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Suppression of malignant rhabdoid tumors through Chb-M'-mediated RUNX1 inhibition

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Abbreviations	
MRT	Malignant Rhabdoid tumor
Chb-M'	Chlorambucil-M prime

Abstract

Malignant rhabdoid tumor (MRT) is a rare and highly aggressive pediatric malignancy primarily affecting infants and young children. Intensive multimodal therapies currently given to MRT patients are not sufficiently potent enough to control this highly malignant tumor. Therefore, additive or alternative therapy for these patients with a poor prognosis is necessary. We herein demonstrated that the inhibition of RUNX1 by novel alkylating-conjugated pyrrole-imidazole (PI) polyamides, which specifically recognizes and binds to RUNX-binding DNA sequences, was highly effective in the treatment of rhabdoid tumor cell lines *in vitro* as well as *in vivo*. Therefore, the suppression of RUNX1 activity may be a novel strategy for MRT therapy.

Background

Malignant rhabdoid tumor (MRT) is a rare and highly aggressive malignancy, which affects infants and young children ¹. MRT occurs at various anatomical locations including the kidneys, central nervous system (CNS), liver, heart, mediastinum, uterus, adnexa, urinary bladder, and soft tissue. Despite the existing standard intensive multimodal therapy, the long-term survival rate of MRT patients is less than 30% ^{2,3}. Therefore, more effective treatments are highly desired.

The genetic hallmark of rhabdoid tumors are mutations of SMARCB1 (INI1), a core subunit of the SWI/SNF chromatin-remodeling complex. SMARCB1 has potent tumor suppressor activity, and Wang et al. reported that oncogenesis caused by loss of the SMARCB1 might be dependent on the activity of the residual SWI/SNF complex ⁴. In the other report, the SWI/SNF complex interacts with the Runt-related transcription factors

(RUNX)1, a member of runt-related transcription factor family ⁵. Thus, we focus on RUNX1 as a potential target of therapy.

RUNX1 functions as an indispensable regulator of various developmental processes, but also plays critical roles in the development and maintenance of a number of malignant tumors^{6–9}. We recently reported that the inhibition of RUNX cluster activities using the newly investigated inhibitor, Chb-M', resulted in tumor suppression in a wide variety of cancer cell lines ¹⁰. Chb-M' consists of two functional components: pyrrole-imidazole (PI) polyamides targeting the RUNX core DNA consensus sequence (5'-TGTGGT-3') and the alkylating agent, chlorambucil (Chb). PI polyamides are known to recognize and bind to the minor groove of specific DNA sequences non-covalently through the interaction between their pyrrole and imidazole pairs interlocked by a hairpin linking ^{11,12}. Once Chb-M' specifically binds to RUNX-binding sequences, the down-regulation of various RUNX signaling pathways induces apoptosis in cancer tumor cells. This inhibitory effect was examined and confirmed in acute myeloid leukemia cells as well as multiple solid tumors with a poor prognosis in xenograft mouse models ^{10,13–15}. We herein propose the efficient suppression of MRT using Chb-M', which has the potential to become an alternative therapeutic strategy for MRT patients.

Procedure

Cell lines

The human MRT cell lines MP-MRT-AN, KP-MRT-RY, and KP- MRT-YM were established as previously reported ^{16–18}. They were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal

bovine serum (FBS) and 1% Penicillin-Streptomycin (PS). HEK293T cells were purchased from RIKEN BRC, Japan and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 1% PS. Cells were cultured at 37 °C and 5% CO₂.

Mice

NOD/Shi-scid, IL-2RγKO (NOG) mice were purchased from Central Institute for Experimental Animals, Japan and were experimented at 8–12 weeks of age. Littermates were used as controls in all experiments. Mice were housed in sterile enclosures under specific pathogen-free conditions. These mice were randomly divided into each group before experiment and were anesthetized with isoflurane for all procedures and killed by cervical dislocation at the end of each experiment. Tumor size was measured every week and tumor volume was determined according to the following equation: tumor size = width² × length × (π/6).

