [11]. CCR1 are considered to promote multiple myeloma cell dissemination in bone and spleen [12]. Overall, these findings make c-Maf an attractive target for the treatment of multiple myeloma [13]. Indeed, a retroviral transduction of dominant-negative form of c-Maf whose basic region of DNA binding region was replaced with an acidic region [14] reduced the proliferation of c-Maf-expressing multiple myeloma cells

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when inoculated into mice [11]. For another instance, the colony formation of c-Maf-expressing multiple myeloma cells was inhibited by lithium chloride that block GSK3-dependent c-Maf phosphorylation [15]. However, a strategy directly targeting the transcription activity of c-Maf has not been developed.

Here, we established a screening pipeline to test small compounds against c-Maf transcriptional activity in high-throughput by using *Ccl8*-promoter-driven luciferase intensity as an indicator. Using this system, we identified small compounds that inhibit the transcriptional activity of c-Maf. Among such molecules, IBS003214 and IBS007125 inhibited the proliferation of c-Maf-expressing multiple myeloma cells but not c-Maf-negative multiple myeloma cells. Some of c-Maf target genes were also repressed in multiple myeloma cells that were cultured in the presence of IBS003214 or IBS007125. In conclusion, these small compounds may be used to develop a new c-Maf inhibitor for the therapy of multiple myeloma.

2. Materials and methods

2.1. Plasmids

Ccl8-firefly luciferase reporter plasmid (CCL8-pGL4) was constructed previously [8]. Briefly, an approximately 300-bp DNA fragment of the mouse *Ccl8* gene promoter that contains 3 MARE-half sites was amplified by PCR using mouse genomic DNA as template. The amplified DNA fragment was subcloned into the pGL4.10 vector (Promega). c-Maf-expression plasmid (Maf-BOS-EX) was constructed previously [8]. To construct MafB-expression plasmid (MafB-BOS-EX), MafB cDNA was amplified by PCR using mouse genomic DNA as template and the following primers: (forward) 5′-GGAATTCTTTAGCGATGGCCGCGGA-3′, (reverse) 5′-GCTCTAGATCACAGAAAGAACTCAGGAGAGGAGG-3′. The PCR product was digested with EcoRI and XbaI and then subcloned into an EcoRI- and XbaI-digested BOS-EX plasmid.

2.2. Small compound preparation

Chemical library (20,000 compounds) was provided from Screening Unit, Research Core Center, Tokyo Medical and Dental University (TMDU). For assays, the test small compounds were used at a maximum final concentration of 100 μM. Data were analyzed by analysis of variance (ANOVA) followed by multiple comparison with GraphPad Prism (GraphPad Software, CA, U.S.A.). $P < 0.05$ was considered statistically significant.

2.3. Cells and animals

RMPMI-8226 and U266 cells were cultured in RPMI/10% FBS/1% penicillin–streptomycin. Bone marrow (BM) cells from C57BL/6 mice were cultured in MEMα/10% M-CSF (CMG14-12 culture medium)/10% FBS/1% penicillin–streptomycin to induce macrophages *in vitro*. BMDMs were harvested on days 4 or 5 and seeded on a 96-w flat bottom plate at the concentration of 4×10^4 cells/well and cultured for another 24h. Then, BMDMs were stimulated with 100 ng/mL LPS in the presence or absence of different concentrations of small compounds for overnight. Animals were maintained in specific-pathogen-free conditions. Animal experiments were performed in accordance with the applicable guidelines and regulations, and were approved by the Tokyo University of Pharmacy and Life Sciences (TUPLS) animal use committee (Approval numbers: L17-24).

2.4. Luciferase reporter assay

HEK293T cells were grown in DMEM/10% FBS/1% penicillin–streptomycin. Twenty-to twenty-five-thousand cells were seeded in a 96-well plate and incubated for 24 h. The cells were transfected with 20 ng of c-Maf expression plasmid (Maf-BOS-EX) or MafB expression plasmid (MafB-BOS-EX) along with reporter plasmids; CCL8-pGL4 and Renilla luciferase plasmid (pRLTK), by PEI-Max (Polysciences, PA, U.S.A.) according to the manufacturer's protocol. The luciferase assay was performed with Dual-Luciferase Reporter Assay kit (Promega, WI, U.S.A.) according to the manufacturer's protocol. Twenty-four hours after the transfection, the luciferase activity was measured by a Luminometer MicroLumat Plus LB96V (Berthold, Germany) or a GloMax-20/20 Luminometer (Promega). Transfection efficiency was normalized to the co-transfected Renilla luciferase activity.

