distribution between two groups using Prism 5 (GraphPad Software Inc., La Jolla, CA). For all analyses, the *P* values were two-tailed, and a P value < 0.05 was considered significant.

3. Results

We identified two *GATA2* gene mutations. One mutation (p.R308P-GATA2) was a p.Arg308Pro substitution within the N-terminal zinc finger (ZF)-1 domain in a FAB M6 patient (Fig. 1A and B). The other mutation (p.A350_N351ins8-GATA2) was a 24-nucleotide insertion resulting in an eight-amino-acid insertion between A350 and N351 residues within the C-terminal ZF-2 domain in a FAB M1 patient. This insertion mutation consisted of a one nucleotide insertion and a 23-nucleotide corresponding to N337-R344 residues duplication (Fig. 1A and C). In both patients, each *GATA2* mutation was heterozygous. Unfortunately, we could not analyze germ-line sequence of both patients because of the lack of appropriate material. Therefore, we could not completely determine whether identified mutations were somatic or germ-line mutations.

Both patients revealed normal karyotype and no mutation in *FLT3*, *KIT*, *NRAS*, *TP53*, *NPM1*, *CEBPA*, *RUNX1*, *MLL-PTD* and *IDH1/2* genes.

The expression ratios of Wt- and Mt-GATA2 transcripts of AML cells harboring the p.A350_N351ins8 mutation were semi-quantitatively examined using a gene scanning system with fluorescent-labeled PCR product. Although the p.A350_N351ins8 mutation was heterozygous, little expression of Wt-GATA transcript was observed in the primary AML cells (Fig. 1D).

We transiently expressed Wt- and mutant-GATA2 in 293T cells, and nuclear lysates were extracted 48 h later. In EMSA using an oligonucleotide containing GATA recognition sites, DNA-binding activity of p.A350_N351ins8-GATA2 was reduced to about 40% of that of Wt-GATA2, although that of p.R308P-GATA2 was almost the same as that of Wt-GATA2 (Fig. 2A). Consistent with the DNA-binding activity, the transcriptional activity of p.A350_N351ins8-GATA2 was significantly lower than that of Wt-GATA2 ($P=0.006$), while that of p.R308P-GATA2 was not affected ($P=0.11$) (Fig. 2B). Upon co-expression of Wt-GATA2 with p.A350_N351ins8-GATA2 at a 1:1 ratio, the transcriptional activity of Wt-GATA2 was not affected by p.A350_N351ins8-GATA, indicating that p.A350_N351ins8-GATA2 did not have a dominant-negative effect over Wt-GATA2 (Fig. 2C).

We established Wt- and two Mt- (p.R308P and p.A350_N351ins8)-GATA2-expressing 32D cells to analyze the cellular effects of Mt-GATA2 (Fig. 3A). Immunofluorescence analysis revealed that Mt-GATA2 showed similar localization in the nucleus as Wt-GATA2 (Fig. 3B). Both Wt- and mutant-GATA2-expressing 32D cells did not show autonomous proliferation without the presence of IL3. Furthermore, there was no difference of proliferation abilities at lower concentrations of IL3 between them, indicating that mutant GATA2 did not provide a growth advantage (Fig. 3C). Since mutant GATA2 did not affect the proliferation ability, we examined the G-CSF-mediated granulocytic differentiation in Wt- and Mt-GATA2-expressing 32D cells. Mock- and Wt-GATA2-expressing 32D cells were differentiated to mature neutrophils in the culture with G-CSF for 10 days, while Wt-GATA2 overexpression moderately impaired the granulocytic differentiation. In p.R308P-GATA2-expressing 32D cells, mature neutrophil counts and promyelocyte to neutrophil counts were significantly lower than those of Wt-GATA2-expressing 32D cells after the 10-day culture ($P=0.020$ and $P=0.003$, respectively), while promyelocyte to neutrophil counts were the same after the 14-day culture. However, in p.A350_N351ins8-GATA2-expressing