Apoptosis assay

In the apoptosis assay, apoptotic cells were detected by Annexin V Apoptosis Detection Kit APC (eBioscience Inc.). In brief, approximately 2×10^5 cells of the indicated control and experimental groups were washed in PBS, suspended in annexin-binding buffer, and then mixed with 5 μL of annexin V. Reaction mixtures were incubated for 30 min. After the incubation, cells were diluted, stained with 7-ADD, and processed for a flow cytometric analysis by FACS CANTO II (BD Biosciences).

Measurement of the half maximal inhibitory concentration (IC₅₀)

In the cell survival assay, cells were seeded at a density of 1×10^5 cells/mL. Cells were treated with the indicated concentrations of each compound in DMSO and then incubated for 72 hours. Cell viability was examined by Cell Count Reagent SF (Nacalai Tesque, Inc.) and the Infinite® 200 PRO multimode reader (TECAN) according to the manufacturer's instructions. Percent inhibition curves were drawn and IC_{50} of the indicated compounds were calculated based on the median-effect method.

Small interfering RNA (siRNA) knockdown of RUNX1

siRNA against human RUNX1 transcript (sc-37677, Santa Cruz Biotechnology, Dallas, TX, USA) and control siRNA (sc-37007, Santa Cruz Biotechnology, Dallas, TX, USA). MRT cell lines (2×10^5 cells/mL) were cultured in RPMI 1640 medium containing 10% fetal bovine serum without 1% penicillin/streptomycin at 37°C. MRT cell lines were transfected with siRNA/Lipofectamine RNAiMAX (Invitrogen) complexes diluted in Opti-MEM Reduced Serum Medium (Gibco) (final siRNA concentration, 16.7 nM) according to the manufacturer's protocol. 24 h after transfection, trypan blue dye exclusion assays were performed for 3 days.

Immunoblotting

Immunoblotting was conducted as previously described ¹⁹. Briefly, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer. Whole cell extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride membranes. Membranes were probed with the following primary antibodies: anti-RUNX1 (A-2, Santa Cruz Biotechnology,

Inc.), anti-p53 (1C12, Cell Signaling Technology), anti-PARP (46D11, Cell Signaling Technology), and anti-GAPDH (FL-335, Santa Cruz Biotechnology, Inc.). HRP-conjugated anti-rabbit IgG and anti-mouse IgG (#7074 and #7076, Cell Signaling Technology) were used as secondary antibodies. Blots were visualized using Chemi-Lumi One Super (Nacalai Tesque, Inc.) and the ChemiDoc™ XRS+ Imager (Bio-Rad Laboratories, Inc.) according to the manufacturers' recommendations.

Statistical analysis

Data are expressed as means $\pm$ s.d.. Differences in mean values between groups were analyzed by the Student's *t*-test using JMP® 13 (SAS Institute Inc., Cary, NC, USA). P-values < 0.01 or 0.05 were considered to be significant.

Study approval

All animal studies were properly conducted according to the Regulations on Animal Experimentation at Kyoto University, based on International Guiding Principles for Biomedical Research Involving Animals. All procedures employed in this study were approved by Kyoto University Animal Experimentation Committee (Permit Number: Med Kyo 14332).

Results

Chb-M' exhibits anti-tumor activity against MRT cell lines.

Chb-M' was synthesized as previously reported. We initially measured the IC₅₀ of Chb-M' in three different MRT cell lines. As shown in Fig. 1, Chb-M' was highly effective in all

three MRT cell lines. In contrast to Chb-M', Chb and Chb-Scramble (Chb-S), PI polyamides targeting the 5'-WGGCCW-3' sequence, were completely ineffective in all three lines. Next, we investigated whether depletion of RUNX1 could have an antitumor effect on MRT cells by si-RNA-mediated RUNX1-knockdown system. As shown in Fig. 2, the depletion of RUNX1 by si-RNA is highly effective against the proliferation of MRT cells.

