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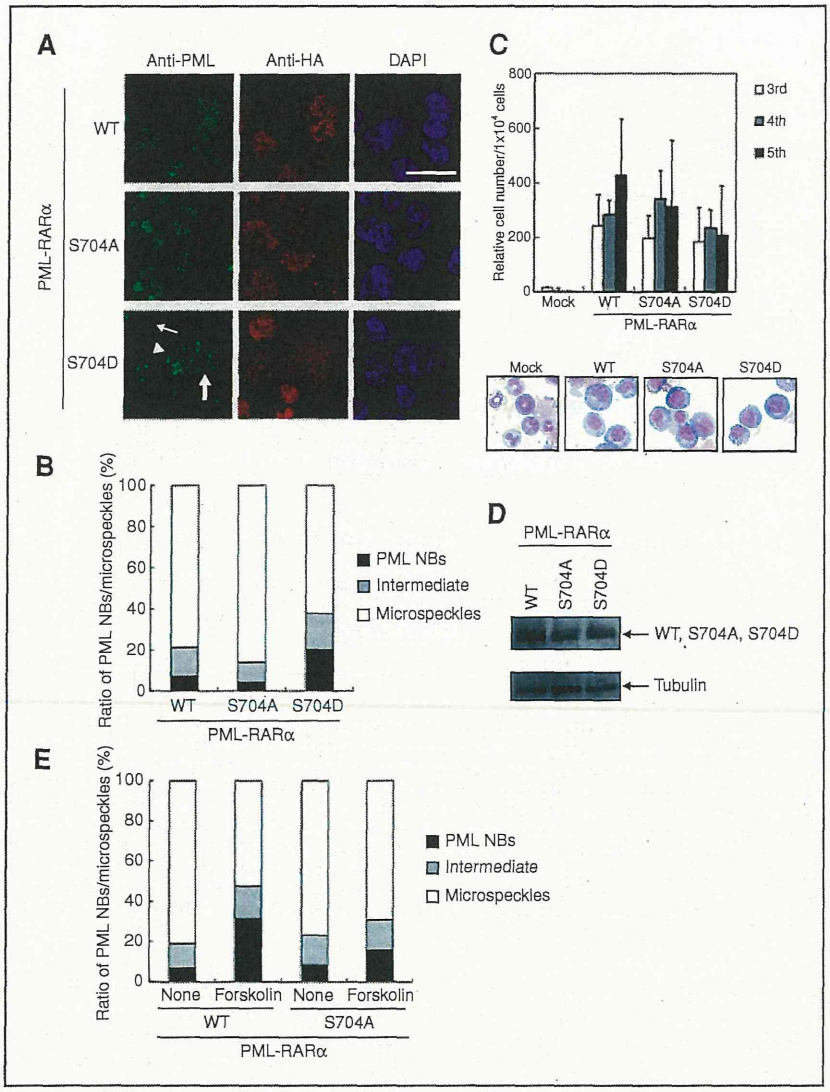

Figure 4. Nuclear body formation is maintained in cells stably expressing PML-RARα S704D. A, PML-RARα S704D does not inhibit nuclear body formation. C-kit$^+$ mouse bone marrow cells were infected with pMSCV-HA-PML-RARα wild-type, S704A, or S704D. Endogenous murine PML nuclear bodies of cells at the third round of colonies were analyzed using mouse Pml-specific antibody (16.1–104). The white bar represents 10 μm. The thick arrow represents PML nuclear bodies, arrowhead represents intermediate nuclear bodies, and thin arrow represents microspeckles. B, quantification of nuclear body formation. The number of cells with PML nuclear bodies, intermediate nuclear bodies, and microspeckles was counted. Values represent the average of 4 independent experiments. C, the cells expressing PML-RARα S704D are immortalized. C-kit$^+$ mouse bone marrow cells were infected with empty vector (mock), pMSCV-HA-PML-RARα wild-type, S704A, or S704D and cultured in methylcellulose medium. The colony number from the third to the fifth round of colonies is indicated (top). Values represent mean ± SEM from 3 independent experiments. The cells at the third round of colonies were stained with May–Giemsa stain (bottom). D, expression of PML-RARα wild-type, S704A, and S704D. The expression of wild-type PML-RARα and mutants and of tubulin in cells at the third round of colonies was analyzed by immunoblotting using anti-PML and anti-tubulin antibodies, respectively. E, quantification of nuclear body restoration. The cells expressing wild-type PML-RARα and S704A at the third round of colonies were collected and exposed to 50 μmol/L forskolin for 24 hours. Endogenous murine PML nuclear bodies were analyzed as described in A. The number of cells with PML nuclear bodies, intermediate nuclear bodies, or microspeckles was counted. Values represent the average of 4 independent experiments. DAPI, 4′, 6-diamidino-2-phenylindole; NB, nuclear body; WT, wild-type.

PML nuclear body disruption and restoration

Nuclear bodies are disrupted in APL cells harboring the t (15;17) chromosomal translocation (16, 17, 19). Results showed that wild-type PML-RARα blocked PML oligomerization (Supplementary Fig. S2C). Deletion analysis showed that PML-RARα mutants that block PML oligomerization