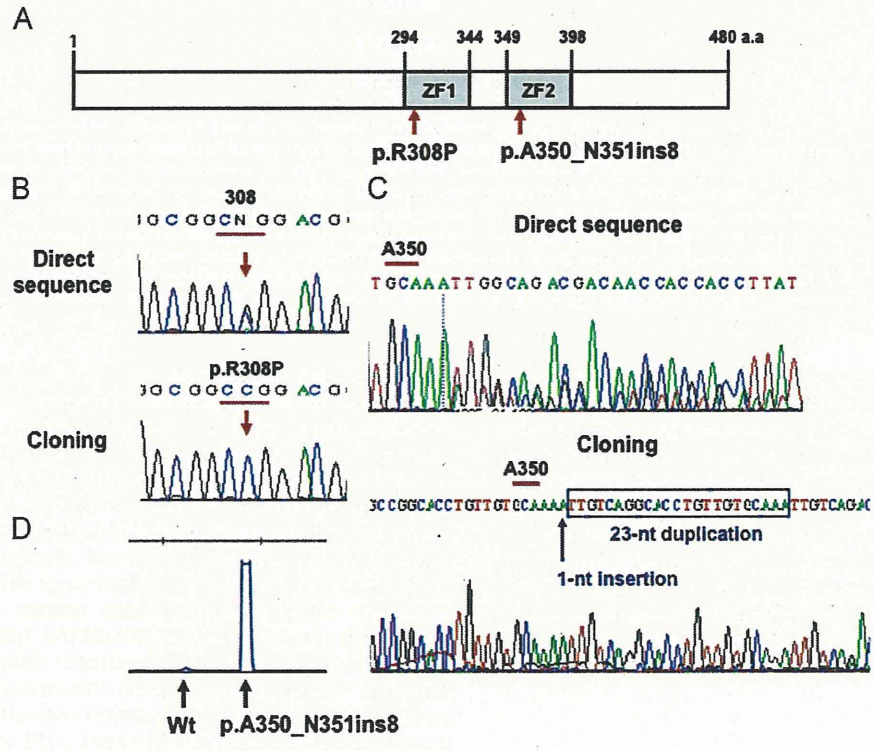

Fig. 1. GATA2 mutations identified in AML. (A) Two novel GATA2 mutations that were identified are shown in the domain structure of GATA2. Two zinc finger domains are indicated by ZF-1 and ZF-2. (B) The sequence diagram of p.R308P mutation. The upper and lower panels show the direct-sequence result and the sequence result after cloning procedure, respectively. (C) The sequence diagram of p.A350_N351ins8 mutation. The upper and lower panels show the direct-sequence result and the sequence result after cloning procedure, respectively. (D) Expression ratio of Wt- and p.A350_N351ins8-GATA2 transcripts of primary AML cells. Little expression of Wt-GATA transcript was observed in the primary AML cells.

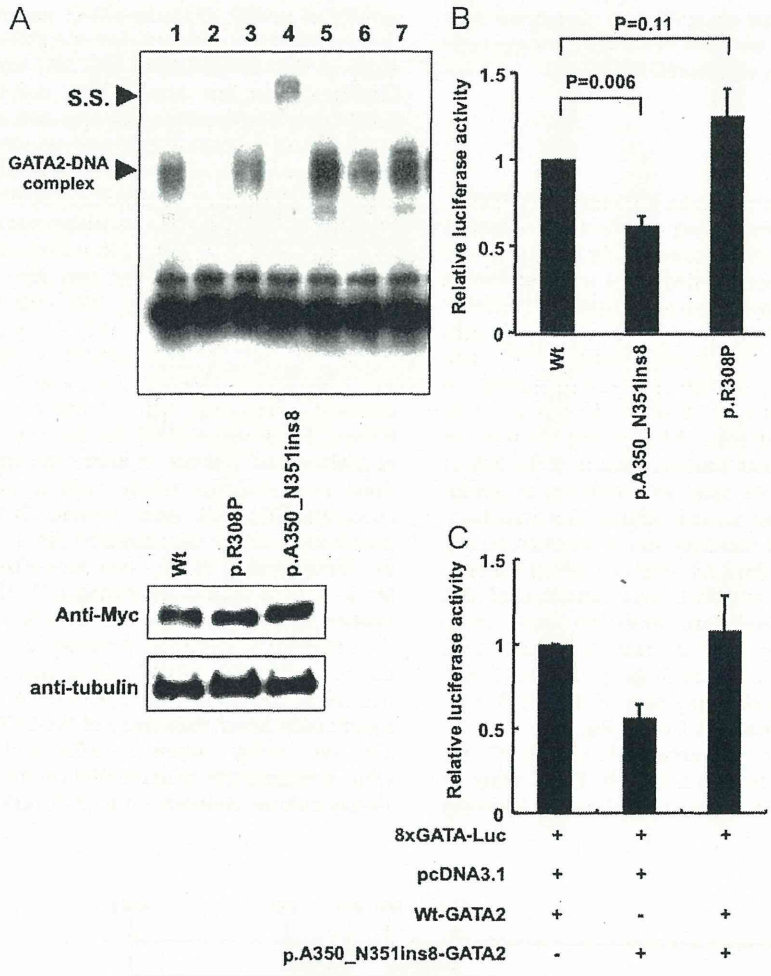

Fig. 2. DNA-binding and transcriptional activities of mutant GATA2. (A) The DNA-binding activities of Wt- and Mt-GATA2 were evaluated by EMSA using an oligonucleotide containing GATA recognition sites. Wt-GATA2 showed the GATA2-DNA complex band (lanes 1 and 5). This band was competed by a 200-fold excess of the unlabeled oligonucleotide (lane 2), and was super-shifted by the addition of anti-GATA2 antibody (lane 4), but not control IgG (lane 3). DNA-binding activity of p.A350_N351ins8-GATA2 was reduced to about 40% of that of Wt-GATA2 (lane 6). In contrast, p.R308P-GATA2 showed almost the same DNA-binding activity as Wt-GATA2 (lane 7). Lower panel shows expression levels of Wt- and Mt-GATA2 proteins in 293 T cells. (B) Transcription activities of Wt- and Mt-GATA2 were evaluated by luciferase reporter assay. 293 T cells were transfected with Wt- or Mt-GATA2 cloned pcDNA3.1 vectors together with a luciferase reporter plasmid containing eight GATA consensus motifs. The transcription activity of p.A350_N351ins8-GATA2 was significantly lower than that of Wt-GATA2 ($P=0.006$), while that of p.R308P-GATA2 was not affected. Mean ± SEM of three independent analyses are shown. (C) Upon co-expression of Wt-GATA2 with p.A350_N351ins8-GATA2 at a 1:1 ratio, the transcriptional activity of Wt-GATA2 was not affected by p.A350_N351ins8-GATA2, indicating that p.A350_N351ins8-GATA2 did not have a dominant-negative effect over Wt-GATA2. Mean ± SEM of three independent analyses are shown.