2.5. Cytokine ELISA

Mouse IL-6, IL-10, and TNF-a concentrations were quantitated by ELISA MAX (BioLegend, CA, U.S.A.), according to the manufacturer's protocols. CCL8 concentration was quantitated by ELISA that was established in our laboratory as reported before [16].

2.6. Myeloma cell growth inhibition assay

RPMI-8226 and U266 cells were cultured in the presence or absence of small compounds for 5 days. Cell number was quantitated by using WST-8 assay kit (Dojindo, Japan).

2.7. Total RNA extraction for quantitative reverse transcription PCR (qRT-PCR)

Total RNA from cultured cells was extracted with an RNeasy Mini Kit (Qiagen, Germany) or a FavorPrep Total RNA Extraction Column (Favorgen, Taiwan) according to the manufacturers' protocols. For qRT-PCR, complementary DNAs (cDNAs) were synthesized using ReverTra Ace (Toyobo, Japan). qRT-PCR was performed on cDNA using a THUNDERBIRD SYBR qPCR Mix (Toyobo). Expression levels were normalized to β-actin. Primer sequences were as follows: *c-MAF*: (forward) 5′-ATCACCAACACAGACGTGGA-3,′(Rev) 5′-ACGGTACTGCTGCAGGCTAT-3′; *ITGB7*: (forward) 5′-GCAGCAACAACTCAACTGGA-3′, (reverse) 5′-CCTCTTGAAAGCGAGGATTG-3′; *CCR1*: (Forward) 5′-CCAATGGGAATTCACTCACC-3′, (reverse) 5′-GAGCCTGAAACAGCTTCCAC-3′; and *ACTB*: (forward) 5′-AGAGCTACGAGCTGCCTGAC-3′, (reverse) 5′-AGCACTGTGTTGGCGTACAG-3′.

2.8. Western blotting and intracellular flowcytometry

Myeloma cells cultured for 2–3 days in the presence of 0.1% DMSO, or 10 μM small compounds were lysed with RIPA buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin). The protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, MA, U.S.A.) according to the manufacturer's protocol. Whole cell lysates were subjected to SDS-PAGE on a 12% acrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore, MA, U.S.A.). After the transfer, the membrane was probed with anti-*c*-MAF antibody (Proteintech, IL, U.S.A.) at the concentration of 353 ng/mL (1:1000), followed by incubation with HRP-conjugated anti-rabbit immunoglobulins (1:2000, DAKO). The membrane was processed with Super Signal West Pico Chemiluminescent substrate (Thermo Fisher Scientific). After antibody removal with 2% SDS/2-ME/62.5 mM Tris-HCl, pH 6.7, the membrane was reproved with HRP-conjugated anti-GAPDH antibody (1:2000, MBL, Japan). Band intensities were measured using Fusion chemiluminescence imaging system (VILBER, France).

For the detection of c-Maf by intracellular flowcytometry, myeloma cells cultured for 2–3 days in the presence of 0.1% DMSO, or 10 μM small compounds were incubated with ethidium monoazide (EMA, Invitrogen/Thermo Fisher Scientific) under a bright fluorescent light for 10 min on ice to allow covalent binding of the EMA to the DNA. Cells were then fixed with True-Nuclear 1x Fix (True-Nuclear Transcription Factor Buffer Set, BioLegend) for 30 min at room temperature followed