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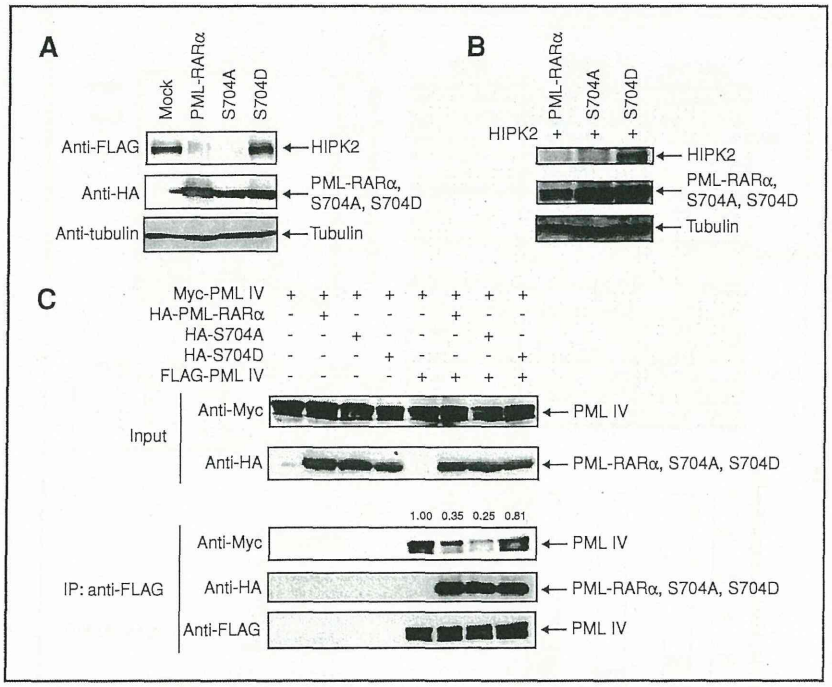

Figure 5. HIPK2 destabilization and inhibition of PML oligomerization are correlated with PML nuclear body disruption by PML-RARα. A, effect of the PML-RARα point mutants on HIPK2 stability. FLAG-tagged HIPK2 was expressed with either empty vector or HA-tagged PML-RARα point mutants. The expression of HIPK2 (top), PML-RARα point mutants (middle), and tubulin (bottom) was detected by immunoblotting using anti-FLAG, anti-HA, and anti-tubulin antibodies, respectively. B, effect of the PML-RARα point mutants on HIPK2 stability in a stable expression system. C-kit$^+$ mouse bone marrow cells were infected with pMSCV-HA-HIPK2 and pMSCV-HA-PML-RARα encoding either wild-type, S704A, or S704D. The expression of HIPK2, PML-RARα (wild-type and mutants), and tubulin at the third round of colonies was analyzed by immunoblotting using anti-HIPK2, anti-PML, and anti-tubulin antibodies, respectively. C, effect of the PML-RARα point mutants on PML oligomerization. 293FT cells were transfected with pLNCX-Myc-PML IV and either pLNCX-HA-PML-RARα substitution constructs or pLNCX-FLAG-PML IV (empty vectors were used as negative constructs). The expression of Myc-tagged PML IV and HA-tagged PML-RARα substitution mutants in the lysates of transfectants was detected by immunoblotting using anti-Myc and anti-HA antibodies, respectively (Input). The lysates of transfectants were incubated with anti-FLAG antibody. The immunoprecipitates were analyzed by immunoblotting using anti-Myc, anti-HA, and anti-FLAG antibodies (IP). The values of IP/Input intensity of Myc-PML IV were quantified with ImageGauge, and normalized to the value of Myc-PML IV/empty vector/FLAG-PML IV. IP, immunoprecipitation.

also disrupt nuclear bodies (Figs. 2B and G, 3B, and 5C). The blocking of PML oligomerization by PML-RARα occurs independent of their interaction (Figs 2G and 5C). These results suggest that the inability of inhibition of PML oligomerization by PML-RARα mutants is not due to the inability of interaction with PML IV, and imply that PML-RARα–induced nuclear body disruption is due to impaired PML oligomerization.

RARα and PML-RARα are phosphorylated by the cAMP/PKA pathway at a site located within the ligand-binding domain (29, 30). The PML-RARα S704D mutant, which is expected to simulate phosphorylated PML-RARα, did not block PML oligomerization and did not disrupt nuclear bodies. Moreover forskolin restored nuclear bodies disrupted in U2OS cells expressing wild-type PML-RARα (Fig. 3B and D), in mouse myeloid stem/progenitor cells expressing wild-type PML-RARα (Fig. 4E), or in APL-derived NB4 cells (Fig. 6D). Our results indicate that the ligand-binding domain of PML-RARα is key to the inhibition of PML oligomerization and to the disruption of PML nuclear bodies, and that PKA-dependent phosphorylation of the serine residue in that region reverses those effects and restores nuclear body formation. Phosphorylated PML-RARα is known to be easily degraded by ATRA (30); however, in the absence of ATRA, phosphorylated PML-RARα was stable (Figs. 4D, 5A and B, and Supplementary Fig. S6C). The mechanism by which phosphorylation of PML-RARα prevents its inhibition of PML oligomerization remains unclear. However, there are precedents for this; the phosphorylation of RARα by PKA enhances the interaction between RARα and cyclin H/cdk2 (29), suggesting that phosphorylation of RARα increases its interaction with cyclin H/cdk2. Thus, PML might find it easier to access a complex of phosphorylated PML-PML-RARα than a complex of dephosphorylated PML-PML-RARα.