32D cells, mature neutrophil counts and promyelocyte to neutrophil counts were significantly lower than those of Wt-GATA2-expressing 32D cells both after the 10-day and the 14-day cultures ($P=0.016$ and $P=0.0005$ after 10 days, and $P=0.009$ and $P=0.007$ after 14 days, respectively), indicating that p.A350_N351ins8-GATA2 impaired the G-CSF-mediated granulocytic differentiation (Fig. 4A and B). These morphological results were confirmed by the surface expression of CD11b after G-CSF stimulation (Fig. 4C). CD11b expression levels of p.R308P-GATA2-expressing 32D cells were the same as Wt-GATA2-expressing 32D cells, while that of p.A350_N351ins8-GATA2-expressing 32D cells was significantly lower than those of Wt-GATA2-expressing 32D cells on day 14 ($P=0.04$).

4. Discussion

In this study, we identified two novel *GATA2* gene mutations (p.R308P in the ZF-1 domain and p.A350_N351ins8 in the ZF-2 domain) in adult *de novo* AML patients. Most mutations in the ZF-2 domain of the *GATA2* gene are reportedly missense mutations.[8] Although three types of in-frame deletion mutations were reported, p.A350_N351ins8 mutation was firstly identified as an in-frame insertion mutation in the ZF-2 domain. L359V mutation, which was recurrently identified in CML-BC, increased transactivation activity and inhibited myelomonocytic differentiation and proliferation.[2] In contrast, T354M and T355del mutations, which were identified in families with hereditary MDS/AML, dominant-negatively reduced transactivation activity over Wt-GATA2.[3] However, it is notable that T354M-GATA2 inhibited the all-*trans* retinoic acid (ATRA)-induced granulocytic differentiation of HL-60 cells, but T355del-GATA2 did not. Consistent with the T354M mutation, p.A350_N351ins8 mutation reduced DNA-binding and transcriptional activities and impaired G-CSF-induced granulocytic differentiation of 32D cells. However, in contrast to T354M-GATA2, p.A350_N351ins8-GATA2 did not show a dominant-negative effect over Wt-GATA2 by transcriptional assay. Since p.A350_N351ins8 mutation was heterozygous in the clinical

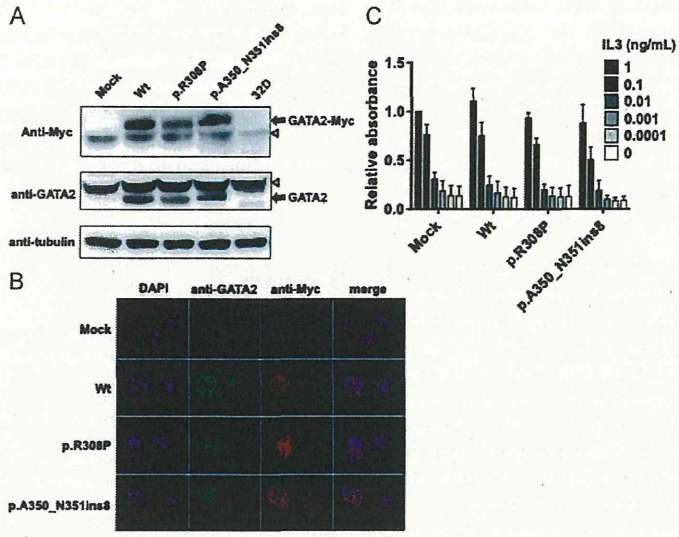

Fig. 3. Establishment of Wt- and Mt-GATA2-expressing 32D cells. (A) Stable expression of GATA2 protein in each cell line was confirmed by Western blotting using anti-Myc (upper panel) and anti-GATA2 (lower panel) antibodies. Of note is that no expression of endogenous GATA2 was observed in 32D cells. White arrowhead indicates non-specific band. (B) Immunofluorescence analysis showed the same localization of Wt- and Mt-GATA2. C. Ratio of cell viability of each GATA2-expressing 32D cell to mock-32D cells after 72-h culture at variable concentrations of IL3 is presented. Cell viability was measured using the CellTiter96 Proliferation Assay (Promega). Mean ± SEM of three independent analyses are shown. There was no significant difference of proliferation abilities between Wt- and Mt-GATA2-expressing 32D cells.