The Chb-M' treatment activates the p53 apoptotic pathway.

We previously confirmed that RUNX cluster regulation-mediated apoptosis is related to the p53 apoptosis pathway [8]. Therefore, we investigated whether the Chb-M' treatment induces p53-mediated apoptosis in three MRT cell lines. As shown in Figs. 3A, 3C and 3E, the Chb-M' treatment significantly increased the rate of apoptotic cells. Furthermore, Chb-M'-treated cells had higher expression levels of p53 and also had the cleaved form of poly ADP-ribose polymerase (PARP) (Fig. 3B, 3D, 3F). These results suggest that the inhibition of RUNX-mediated apoptosis is mediated by the activation of the p53 pathway.

Chb-M' exhibits anti-tumor activity in an *in vivo* mouse model.

Since Chb-M' suppressed RUNX1 activities in MRT *in vitro*, we tested its anti-tumor efficacy *in vivo*. KP-MRT-YM cells were subcutaneously injected into immunodeficient NOG (NOD/Shi-scid/IL-2R γ ^{null}) mice. These mice were treated with Chb-M' (injections of 320 μ g/kg body weight twice a week) or an equivalent amount of dimethyl sulfoxide (DMSO) as a control to observe the growth of transplanted MRT. After 3 weeks of treatments, the growth of rhabdoid tumors was markedly less in Chb-M'-treated mice than

in the controls (Fig. 4A). The difference in the growth of the tumor size confirmed the efficacy of the Chb-M' treatment (Fig. 4B). These tumors were then stained by hematoxylin-eosin (HE) and Ki- 67 to validate the suppression of MRT (Fig. 4C). We also confirmed apoptosis in MRT via an increase in the frequency of TUNEL+ cells in Chb-M'-treated mouse samples (Fig. 4D).

Conclusions

MRT was originally described as a renal sarcomatous tumor of childhood but is currently being highlighted as an independent tumor category. This is due to how this histologically unique tumor has been detected in various extra-renal sites. Although MRT is one of the most aggressive tumors identified to date, a definite therapy has not yet been established. In our laboratory, we demonstrated that the PI-polyamide-based compound, Chb-M', was highly effective at suppressing various cancer tumors, including leukemia, gastric cancer, and lung cancer. Chb-M' was designed to bind to the RUNX core DNA consensus domain in order to control the growth of various malignant tumors. In the present study, we examined the efficacy of Chb-M' in three MRT cell lines as well as in a mouse model. We confirmed that Chb-M' suppressed cell growth in all three cancer lines more strongly than Chb and Chb-Scramble, which randomly bind to DNA. This suggests that targeting the RUNX signaling pathway in MRT is efficient for suppressing tumor growth. Based on the inhibition of the RUNX signaling pathway by Chb-M', we also suggest that a p53-dependent cell death signaling pathway was activated, resulting in the suppression of tumor growth. Furthermore, the efficacy of Chb-M' against MRT was verified using an MRT xenograft mouse model.

Considering that the genetic hallmark of MRT are the mutations of SMARCB1, Chb-M' inhibition pathways were thought to be related to the activity of SWI/SNF complex. However, the mechanisms underlying Chb-M' inhibition pathways in MRT are unclear regarding SWI/SNF complex. R. Bakshi *et al.* reported that the SWI/SNF complex associates with RUNX1 to control hematopoietic-specific gene expression ⁵ and D. Lee *et al.* reported the SWI/SNF complex interacts with p53 in the other literature ²⁰. But the tripartite relation between RUNX1, p53 and the SWI/SNF complex in MRT is still unknown. Even though further investigations are needed to elucidate the mechanisms underlying Chb-M' inhibition pathways in MRT, the present results are the first step in the development of MRT treatments. We assume that Chb-M' has potential in the treatment of other cancers with currently unmet medical needs.