Nuclear body restoration is important for differentiation of APL cells

The PKA phosphorylation site of PML-RARα regulates nuclear body formation. Forskolin restored nuclear bodies in

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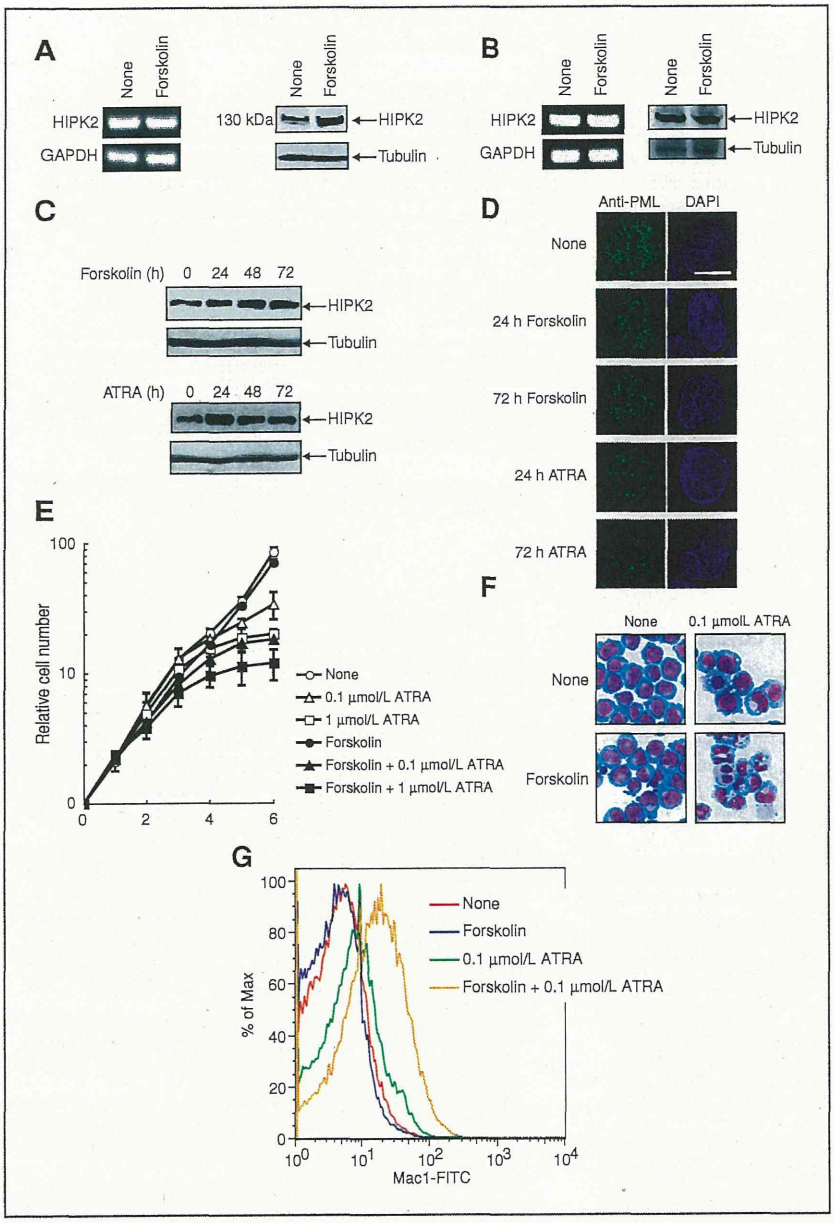

Figure 6. Nuclear body restoration promotes ATRA-induced APL cell differentiation. A, forskolin increases HIPK2 protein expression in NB4 cells. NB4 cells were treated with forskolin for 72 hours. The expression of HIPK2 and tubulin was analyzed by immunoblotting (right) using anti-HIPK2 and anti-tubulin antibodies, respectively. B, forskolin does not increase HIPK2 protein expression in K562 cells. The expression of HIPK2 and tubulin in K562 cells was analyzed as described in A. C, effect of forskolin or ATRA on HIPK2 expression. NB4 cells were treated with forskolin or ATRA for 24, 48, and 72 hours and were harvested. The lysates were analyzed as described in A. D, effect of forskolin or ATRA on nuclear body restoration. NB4 cells were exposed to forskolin or ATRA for 24 or 72 hours and stained with anti-PML antibody. The white bar represents 10 μm. E, growth curve of NB4 cells exposed to ATRA and/or forskolin. Cells were counted every other day for 6 days. Values were normalized to the value obtained at the zero time point. Values represent the mean ± SEM of 4 independent experiments. F, forskolin increases ATRA-induced NB4 cell differentiation. NB4 cells were stained with May–Giemsa stain 5 days after adding ATRA and/or forskolin. G, the combination of ATRA and forskolin increases the expression of Mac-1 in NB4 cells. NB4 cells were treated with ATRA and/or forskolin for 5 days, and incubated with anti-Mac-1-FITC. The cells were analyzed by flow cytometry.

APL-derived NB4 cells (Fig. 6D). Although forskolin alone was not sufficient to induce NB4 cell differentiation, it promoted ATRA-induced differentiation (Fig. 6G). Published studies showed that cAMP enhances retinoic acid-induced APL differentiation and PML-RARα transactivation (32–35). These studies also showed that PKA dissociates RARα from SMRT and activates transcription. Moreover, a recent report indicated that cAMP-dependent phosphorylation of PML-RARα was crucial for the eradication of APL-initiating cells (30). Taken together, these reports and the present data suggest that nuclear body restoration is one of the reasons why cAMP/PKA could be useful as an APL therapy.

We previously showed that HIPK2 is stabled in PML nuclear bodies and degraded outside of nuclear bodies by SCFFbx3, suggesting that HIPK2 is destabilized by disruption of nuclear bodies (15). In this paper, we showed that PML IV ΔCC, wild-type PML-RARα, PML-RARα 1–748, and S704A, which disrupted PML nuclear bodies, also destabilized HIPK2. In contrast, PML, PML-RARα 1–567, 1–492, 1–420, ΔE, and S704D, which did not disrupt nuclear bodies,