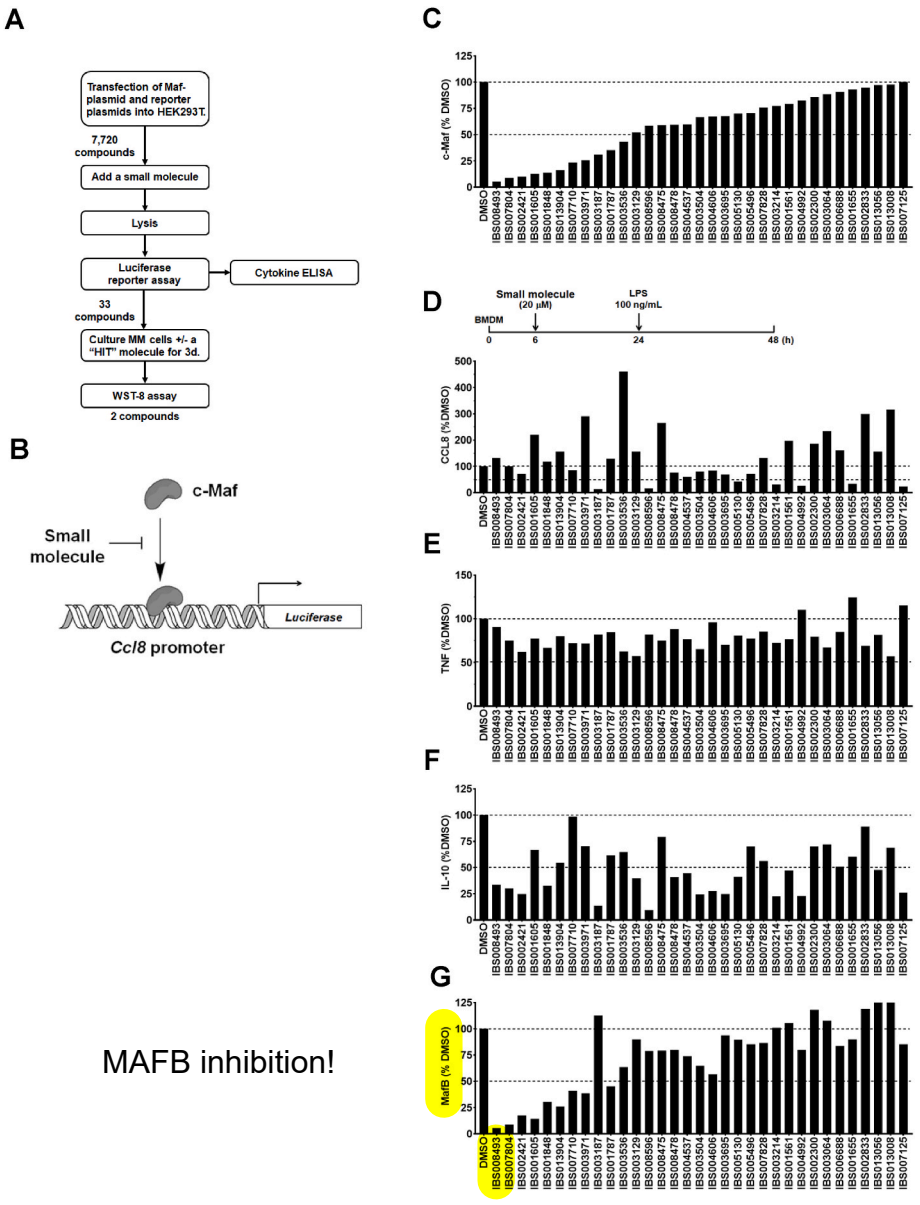

Fig. 1. Development of screening pipeline to test small compound against c-Maf transcriptional activity *in vitro*.
(**A**) Workflow of screening strategy. **(B)** HEK293T cells were transfected with Maf-BOS-EX, CCL8-pGL4, and pRLTK, and were cultured in the presence of one of 7,720 small compounds or DMSO for 24h. Luciferase reporter activity driven by c-Maf was quantitated. Non-specific cytotoxic molecules were excluded by Renilla luciferase intensity. **(C)** Thirty-three small compounds that inhibited c-Maf activity in more than 3 repeated screenings were selected. The "HIT" compounds were tested for their ability to suppress c-Maf-dependent cytokine (CCL8 and IL-10) production, and a c-Maf-independent cytokine (TNFα) production by LPS-stimulated BMDMs. BMDMs were pre-treated with one of the hit compounds 18 h before the stimulation with 100 ng/mL LPS. Concentrations of CCL8 (**D**), TNFα (**E**), and IL-10 (**F**) in the culture medium 24 h after LPS stimulation was quantitated by ELISA. Values are relative to DMSO control. Representative data of at least two independent experiments are shown. **(G)** Suppression of MafB-driven luciferase activity was quantitated as in (**A**) in HEK293T cells transfected with MafB-BOSEX and reporter plasmids. Representative result of 2 independent experiments is shown.

by permeabilization with True Nuclear 1x Perm. The permeabilized cells were stained with either PE-mouse IgG (BioLegend) or PE-anti-c-Maf (BD Biosciences, NJ, U.S.A.) for 30 min at room temperature. Cell suspensions were analyzed with a BD FACSCelesta (BD Biosciences).