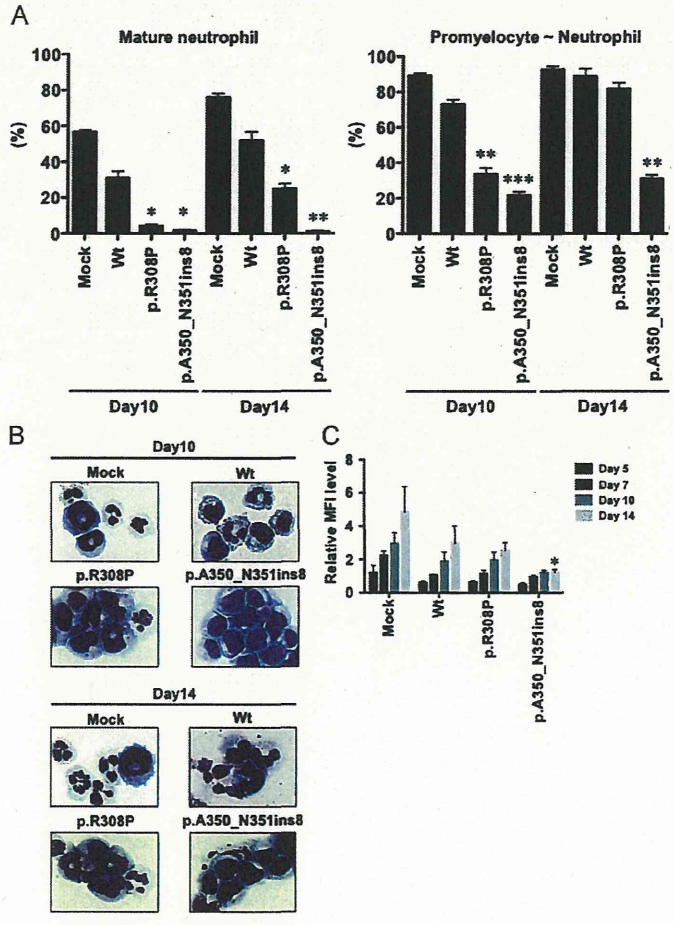

Fig. 4. G-CSF-induced granulocytic differentiation. (A) Granulocytic differentiation of G-CSF-treated 32D cells was morphologically determined. One hundred cells were counted in each experiment. Mean ± SEM of three independent analyses are shown. Mature neutrophil counts (left panel) and promyelocyte to neutrophil counts (right panel) on Day 10 and Day 14 were compared with Wt-GATA2-expressing 32D cells. (B) Morphological features of Wt- and Mt-GATA2-expressing 32D cells are shown. (C) The relative MFI level of CD11b to isotype after the G-CSF treatment in Mt-GATA2-expressing 32D cells were compared with Wt-GATA2-expressing 32D cells. Mean ± SEM of three independent analyses are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

sample, this result raised the question of how this mutation is involved in leukemogenesis, particularly in the impairment of granulocytic differentiation. In 32D cells, endogenous GATA2 expression was very faint by western blot and immunohistochemical analyses. Furthermore, Wt-GATA2 transcript was expressed little in the primary AML cells harboring p.A350_N351ins8-GATA2 mutation. These results suggested that p.A350_N351ins8-GATA2 might act as a loss of function of GATA2 in the absence of Wt-GATA2, resulting in the impairment of granulocytic differentiation.

Recently, several kinds of missense mutations within the ZF-1 domain were reported in AML.[5] Although transcriptional activities of GATA2 ZF-1 mutants were different among the mutation types, all of them reduced the capacity to enhance CEBPA-dependent activation of transcription.[5] p.R308P-GATA2 did not show significant reduction of DNA-binding and transcriptional activities, while p.R308P-GATA2-expressing 32D cells revealed the delay of G-CSF-induced granulocytic differentiation, suggesting a possible effect on maturation machinery through the reduction of CEBPA-dependent transcriptional activity.

In addition, further study is required to evaluate whether identified mutations influence post-translational modifications because they are important mechanism of transcription factors for regulating normal and leukemic hematopoiesis.[6,9–11]

Authors' contributions

K. N., Y. I., F. H., S. K. and R. K. performed experiments. K. N., H. K. and A. T. interpreted the data. H. K. and T. N. designed the study and wrote the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

All authors have no conflicts of interest to declare.

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ORIGINAL ARTICLE

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

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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

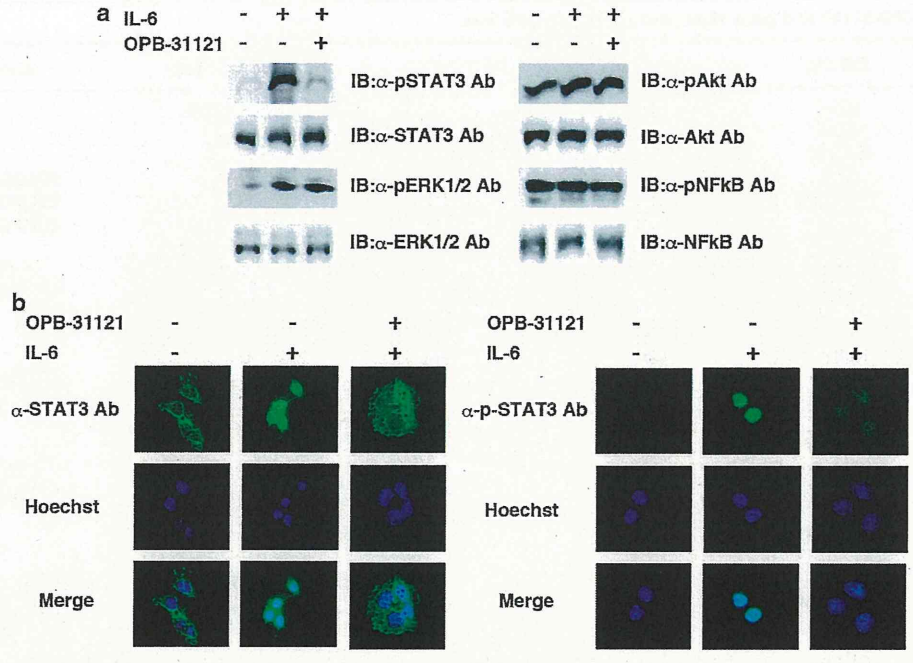


Figure 1. OPB-31121 selectively inhibited STAT. (**a**) Selective inhibition of STAT. Hep G2 cells were treated with or without 100 nM OPB-31121 for 4 h and stimulated with or without 100 ng/ml IL-6 for 10 min as indicated. Then, cells were lysed and subjected to immunoblotting (IB) with the indicated 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).