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did not destabilize HIPK2. Forskolin and ATRA, which restore nuclear bodies in NB4 cells, increased HIPK2 expression (Fig. 6A and C), and the increase in HIPK2 expression was correlated with nuclear body restoration (Fig. 6D). These data indicate that HIPK2 destabilization is strongly correlated with nuclear body disruption and that nuclear body formation is important for HIPK2 stabilization. HIPK2 is important for PML-dependent transcriptional activation (15). We have also found that PML stabilizes the PU.1/p300 complex to regulate PU.1-dependent transcription and myeloid differentiation (26). Mutations of HIPK2 are found in AML and myelodysplastic syndrome (36). PML-RARα also disrupts PU.1/p300 complexes and inhibits myeloid differentiation (26). Therefore, nuclear body formation by PML oligomerization may lead to the recruitment of transcription factors/coactivators and to their stabilization for transcriptional activation and regulation of granulopoiesis. As suggested by the results of the present study, this might be because cAMP/PKA-dependent nuclear body restoration enhances APL cell differentiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Shima, I. Kitabayashi
Development of methodology: Y. Shima
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Shima, Y. Honma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Shima, I. Kitabayashi
Writing, review, and/or revision of the manuscript: Y. Shima, I. Kitabayashi

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ORIGINAL ARTICLE

A novel STAT inhibitor, OPB-31121, has a significant antitumor effect on leukemia with STAT-addictive oncokinases

F Hayakawa[1], K Sugimoto[1,2], Y Harada[2], N Hashimoto[2], N Ohi[2], S Kurahashi[1] and T Naoe[1]

Signal transduction and activator of transcription (STAT) proteins are extracellular ligand-responsive transcription factors that mediate cell proliferation, apoptosis, differentiation, development and the immune response. Aberrant signals of STAT induce uncontrolled cell proliferation and apoptosis resistance and are strongly involved in cancer. STAT has been identified as a promising target for antitumor drugs, but to date most trials have not been successful. Here, we demonstrated that a novel STAT inhibitor, OPB-31121, strongly inhibited STAT3 and STAT5 phosphorylation without upstream kinase inhibition, and induced significant growth inhibition in various hematopoietic malignant cells. Investigation of various cell lines suggested that OPB-31121 is particularly effective against multiple myeloma, Burkitt lymphoma and leukemia harboring BCR–ABL, FLT3/ITD and JAK2 V617F, oncokinases with their oncogenicities dependent on STAT3/5. Using an immunodeficient mouse transplantation system, we showed the significant antitumor effect of OPB-31121 against primary human leukemia cells harboring these aberrant kinases and its safety for normal human cord blood cells. Finally, we demonstrated a model to overcome drug resistance to upstream kinase inhibitors with a STAT inhibitor. These results suggested that OPB-31121 is a promising antitumor drug. Phase I trials have been performed in Korea and Hong Kong, and a phase I/II trial is underway in Japan.

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Keywords: novel STAT inhibitor; OPB-31121

INTRODUCTION

Signal transduction and activator of transcription (STAT) proteins are extracellular ligand-responsive transcription factors that mediate a wide range of biological processes such as cell proliferation, apoptosis, differentiation, development and the immune response.[1,2] Stimulation with cytokines or growth factors results in the tyrosine phosphorylation of STAT proteins via the activation of upstream tyrosine kinases such as JAK family kinases (JFKs) and Src family kinases (SFKs).[3] Activated STAT proteins translocate to the nucleus and regulate gene expression through direct binding to the promoters of responsive genes.[4,5]

Out of 7 STAT family members, STAT3 and STAT5 are widely recognized as being master regulators of the cellular functions that lead to the cancer phenotype. Constitutive STAT3 activation is required for oncogenic transformation by oncokinases such as v-Src,[6,7] v-Eyk[8] and v-Ros.[9] In addition, constitutive STAT3 activation is associated with transformation by tumor viruses, including HTLV-1[10] and EBV.[11] Constitutive activation of STAT5 is essential for oncogenesis by the v-Abl tyrosine kinase,[12,13] BCR–ABL fusion protein,[14–16] FLT3 with internal tandem duplication (FLT3/ITD)[17,18] and JAK2 V617F mutation.[19] Moreover, a constitutive activation mutant of STAT3 or STAT5 alone is enough to induce oncogenic transformation.[20,21] These results indicate that STAT3 and STAT5 have intrinsic oncogenic potential and are strongly associated with cancer development.

Considering the strong association of STAT signaling with cancer development and the observed constitutive activation of STAT3/5 in various cancers, STAT3/5 have been identified as promising targets for antitumor drugs; however, to date most trials to block STAT signaling have not been fully successful.[22] Many trials aimed to inhibit upstream kinases such as JAK2; however, specific JAK2 inhibition was overcome by alternative activation of other JFKs.[23] Several JFK inhibitors are under development, but no significant clinical effect has been achieved. Other approaches that directly inhibit STAT function, such as STAT dimerization inhibitors and STAT phosphorylation inhibitors, are under development, but none has undergone a clinical trial yet.

Here, we demonstrated that a novel STAT3 inhibitor, OPB-31121, strongly inhibited not only STAT3 but also STAT5 phosphorylation. OPB-31121 did not inhibit activities of kinases including JFKs and SFKs and its exact mechanism of action is under investigation; however, it induced significant growth inhibition in a wide range of hematopoietic malignant cells. Investigation among various cell lines indicated that this compound was particularly effective against multiple myeloma and Burkitt lymphoma, and leukemia harboring BCR–ABL, FLT3/ITD and JAK2 V617F, oncogenic kinases with their oncogenicities dependent on STAT3/5. Using an immunodeficient mouse transplantation system, we also showed the significant antitumor effect of this compound against primary human leukemia cells harboring these aberrant kinases and its safety for normal human cord blood cells. Finally, we demonstrated a model to overcome drug resistance to upstream kinase inhibitors with a STAT inhibitor. These results suggested that OPB-31121 is a promising antitumor drug. Phase I trials have been performed in Korea (NCT00955812) and Hong Kong (NCT00511082), and a phase I/II trial is underway in Japan (NCT1406574).