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Contributions

T.D. performed experiments, analyzed data, and wrote the manuscript. M.M. M.H., and M.N. performed experiments and analyzed data. H.H., A.I. K.U., H.K., T.M., K.F., S.T., Y.N., K.M., T.B., T.R.K., T.N., J.T. and S.A. participated in discussions and the interpretation of data and results and commented on the research direction. Y.K, T.I. and H.H. established and characterized the human MRT cell lines. H.S. synthesized and designed the PI polyamides. Y.K. designed and initiated the study, supervised research, and gave the final approval for submission.

Conflict of Interest statement

The authors have no conflicting financial interests.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legend

Fig. 1. IC₅₀ of Chb-M' in three rhabdoid cell lines *in vitro*.

The efficacies of Chb, Chb-M', and Chb-S were examined in MP-MRT-AN (A), KP-MRT-RY (B), and KP-MRT-YM (C). IC₅₀ values are described in (D). These examinations were performed in triplicate. The results obtained were represented as the average $\pm$ standard error of the mean (SEM) of three independent experiments.

Fig. 2. The depletion of RUNX1 by si-RNA in three rhabdoid cell lines.

Western blotting of RUNX1 and GAPDH and the growth curves of MRT cell lines transduced with si-RNA (RUNX1 and control). (A) MP-MRT-AN. (B) KP-MRT-RY. (C) KP-MRT-YM. ** $P < 0.01$, *** $P < 0.001$, $n = 3$.

Fig. 3. The Chb-M' treatment induces apoptosis in MRT.

(A) (C) (E) Apoptosis in MRT cells was assessed by a flow cytometry assay using PE-Annexin-V/7-ADD staining. Cells were initially seeded and incubated at 37 °C for 24 h. Chb-M' dissolved in DMSO was then added at 10 μ M. Forty-eight hours after the treatment, cells were harvested and subjected to a flow cytometric analysis. * $P < 0.05$, $n = 3$, (B) (D) (F) Immunoblotting of RUNX1 and apoptotic factors (p53 and cleaved PARP). Forty-eight hours after the treatment, cells were harvested, and cell lysates were analyzed by immunoblotting with the indicated antibodies