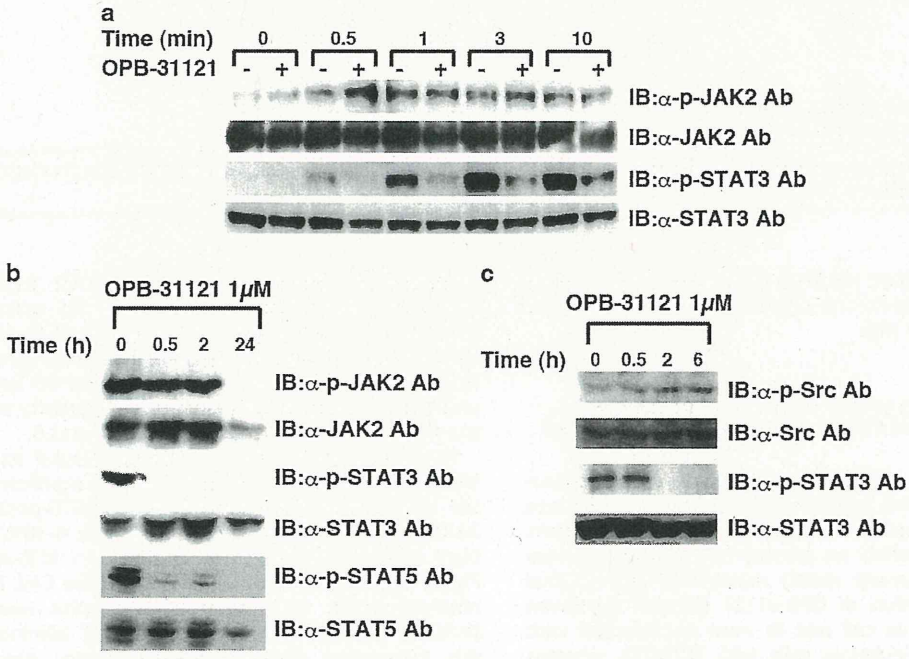


Figure 2. OPB-31121 inhibited STAT3 and STAT5 phosphorylation without upstream kinase inhibition. (**a**) OPB-31121 inhibited.IL-6-induced STAT3 phosphorylation without JAK2 inhibition. Hep G2 cells were treated with OPB-31121 and IL-6, as described in Figure 1a, except that the concentration of OPB-31121 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**).

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.

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

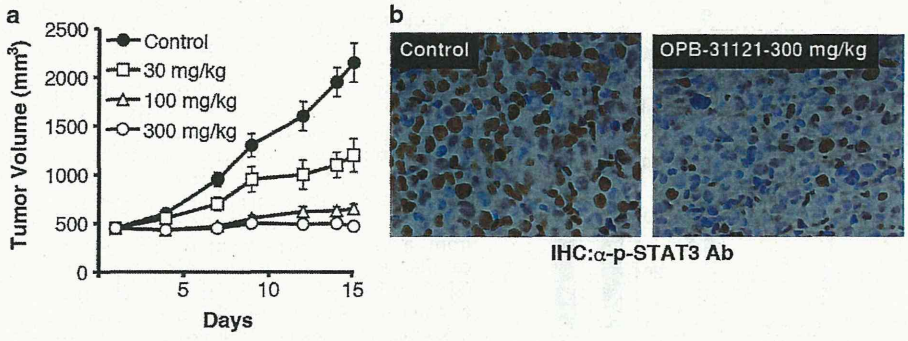
Disease	*Cell line*	*SAO*	*IC_{50} (nM)*
AML	MV 4-11	FLT3/ITD	4.0
	MOLM13	FLT3/ITD	10.0
	HEL	JAK2 V617F	9.5
CML	KU812	BCR–ABL	0.6
	K562	BCR–ABL	21.0
B-ALL	ALL-1	BCR–ABL	2.5
	TCC-Y/sr	BCR–ABL T315I	24.7
AML	KG-1	Unknown	0.3
	U937	Unknown	3.2
	NB4	Unknown	19.0
	HL-60	Unknown	95.0
	UT-7	Unknown	95.0
	THP-1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

another FLT3/ITD-positive AML cell line (top panel of Figure 5a lane 4 and 6 vs lane 1 and 3), although STAT5 phosphorylation was inhibited similarly to the condition without FLT3 ligand (third panel of Figure 5a lane 4 and 6 vs lane 1 and 3). Consistently, the sensitivity of MOLM13 cells to sunitinib fell with FLT3 ligand (Figure 5b). On the other hand, OPB-31121 induced strong inhibition of both STAT3 and STAT5 phosphorylation independently of FLT3 ligand presence (Figure 5a lane 1 and 2 vs lane 4 and 5), and the growth inhibitory effect of this compound on MOLM13 was hardly affected by the addition of FLT3 ligand (Figure 5c). These results indicated that this compound overcame FLT3 ligand-induced FLT3 inhibitor resistance.