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MATERIALS AND METHODS

Cells and reagents

TCC-Y/sr was described previously.[24] OCI-Ly1, OCI-Ly3, OCI-Ly7 and OCI-Ly10 were kind gifts from Dr K Takeyama (Dana-Farber Cancer Institute, MA, USA) and were cultured in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum. Other cells were purchased from the American Type of Culture Collection (ATCC, Rockville, MD, USA) and cultured according to the recommendation of ATCC. Sunitinib was purchased from Wako Chemicals (Osaka, Japan). OPB-31121 was described previously[25] and provided by Otsuka Pharmaceuticals Co. Ltd. (Tokushima, Japan).

Antibodies

The following antibodies were purchased from Cell Signaling Inc. (Beverly, MA, USA): anti-phospho-STAT5 (Y694) antibody, anti-phospho-JAK2 antibody, anti-phospho-Src antibody, anti-phospho-Akt antibody, anti-phospho-MAPK p44/p42 antibody, anti-phospho-NFκB antibody, anti-STAT3 antibody, anti-STAT5 antibody, anti-JAK2 antibody, anti-Src antibody and anti-IKBα antibody. Anti-phospho-STAT3 (Y705) antibody was obtained from EPIT MICS (Burlingame, CA, USA). Anti-human CD45 antibody and anti-mouse CD45 antibody were from Becton Dickinson (San Jose, CA, USA).

Cell proliferation assay

Cell proliferation was analyzed by the MTT assay using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) or TetraColor One (Seikagaku Co., Tokyo, Japan) according to the manufacturer's instructions.

Immunohistochemistry, immunoblotting, immunofluorescence and flow cytometry

These were performed as described previously.[26,27]

Subcutaneous xenotransplantation of cell lines into SCID mice

This was performed as described previously[28] except that 8-week-old male SCID mice purchased from Clea Japan (Tokyo, Japan) were used. Tumor size was monitored twice a week. Tumor volume was calculated using the following formula: Tumor volume (mm^3) = ($d^2 \times D$)/2, where D (mm) and d (mm) are the longest and shortest diameters of the tumor, respectively.

Primary leukemia cell xenotransplantation into NOD/SCID/IL2-Rγc$^{-/-}$ (NOG) mice

Primary leukemia cells from patients were collected after obtaining written informed consent, preserved and transplanted into NOG mice as described previously.[27] Patients' characteristics are shown in Supplementary Table 2.

Human cord blood cells

Human cord blood cells were obtained from RIKEN BRC (Tsukuba, Japan). The use of human cord blood cells in this study was permitted by the ethics committee of Nagoya University Graduate School of Medicine.

RESULTS

OPB-31121 selectively inhibits STAT phosphorylation without upstream kinase inhibition

OPB-31121 was identified by Otsuka pharmaceuticals Co. Ltd. as a chemical that induced strong growth inhibition of various kinds of tumor cell lines. It was reported that OPB-31121 inhibited growth of gastric cancer cell lines and phosphorylation of STAT1, STAT3 and STAT5 in those cell lines; however, the exact mechanism of action is yet to be clarified.[25] In the phase I study performed in Korea, 21 patients with advanced solid tumor were enrolled. The most common toxicities were nausea, vomiting, diarrhea, fatigue and anorexia. Those were predominantly grade 1 or grade 2.[29]

We first analyzed the signal transduction pathway inhibited by OPB-31121. Four major growth signal components, STAT3, ERK1/2, Akt and NFk-B, were analyzed. Among them, tyrosine phosphorylation of STAT3 was selectively inhibited by this compound (Figure 1a). Inhibition of STAT3 nuclear translocation by this compound was investigated by immunofluorescent staining. Inhibition of STAT3 nuclear translocation was observed by immunofluorescent staining with anti-STAT3 antibody (Figure 1b, upper-right corner panel of left panels). On the other hand, residually phosphorylated STAT3 completely translocated to the nucleus under OPB-31121 treatment (Figure 1b, upper-right corner panel of right panels), indicating that this compound did not inhibit nuclear translocation of phosphorylated STAT3. Observed inhibition of nuclear translocation seemed to be the consequence of the inhibition of STAT3 phosphorylation by this compound.

Next, we examined whether OPB-31121 inhibited the upstream kinases of STAT. In Hep G2 cells, JAK2 phosphorylation was induced by IL-6 stimulation and was not inhibited by this compound, whereas STAT3 phosphorylation was strongly inhibited (Figure 2a). In HEL cells with active mutation of JAK2, phosphorylation of STAT3 and STAT5 was inhibited at early time points when JAK2 phosphorylation was not inhibited, although phosphorylated JAK2 was reduced 24 h after OPB-31121 administration, probably due to cell death-related degradation of JAK2 (Figure 2b). In H1650 cells, where mutated epidermal growth factor receptor (EGFR) constitutively activated STAT3 via SFKs, this compound reduced STAT3 phosphorylation without reduction of SFK phosphorylation, indicating that this compound could inhibit STAT3 phosphorylation independently of the type of upstream kinases (Figure 2c). These results strongly suggested that this compound was not an inhibitor of upstream kinases such as JFKs and SFKs. Consistent with this, *in vitro* screening of kinase inhibitory activity demonstrated that this compound had almost no kinase inhibitory activity against any of the 31 kinases examined (Supplementary Table S1). To further investigate whether STAT was directly inhibited by this compound, we set up *in vitro* kination assays using STAT3 immunoprecipitated from cells as a substrate and recombinant JAK2 or Lyn as a kinase, and examined whether this compound could inhibit STAT3 phosphorylation *in vitro*; however, this compound did not inhibit STAT3 phosphorylation *in vitro*, suggesting that another cellular protein was required for this compound to inhibit STAT phosphorylation (Supplementary Figure S1).