2.9. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by multiple comparison with GraphPad Prism (GraphPad Software, CA, U.S.A.). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Development of screening pipeline to test small compound against c-Maf transcriptional activity in vitro

Fig. 1A is an overview of the small compound-screening pipeline that was developed and implemented in this study. To evaluate inhibition of c-Maf transcriptional activity in high throughput, we established a luciferase reporter assay-based protocol in 96-w plate format (Fig. 1A). In HEK293T cells co-transfected with Maf-BOS-EX (c-Maf-expression plasmid), CCL8-pGL4 (firefly luciferase reporter plasmid), and pRLTK (Renilla luciferase reporter plasmid), c-Maf binds to MARE-half sites in the *Ccl8* promoter region and enhance the firefly luciferase intensity

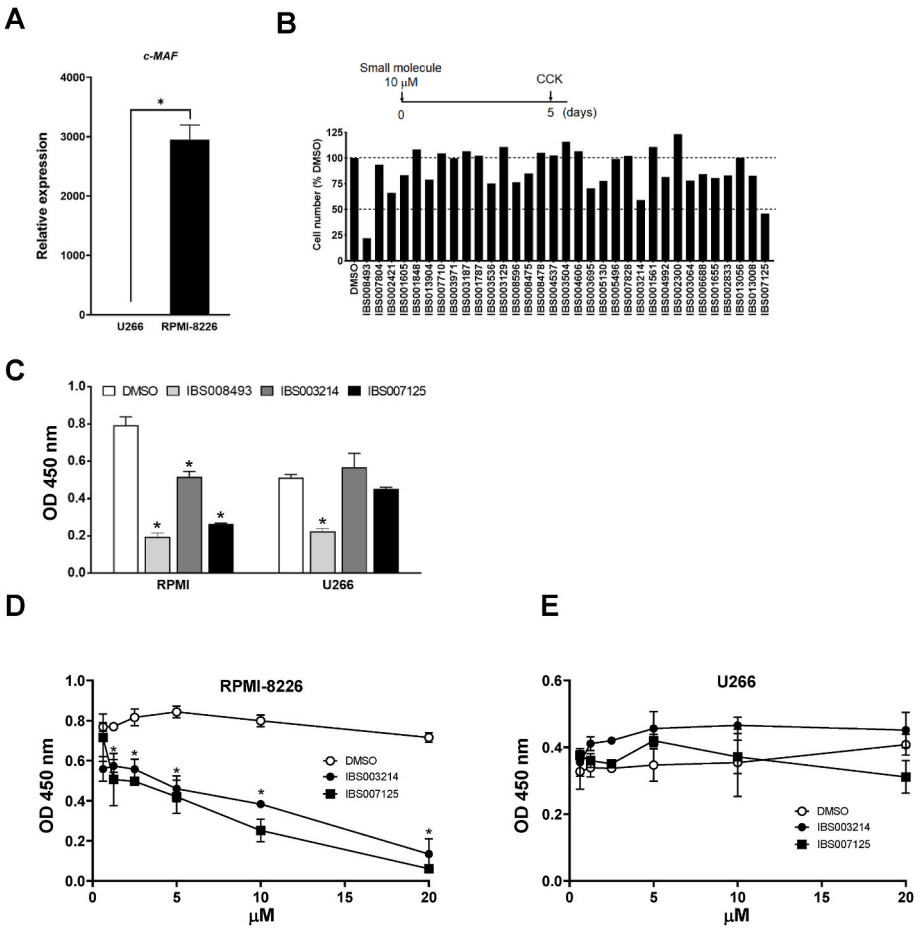

Fig. 2. Identification of small compounds that suppress the proliferation of c-Maf-expressing multiple myeloma cells.
(**A**) c-Maf expression levels in U266 and RPMI-8226 cells were measured by qRT-PCR. n = 3/cell type. Average values are shown with SD. *$P < 0.05$, *t*-test. Representative data of two independent experiments with similar results is shown. (**B**) One-thousand RPMI-8226 cells were seeded in a 96-w flat plate and cultured in the presence of one of hit molecules (10 μM) or DMSO for 5 days. Cell number was quantitated by WST-8 assay. Small compounds that inhibited multiple myeloma cells proliferation by more than 50% were selected. Representative data of 2 independent experiments with similar results is shown. (**C**) One-thousand RPMI-8226 cells or U266 cells were cultured as in (**B**) in the presence of indicated small compounds or DMSO for 5 days. Cell number was quantitated by WST-8 assay. Representative data of 2 independent experiments performed in triplicate is shown. Average values are shown with SD. *$P < 0.05$, one-way ANOVA. n = 3/condition. One-thousand RPMI-8226 cells (**D**) or U266 cells (**E**) were cultured in the presence of different concentrations of indicated small compounds or DMSO. Cell number at day 5 was quantitated by WST-8 assay. Representative data of 2 independent experiments performed in triplicate are shown. n = 3/condition. Average values are shown with SD. *$P < 0.05$, two-way ANOVA.