DISCUSSION

Constitutive activation of STAT3 and STAT5 has been reported in various cancers and the inhibition of STAT signaling has been thought to be a promising strategy for cancer therapy.[33–35] Many trials using several approaches, such as small molecules including upstream kinase inhibitors, STAT dimerization inhibitors, and STAT phosphorylation inhibitors, neutralizing antibody against upstream receptors and ligands, and decoy oligonucleotides, have been performed; however, very few of them demonstrated an *in vivo* effect in mouse models and none has undergone clinical trials, except upstream kinase inhibitors such as JAK inhibitors.[22] To the best of our knowledge, OPB-31121 is the first STAT inhibitor to undergo phase I trials.

The mechanism of action of OPB-31121 has not been fully revealed. This compound did not have kinase inhibitory activity against any kinase (Supplementary Table S1) and inhibited STAT3 and STAT5 phosphorylation without inhibition of JAK2 and SFKs (Figures 2a–c). This compound did not inhibit nuclear translocation of STAT3 after it was phosphorylated (Figure 1c) and did not disrupt the dimerization of STAT3 (data not shown). In addition, this compound did not induced the protein expression of JAK-STAT pathway negative regulators such as suppressor or cytokine signaling (SOCS)3, protein inhibitor of activated Stats (PIAS)4 and LNK (data not shown). These data indicated that this compound did not inhibit the phosphorylation of upstream kinases but inhibited STAT phosphorylation, most likely by inhibiting the association of STAT with JAK or cytokine receptors; however, this

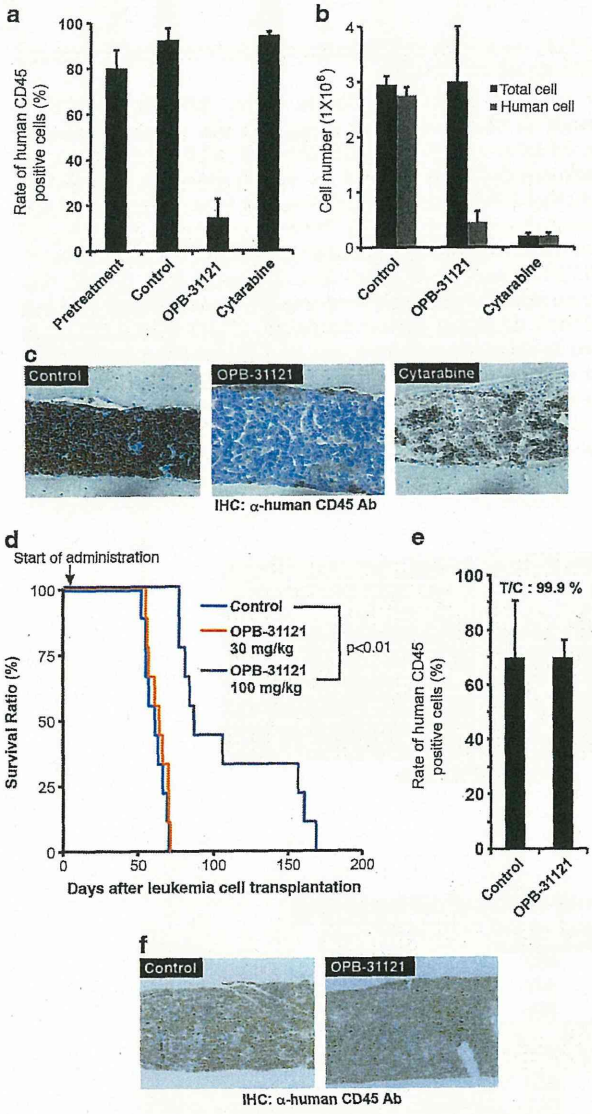


Figure 4. OPB-31121 selectively reduced human primary leukemia cells in mice. MAE cells (1×10^6), primary FLT3/ITD-positive human AML cells, were intravenously transplanted into NOG mice. On day 29, the rate of human leukemia cells in femoral bone marrow of the pretreatment mice was measured by flow cytometry using anti-human CD45 antibody, and daily oral administration of 5% gum arabic or OPB-31121 (200 mg/kg) for 10 days was started. On day 32, daily intraperitoneal injection of cytarabine (400 mg/kg) for 7 days was started. On day 38, bone marrow cells were collected from one femur and the other femur was fixed and subjected to immunohistochemistry with anti-human CD45 antibody. The rate of human leukemia cells in bone marrow was analyzed as above. (**a**) The average human leukemia cell rate of three mice was plotted on the bar chart with standard deviation. (**b**) Bone marrow cells collected from a femur were counted. The leukemia cell number was calculated by multiplying the total cell number by the human CD45-positive cell rate. The average value of three mice was plotted on the bar chart with standard deviation. OPB-31121 did not reduce the total cell number. (**c**) Immunohistochemistry of bone marrow with anti-human CD45 antibody. Cells stained in brown and blue were human CD45-positive cells (leukemia cells) and negative cells (mouse normal hematopoietic cells), respectively. Normal hematopoietic cells recovered by OPB-31121 treatment. (**d**) Survival benefit of OPB-31121 treatment in mice transplanted with primary CML BC cells harboring BCR–ABL T315I. INH cells (5×10^6), primary BCR–ABL T315I-positive CML BC cells, were intravenously transplanted into NOG mice. Daily oral administration of 5% gum arabic or the indicated dose of OPB-31121 was started on day 2 and continued until mice died. Each group consisted of nine mice. Survival curve were plotted according to the Kaplan–Meier method. Statistical difference of survival was analyzed by the log-rank test. (**e**) OPB-31121 did not affect the growth of normal hematopoietic cells. CD34-positive human cord blood cells (1×10^5) were transplanted, treated and analyzed as described above except that mice were irradiated (2.5 Gy) the day before transplantation and that OPB-31121 and gum arabic were administered daily from days 50–59. The average human cell rate of two mice was plotted on the bar chart with standard deviation. (**f**) Immunohistochemistry of bone marrow of mice transplanted with cord blood cells were performed as in (**c**). (**g**) Summary of OPB-31121 effect on primary leukemia and normal hematopoietic cells.