OPB-31121 had a strong growth inhibitory effect against a wide range of hematopoietic malignant cells

We next investigated the effect of OPB-31121 on the cell growth of various hematopoietic cell lines. Among 35 hematopoietic cell lines, IC_{50} of OPB-31121 was $\leqslant$10 nM in 20 cell lines (57%), but $>$100 nM in 8 cell lines (23%, Table 1). Multiple myeloma, Burkitt's lymphoma and chronic myeloid leukemia (CML) seemed to be generally sensitive to this compound. Concerning to other leukemias and lymphomas, all disease types contained both sensitive and insensitive cell lines. Sensitivity to this compound was independent of the strength of STAT3/5 phosphorylation detected by immunoblotting (data not shown); however, looking at gene aberrations, leukemia cells harboring gene aberrations such as BCR–ABL, FLT3/ITD and JAK2 V617F were all sensitive to this compound (Table 2). It has been established that these three mutated kinases are oncokinases and cause constitutive activation of STAT3 and/or STAT5, and that the oncogenicities of these kinases depend on STAT3/5 signal.[17,30,31] We designated these oncokinases as STAT-addictive oncokinases (SAO).

We further investigated the growth inhibitory effect of OPB-31121 against tumors of cell lines inoculated into SCID mice. Orally administered OPB-31121 suppressed the tumor growth of HEL cells significantly in mice, and consistently, STAT3 phosphorylation was strongly inhibited in the tumors (Figures 3a and b). We further examined using OPB-31121-sensitive cell lines containing myeloma and SAO-positive leukemia cell lines. OPB-31121 demonstrated significant tumor growth suppression or even regression in

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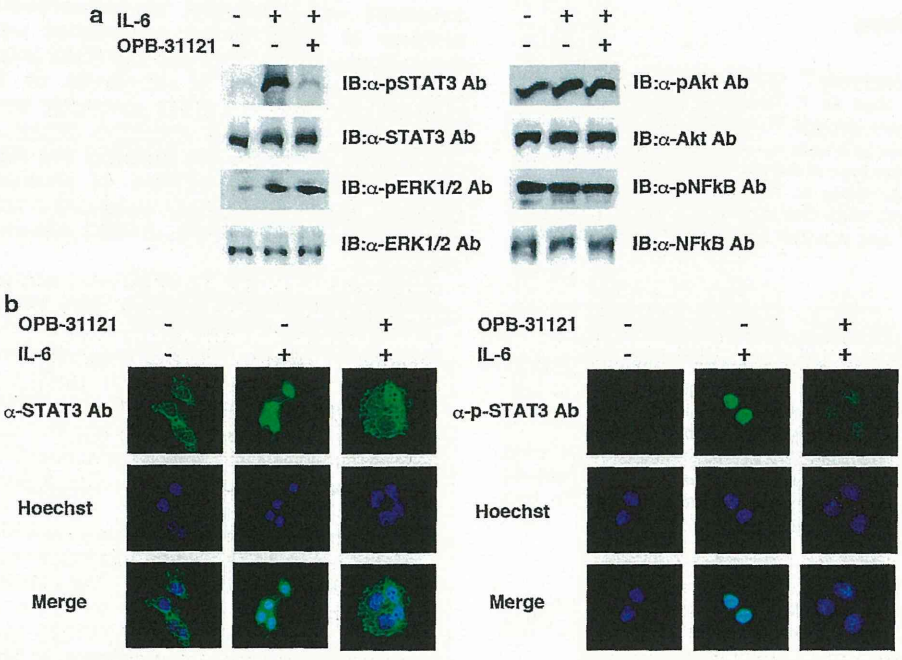

Figure 1. OPB-31121 selectively inhibited STAT. (**a**) Selective inhibition of STAT. Hep G2 cells were treated with or without 100 nM OPB-31121 for 4 h and stimulated with or without 100 ng/ml IL-6 for 10 min as indicated. Then, cells were lysed and subjected to immunoblotting (IB) with the indicated antibodies. (**b**) OPB-31121 did not inhibit nuclear translocation of phosphorylated STAT3. Hep G2 cells were treated with OPB-31121 and IL-6 as in (**a**). Cells were fixed and subjected to immunofluorescent staining with the indicated antibodies. Nuclear translocation of residually phosphorylated STAT3 was not inhibited on immunofluorescence by anti-phospho-STAT3 antibody (upper-right corner panel).