(Fig. 1B). c-Maf-driven firefly luciferase intensity was normalized against Renilla luciferase intensity that positively correlates with transfection efficiency in each well. Using this pipeline, we screened a library of 7,720 compounds for their effects on c-Maf-induced *Ccl8*-promoter-dependent firefly luciferase intensity in HEK293T cells (Fig. 1A). Small compounds that suppressed luciferase intensity without suppressing Renilla luciferase intensity were defined as "HITs". Those suppressed both firefly and Renilla luciferase intensity were considered cytotoxic and therefore excluded from further study. Through three cycles of screenings, thirty-three small compounds that repeatedly suppressed firefly luciferase intensity with a maximum effect of <100% of DMSO control were selected (Fig. 1C).

Next, to examine whether the selected small compounds suppressed firefly luciferase intensity in a c-Maf-specific manner, cytokine concentration in the culture medium of BMDMs that were stimulated with lipopolysaccharide (LPS) in the presence or absence of the selected small compounds was quantitated by ELISA. This analysis showed that 7 out of the 33 small compounds also reduced the concentrations of c-Maf-dependent cytokine CCL8 by more than 50% (Fig. 1D). On the other hand, none of the 33 small compounds inhibited the production of TNFα, which does not require c-Maf (Fig. 1E). We found that 6 out of 7 small compounds that inhibited CCL8 also inhibited another c-Maf-dependent cytokine IL-10 (Fig. 1F). It was reported that c-Maf shares consensus DNA binding sequences with other family members of large Mafs [17]. As shown in Fig. 1G, degree of MafB suppression by the small compounds relatively positively correlated with that of c-Maf suppression, suggesting that most of the small compounds inhibited the binding of c-Maf to MARE. Collectively, these findings demonstrated that our screening pipeline can be used to explore inhibitors of c-Maf transcriptional activity.

3.2. *Identification of small compounds that inhibit the proliferation of c-Maf-expressing multiple myeloma cells*

Having established a c-Maf inhibitor screening pipeline, and selected small compounds that can inhibit transcriptional activity of c-Maf, we next aimed to examine their effect on the proliferation of multiple myeloma cells. To this goal, we used RPMI-8226 cell line that express c-Maf at high level and U266 that barely express c-Maf [11](Fig. 2A). We cultured two lines of multiple myeloma cells in the presence of one of the hit molecules for 5 days. Then, the number of multiple myeloma cells was quantitated by WST-8 assay. This analysis identified three small