g Summary of OPB-31121 effect on primary leukemia and normal hematopoietic cells

Sample	Disease	SAO	Duration	Dose (mg/kg)	T/C (%)
OMR	ALL	BCR-ABL	Day 23-32	200	4
ARK	ALL	BCR-ABL	Day 18-31	300	58
KWI	ALL	BCR-ABL Y253H	Day 45-54	200	57
INH	CML BC	BCR-ABL T315I	Day 21-30	200	87
			Day 1-29	200	15.3
MAE	AML	FLT3/ITD	Day 29-38	200	15.9
MIZ	AML	FLT3/ITD	Day 40-49	200	26.3
CB	Normal	(-)	Day 50-59	200	99.9

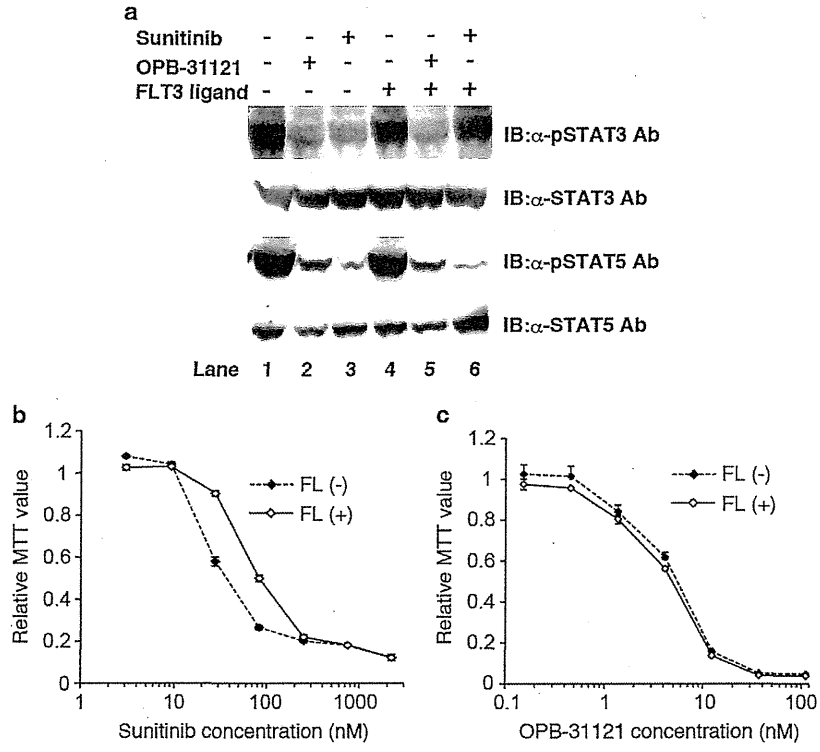


Figure 5. OPB-31121 overcame autocrine-induced FLT3 inhibitor resistance. (**a**) MOLM13 cells were treated with 100 nM sunitinib, 100 nM OPB-31121 and 100 ng/ml FLT3 ligand as indicated for 4 h. Then, cells were lysed and subjected to IB with the indicated antibodies. (**b**) FLT3 ligand reduced sunitinib sensitivity in MOLM13 cells. MOLM13 cells were cultured with the indicated dose of sunitinib with or without 100 ng/ml FLT3 ligand. Cell proliferation was analyzed by MTT assay after 48 h culture. MTT values were plotted as relative values to the value of the cells without FLT3 ligand and sunitinib. The average value of three experiments was plotted with standard deviation. (**c**) FLT3 ligand did not affect the sensitivity to OPB-31121 in MOLM13 cells. Sensitivity of OPB-31121 was measured as in (**b**) using OPB-31121 instead of sunitinib.

compound could not inhibit STAT3 phosphorylation by JAK2 and Lyn *in vitro*, suggesting that another cellular protein was required for this compound to inhibit STAT phosphorylation. This compound may indirectly bind to STAT through an unknown protein that interacts with STAT and disrupts the association of STAT with upstream kinases or receptors. We are now searching for the components of STAT complex that interact with this compound. Very recently, another group reported a decrease in the phosphorylation of STAT3 and JAK2 24 or 48 h after treatment with this compound in gastric cancer cell lines, and claimed that this compound was a JAK2 inhibitor;[25] however, we clearly demonstrated that the decrease in STAT3 phosphorylation occurred much earlier, within 2 h after treatment with this compound, and before the decrease in JAK2 and c-Src phosphorylation (Figures 2b and c). At 24 h after treatment, we also observed a decrease in JAK2 phosphorylation, probably due to the cell death response (Figure 2b). A similar phenomenon might have been observed in gastric cancer cell lines.