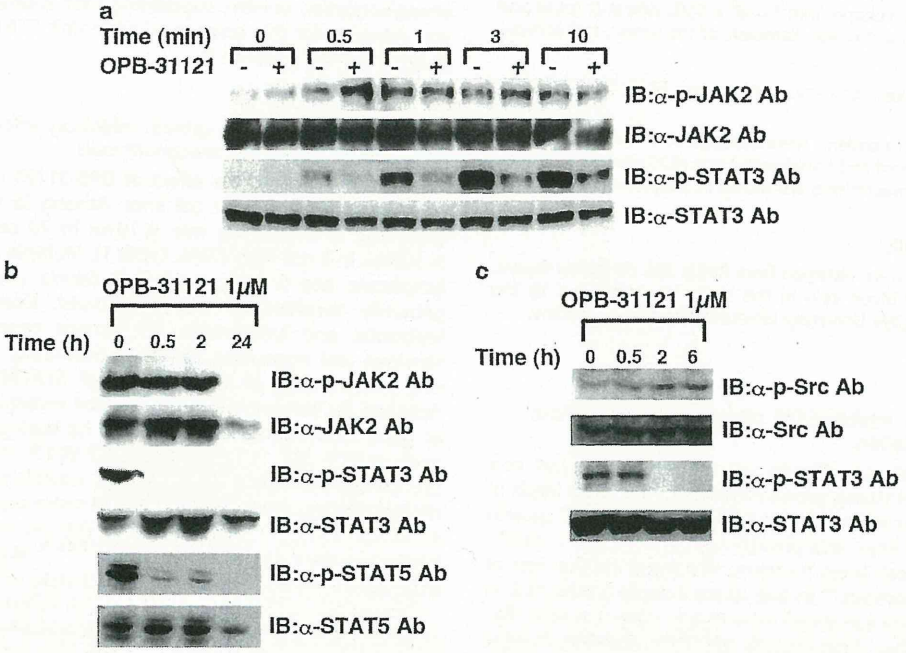

Figure 2. OPB-31121 inhibited STAT3 and STAT5 phosphorylation without upstream kinase inhibition. (**a**) OPB-31121 inhibited IL-6-induced STAT3 phosphorylation without JAK2 inhibition. Hep G2 cells were treated with OPB-31121 and IL-6, as described in Figure 1a, except that the concentration of OPB-31121 was 1 μM. Cells were lysed at the indicated time after IL-6 stimulation and subjected to IB with the indicated antibodies. (**b**) OPB-31121 inhibited constitutive activation of STAT3 and STAT5 without JAK2 inhibition. HEL cells were lysed at the indicated time after 1 μM OPB-31121 treatment and subjected to IB with the indicated antibodies. (**c**) OPB-31121 inhibited constitutively activated STAT3 without Src inhibition. H1650 cells were analyzed as in (**b**).

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Table 1. List of IC_{50} of OPB-31121 and gene aberrations in various cell lines

Disease	*Cell line*	*IC_{50}* (nM)	*Fusion gene*	*Gene mutation*	*Gene deletion*
AML	KG-1	0.3		NRAS, p53	
	U937	3.2	CALM-AF10	p53	
	MV4-11	4.0	MLL-AF4	FLT3	
	HEL	9.5		JAK2	CDKN2A , CDKN2B
	MOLM13	10.0	MLL-AF9	FLT3	CDKN2A , CDKN2B
	NB4	19.0	PML-RARα		CDKN2A , CDKN2B
	HL-60	95.0		p53	
	UT-7	95.0		p53	CDKN2B
	THP-1	>100	MLL-AF9	NRAS, p53	CDKN2A , CDKN2B
	Kasumi-1	>100	AML1-ETO		
CML	KU812	0.6	BCR–ABL	p53	
	K562	21.0	BCR–ABL	p53	CDKN2A , CDKN2B
B-ALL	ALL-1	2.5	BCR–ABL		
	TCC-Y/sr	24.7	BCR–ABL T315I		
	BALL-1	>100	IgH-Myc		CDKN2B
	RS4;11	>100	MLL-AF4		CDKN2A , CDKN2B
B-lymphoblast	CRL8062	7.0			
T-ALL	Jurkat	5.6		p53	CDKN2A , CDKN2B, IFNA1
	CCRF-CEM	70.0	SIL-SCL	p53	CDKN2B, IFNA1, IFNB1
	MOLT-4	>100		NRAS	CDKN2B
DLBCL	OCI-Ly10	2.1			
	RL	2.9	IgH-BCL2	p53	
	OCI-Ly3	4.0			
	OCI-Ly7	7.8			
	RC-K8	7.9			
	IM-9	10.9			
	OCI.LY1	>100	IgH-BCL1		
	SU-DHL4	>100	IgH-BCL2	p53	
	WILL2	>100	Igλ-Myc, IgH-BCL2		
BL	Ramos	2.4		p53	
	Raji	3.1	IgH-Myc	p53	
	Daudi	4.5	IgH-Myc	p53	
MM	U266	3.9		p53	
	LICR-LON-Hmy2	6.0			
	WI-L2-729HF2	7.0		p53	

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; BL, Burkitt's lymphoma; CML, chronic myeloid leukemia; DLBCL, diffuse large B cell lymphoma; MM, multiple myeloma.

mice inoculated with these cell lines (Figure 3c). These results indicated that this compound was effective against myeloma and SAO-positive leukemia *in vivo*.

OPB-31121 caused strong growth inhibition of primary SAO-positive leukemia cells but did not affect the growth of normal cord blood cells in mice

For further analyses, we used a primary leukemia cell xenotransplantation system in which primary human leukemia cells were maintained by serial transplantation into NOG mice. In this system, we could test the drug effect on primary leukemia cells, whose phenotype and heterogeneity mainly maintained their original status.[27] Oral administration of OPB-31121 induced significant reduction of the leukemia cell rate in mice transplanted with primary acute myeloid leukemia cells with FLT3/ITD, whereas cytarabine treatment hardly affected it. The treated group/control group (T/C) tumor cell rate were 15.9 and 102.1%, respectively (Figure 4a). Analyzing in detail, cytarabine reduced the number of both human leukemia cells and mouse normal hematopoietic cells, so the rate of leukemia cells did not change by this treatment. On the other hand, OPB-31121 treatment induced selective leukemia cell reduction and the recovery of mouse hematopoietic cells (Figure 4b). These could be clearly observed by immunohistochemistry of bone marrow (Figure 4c). Representative images of flow cytometric analysis of the leukemia cell rate and larger images of the immunohistochemistry of bone marrow are shown in Supplementary Figure S2A and B.

We examined the effect of this compound on other primary leukemia cells. This compound induced significant reduction of the leukemia cell rate of another FLT3/ITD-positive AML (T/C: 26.3%), three BCR–ABL -positive ALL (T/C: 4–58%) and one CML blast crisis (BC) with T315I mutation in BCR–ABL (T/C: 87%, Figure 4g). The effect of OPB-31121 on the CML BC sample was relatively weak, when drug administration was started after leukemia engraftment; however, starting administration earlier, this compound showed stronger tumor growth inhibition (T/C: 15.3%, Figure 4g) and a significant survival benefit (Figure 4d). These results further confirmed the effectiveness of this compound on SAO-positive leukemia and suggested that the growth inhibitory effect of this compound was selective to tumor cells.