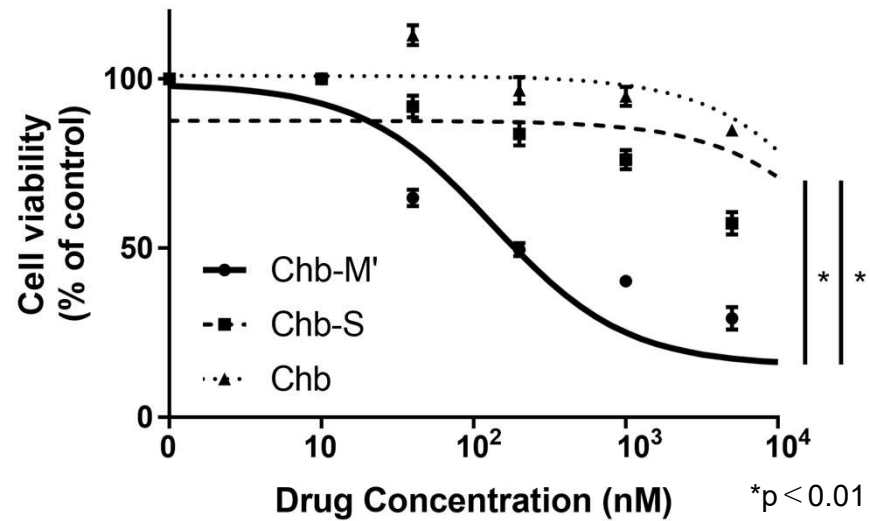
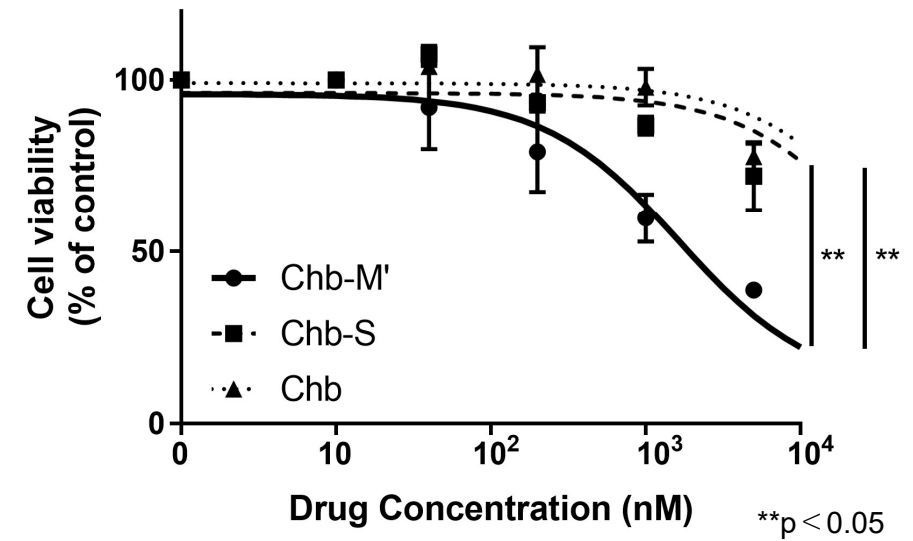
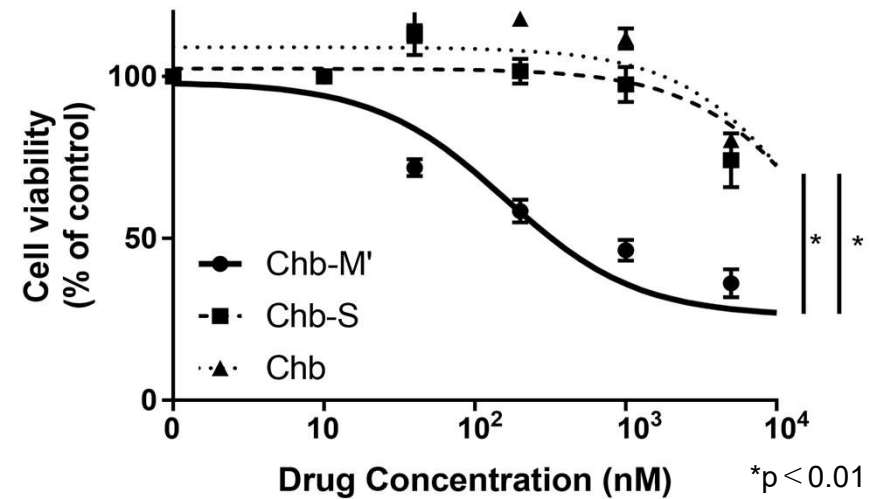
Fig. 4. The Chb-M' treatment suppressed MRT growth *in vivo*.

(A) Antitumor effects were examined by changes in the volume of xenograft tumor cells over time after the Chb-M' injection.

(B) *In vivo* antitumor effects of Chb-M' and dimethyl sulfoxide (DMSO) on tumor volumes when mice were sacrificed. *P < 0.05, n = 6

(C) Tumor histology. HE (Hematoxylin and *eosin*)- and Ki-67-paraffin sections of tumor tissues.

(D) The number of TUNEL-positive cells per microscopic field (×20 magnification). *P < 0.01, n = 10

A**MP-MRT-AN****B****KP-MRT-RY****C****KP-MRT-YM****D**

Drugs	IC ₅₀ (μM)		
	MP-MRT-AN	KP-MRT-RY	KP-MRT-YM
Chb-M'	0.13	1.64	0.16
Chb-S	38.3	36.7	24.4
Chb	34.3	44.2	18.8