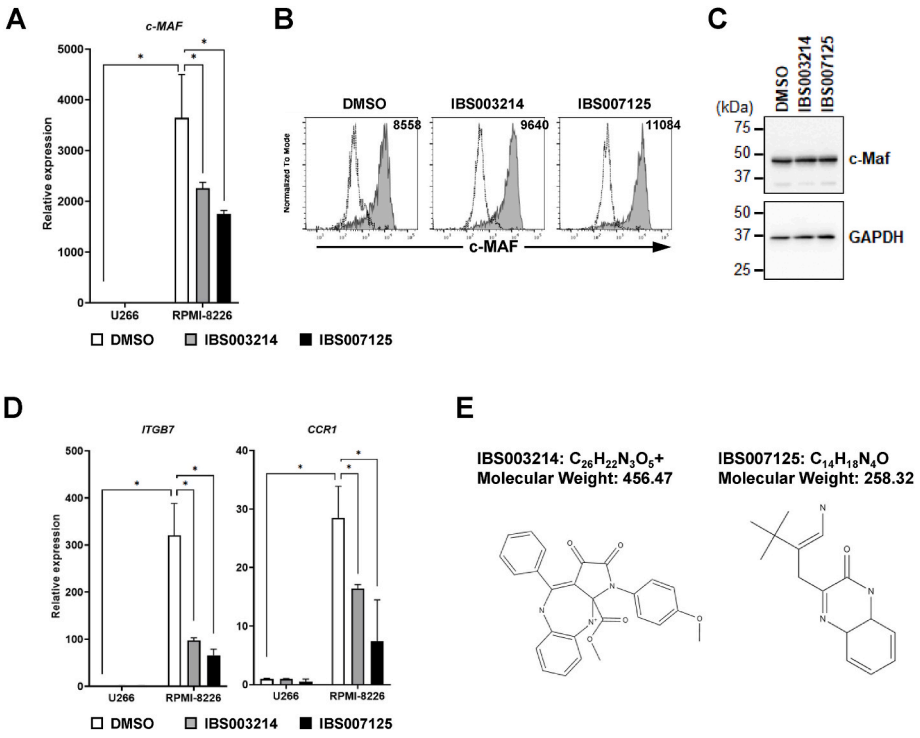

Fig. 3. Suppression of c-Maf-target genes by the small compounds.
(**A**) c-Maf mRNA level in myeloma cells that were treated with DMSO, 10 μM of IBS003214, or IBS007125 for 2 days was quantitated by qRT-PCR. n = 3/condition. Average values relative to DMSO-treated U266 cells is shown with SD. Representative data of two independent experiments with similar results are shown. *$P < 0.05$, one-way-ANOVA. (**B**) c-Maf protein level was quantitated by intracellular flowcytometry. Representative FACS plots of two independent experiments with similar results are shown. Dashed line indicates mouse IgG control. Numbers indicate geometric means. (**C**) Western blotting showing the amount and molecular weight of c-Maf protein in RPMI-8226 cells cultured in the presence of indicated reagents for 2 days. (**D**) Small compounds repress the expression of some c-Maf target genes in RPMI-8226 cells. RPMI-8226 cells were cultured in the presence or absence of 10 μM of indicated small compound for 3 days. Representative data of two independent experiments with similar results are shown. Average values relative to DMSO-treated U266 cells are shown with SD. *$P < 0.05$, one-way ANOVA. (**E**) Chemical structures of IBS003214 and IBS007125.

compounds: 12-(4-methoxyphenyl)-9, 9-dimethyl-2-((naphthalen-1-yloxy)methyl)-9,10-dihydro-8H-chromeno [3,2-e], [1,2,4] triazolo [1, 5-c] pyrimidin-11(12H)-one, named IBS008493, 10a- (methoxycarbonyl)- 1- (4-methoxyphenyl)-2, 3-dioxo-4-phenyl-1, 2, 3, 5, 10, 10a-hexahydrobenzo [b] pyrrolo [2,3-e] [1,4] diazepin-10-ium), named IBS003214, and (Z)-3- (2-hydrazono-3, 3-dimethylbutyl) quinoxaline-2 (1H)-one, named IBS007125, that suppress the proliferation of RPMI-8226 cells by nearly 50% (Fig. 2B). Next, to examine whether inhibition was c-Maf-specific, we cultured RMPI-8226 cells and U266 cells in the presence or absence of the three small compounds. This analysis showed that IBS003214 and IBS007125 did not suppress the proliferation of U266 cells (Fig. 2C). These findings indicated that the inhibition of multiple myeloma proliferation by IBS003214 and IBS007125 was c-Maf-dependent. On the other hand, IBS008493 also strongly suppressed U266 proliferation (Fig. 2C), suggesting that the suppression by IBS008493 was not c-Maf-specific. To estimate the efficiency of RPMI-8226 suppression, we cultured multiple myeloma cells in different concentrations of IBS003214 and IBS007125. We found that the two small compounds suppressed the proliferation of RPMI-8226 in a dose-dependent manner but not U266 cells (Fig. 2D and E).