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Table 2. Comparison of OPB-31121 effect between STAT-addictive oncokinase-positive and unknown leukemia

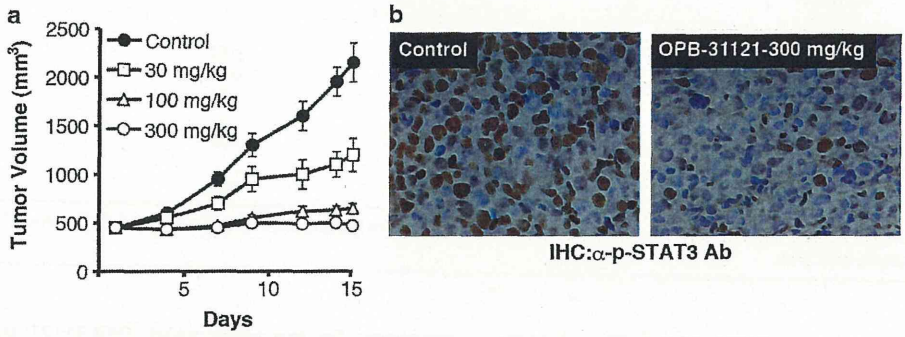
Disease	*Cell line*	*SAO*	*IC_{50} (nM)*
AML	MV 4-11	FLT3/ITD	4.0
	MOLM13	FLT3/ITD	10.0
	HEL	JAK2 V617F	9.5
CML	KU812	BCR–ABL	0.6
	K562	BCR–ABL	21.0
B-ALL	ALL-1	BCR–ABL	2.5
	TCC-Y/sr	BCR–ABL T315I	24.7
AML	KG-1	Unknown	0.3
	U937	Unknown	3.2
	NB4	Unknown	19.0
	HL-60	Unknown	95.0
	UT-7	Unknown	95.0
	THP-1	Unknown	>100
	Kasumi	Unknown	>100
B-ALL	BALL-1	Unknown	>100
	RS4;11	Unknown	>100
T-ALL	Jurkat	Unknown	5.6
	CCRF-CEM	Unknown	70.0
	MOLT-4	Unknown	>100

Abbreviation: SAO, STAT-addictive oncokinase.
IC50 (nM) ⩽ 10
IC50 (nM) > 10 to ⩽ 100
IC50 (nM) > 100.

To further confirm the safety of this compound for normal hematopoiesis, we performed colony formation assay using $CD34^+$ human cord blood cells. OPB-31121 did not significantly affect the number and composition of colonies (Supplementary Figure S3A). Furthermore, we used NOG mice transplanted with healthy human cord blood cells. OPB-31121 administration did not affect the percentage of human $CD45^+$ cells (T/C: 99%, Figures 4e–g). The rate of stem cell fraction ($CD34^+/CD38^-$), *monocytic progenitor fraction* ($CD13^+/CD14^-$) and granulocytic progenitor fraction ($CD13^+/CD14^+$) in $CD45^+$ cells were not significantly changed by this compound (Supplementary Figure S3B). These results indicated the safety of this compound for normal hematopoiesis. Taken together, OPB-31121 is a promising antitumor drug for Burkitt's lymphoma, multiple myeloma and SAO-positive leukemia.

OPB-31121 overcame autocrine-induced FLT3 inhibitor resistance by STAT signal inhibition

T315I mutation in BCR–ABL causes strong ABL kinase inhibitor resistance in cells. OPB-31121 conquered this mutation-induced kinase inhibitor resistance in TCC-Y/sr cells (Table 2 and Figure 3c) and primary CML cells (Figures 4d and g); therefore, we tried to see whether OPB-31121 overcame another type of kinase inhibitor resistance by inhibition of the downstream signal. According to the previous report, a FLT3 inhibitor-resistant subclone of MV4-11, the FLT3/ITD-positive AML cell line, expressed FLT3 ligand. This autocrine signaling enhanced not only STAT5 phosphorylation but also STAT3 phosphorylation. Additional STAT3 phosphorylation seemed to have an important role in FLT3 inhibitor resistance.[32] We therefore examined whether OPB-31121 overcame FLT3 ligand-induced FLT3 inhibitor resistance. Consistent with the previous report, under culture with FLT3 ligand, STAT3 phosphorylation became resistant to sunitinib in MOLM13 cells,



c

Summary of OPB-31121 effect on subcutaneous tumors of cell line in mice

Disease	Cell line	Dose (mg/kg)	T/C (%)
SAO-positive leukemia	HEL	300	21.9
	KU812	100	1.8
	TCCy/sr	100	5.9
SAO-unknown leukemia	KG-1	100	0.4
	U937	300	36.4
Multiple myeloma	WI-L2-729HF2	300	6.9
	RPMI8226	300	21.6
Lymphoma	RL	200	18.5

SAO: STAT-addictive oncokinase
T/C: Tumor volume of treated group/Tumor volume of control group

Figure 3. OPB-31121 inhibited STAT3 phosphorylation and growth of subcutaneous tumors of cell lines in mice. (**a**) Dose-dependent tumor growth suppression in mice. HEL cells (4×10^7 cells/body) were subcutaneously inoculated into SCID mice. Oral administration of OPB-31121 or 5% gum arabic for control was started 14 days after inoculation when tumors had developed detectably. The average tumor volume of five mice was plotted with standard deviation. (**b**) STAT3 inhibition by OPB-31121 in mouse tumor. HEL cells were inoculated into SCID mice as in (**a**). After tumor development, 300 mg/kg OPB-31121 or 5% gum arabic was administered daily for 3 days. On the 4th day, tumors were resected and subjected to immunohistochemistry with anti-phospho-STAT3 antibody. (**c**) Summary of OPB-31121 effect on cell line tumors in mice.

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