Then, we examined the expression of c-Maf and its target genes in myeloma cells that were treated with either IBS003214 or IBS007125. The two small compounds modestly repressed c-Maf expression at the mRNA level (Fig. 3A), however, the amount of protein (Fig. 3B and C) was similar between RPMI-8226 that were treated with either DMSO, IBS003214, or IBS007125. The molecular weight of c-Maf protein was also identical in those RPMI-8226 cells (Fig. 3C). These analyses suggest that the production nor post-transcriptional modifications, such as phosphorylation, of c-Maf may not be inhibited by the two small compounds. Lastly, we asked whether IBS003214 and IBS007125 can repress c-Maf target genes that are responsible for the malignant transformation of multiple myeloma cells. To answer this question, we treated RPMI-8226 cells with IBS003214 or IBS007125 for 3 days and quantitated c-Maf-target gene mRNA levels by qRT-PCR. This analysis showed that the two small compounds repressed *ITGB7* and *CCR1* mRNA levels demonstrating that c-Maf transcriptional activity was indeed repressed by the addition of the two small compounds (Fig. 3D). In conclusion, we identified two lead compounds that can suppress the proliferation of c-Maf-expressing multiple myeloma cells by targeting c-Maf transcriptional activity (Fig. 3E).

4. Discussion

Multiple myeloma is characterized by expansion of clonal B cells in the bone marrow, leading to increased serum or urine monoclonal immunoglobulin and osteolytic bone destruction. One of the most frequent structural abnormalities carried by multiple myeloma karyotypes involves the translocation of immunoglobulin heavy chain locus on 14q32. Unlike the physiological immunoglobulin VDJ rearrangement, 14q32 translocations results in juxtaposition of immunoglobulin heavy chain region with non-immunoglobulin gene sequences, a process called illegitimate switch rearrangements [18]. The immunoglobulin heavy chain translocations are observed in more than 70% of multiple myeloma and related plasma cell malignancy patients [19] leading to dysregulated expression of oncogenes at translocation partner regions. 11q13 (cyclin D1), 4p16.3 (FGFR3), 16q23 (c-Maf), and 6p21 (cyclin D3) are the most common translocation partners of 14q32 in primary multiple myeloma patients [10] among which translocation with 16q23

induces extensive expression of c-Maf [20]. Because of the critical role of c-Maf in cell proliferation, therapeutic targeting of this transcription factor has been explored as a potential strategy for the treatment of multiple myeloma. In this study, we introduced a high-throughput screen pipeline to test c-Maf transcriptional activity. Combination of this pipeline with cytokine ELISA and tumor proliferation assay identified c-Maf inhibitors that can selectively suppress the proliferation of c-Maf-expressing multiple myeloma cells. We showed that IBS003214 and IBS007125 repressed some target genes of c-Maf (*ITGB7* and *CCR1*) that are involved in the malignant transformation of myeloma cells without changing c-Maf protein level or molecular weight. These findings indicate that the two small compounds inhibit the transcriptional activity but not the production or post-transcriptional modification of c-Maf. It remains to be examined whether the inhibition of c-Maf activity secondarily downregulated its transcription. As c-Maf is important for the production of IL-10, c-Maf-inhibitors selected in this study may also inhibit the immunosuppressive capacity of regulatory T cells and macrophages in cancer. Some inhibitors of c-Maf that were selected by our screening pipeline also repressed the transcription activity of MafB. Therefore, side effects of suppressing other members of large Maf family should be carefully monitored *in vivo*. In conclusion, our screening pipeline can be used to explore leads for optimization into c-Maf-targeting molecule that may be applied for the treatment of multiple myeloma.

CRediT authorship contribution statement

Kenichi Asano: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Kenta Kikuchi:** Investigation, Methodology. **Miki Takehara:** Data curation, Investigation. **Manami Ogasawara:** Data curation, Investigation. **Yuki Yoshioka:** Data curation, Investigation. **Kie Ohnishi:** Data curation, Investigation. **Ayaka Iwata:** Data curation, Investigation, Visualization. **Shigeomi Shimizu:** Resources. **Masato Tanaka:** Conceptualization, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kenichi Asano has patent #2021–192959 pending to Tokyo University of Pharmacy and Life Sciences.

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