The sensitivity of cell lines to OPB-31121 varied markedly and was independent of the phosphorylation level of STAT (Table 1 and data not shown). This was probably because the survival dependency on STAT signaling varied among cell lines and was independent of the STAT phosphorylation level; therefore, it was difficult to predict OPB-31121-sensitive cells from the phosphorylation level of STAT. On the other hand, it is reasonable to predict a cancer to be addictive to STAT signaling, when the cancer gets addicted to an oncokinase, and the survival supporting activity of the oncokinase depend on STAT signaling. It is also difficult to clarify whether a mutated kinase observed in a cancer is really responsible for cancer cell survival through STAT signaling, but numerous past studies have proved it in some oncokinaes such as SAO (BCR–ABL, FLT3/ITD and JAK2 V617F).[17,30,31] Therefore, we selected SAO-positive leukemia cells as the target of OPB-31121. Strikingly, SAO-positive leukemia cells, including primary leukemia cells, were generally OPB-31121 sensitive both *ex vivo* and *in vivo*. It is intriguing that OPB-31121 did not cause growth inhibition of normal hematopoietic cells (Figures 5b, c, e and f). In fact, the dose-limiting toxicity of this compound did not show hematological toxicity in phase I trial with a maximum dose of 800 mg/kg.[29] No hematological toxicity was observed in the toxicity study of this compound in monkeys (1000 mg/kg for 14 days, data not shown). Although the importance of STAT3 and STAT5 in various signals from cytokines such as erythropoietin, thrombopoietin and granulocyte-colony stimulating factor has been established, the dependence on STAT signaling will be lower in normal hematopoietic cells than in malignant cells.

Specific inhibitors of kinases aberrantly activated in tumor cells are powerful tools with high antitumor effects and less toxicity; however, cancer cells sometimes develop resistance in various ways. CML cells are reported to develop the resistance to ABL kinase inhibitor by a drug-resistant mutation in BCR–ABL, overexpression of BCR–ABL,[36] overexpression of Lyn as an alternative activator of STAT5[37] and STAT3 activation through an alternative signal from bone marrow stroma cells.[38] In FLT3/ITD-positive AML cells, resistance to FLT3 inhibitor was achieved by a drug-resistant mutation in FLT3, overexpression of FLT3 and autocrine Flt3 ligand stimulation.[32,39] In JAK2 V617F-positive AML or polycythemia vera, specific JAK2 inhibition was overcome by

alternative activation of other JFKs.[23] The mechanisms of drug resistance are various but many of them finally lead to the maintenance of STAT3/5 activation. This is probably because SAO-positive leukemia cells require the maintenance of STAT3/5 activation for drug-resistant survival; therefore, it is a reasonable strategy to overcome SAO inhibitor resistance by a STAT inhibitor. We showed examples of this strategy in the case of a drug-resistant mutation in BCR–ABL and autocrine-induced FLT3 inhibitor resistance (Figures 4d and 5a–c).

Taken together, we conclude that OPB-31121 holds promise as a non-myelosuppressive therapeutic agent against a wide range of hematopoietic malignancies, especially SAO-positive leukemia. As OPB-31121 showed strong antitumor effects also in a wide range of solid tumors (data not shown), two phase I studies have been performed on advanced solid tumors in Korea (NCT00955812) and for non-Hodgkin's lymphoma and multiple myeloma in Hong Kong (NCT00511082), and the results are awaited. A phase I/II study of hepatocellular carcinoma in Japan (NCT1406574) is ongoing.

CONFLICT OF INTEREST

TN received research funding from Otsuka Pharmaceutical Co., Ltd, Kyowa Hakko Kirin Co., Ltd., Wyeth and Chugai Pharmaceutical Co., Ltd. KS, YH, NH and NO are employees of Otsuka Pharmaceutical Co., Ltd., whose product was studied in this work. The other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

FH designed the research, performed experiments and wrote the paper. KS designed the research and performed experiments. YH, NH, NO and SK performed experiments. TN designed the research.

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Saracatinib impairs the peritoneal dissemination of diffuse-type gastric carcinoma cells resistant to Met and fibroblast growth factor receptor inhibitors

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Key words

c-Met, c-src, neoplasm metastasis, saracatinib, stomach neoplasms

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Diffuse-type gastric carcinomas (DGC) exhibit more aggressive progression and poorer prognosis than intestinal-type and other gastric carcinomas. To identify potential therapeutic targets, we examined protein tyrosine phosphorylation in a panel of DGC and other gastric cancer cell lines. Protein tyrosine phosphorylation was significantly enhanced or altered in DGC cell lines compared with that in other gastric cancer cell lines. Affinity purification and mass spectrometry analysis of tyrosine-phosphorylated proteins identified Met as a protein that is preferentially expressed and phosphorylated in DGC cell lines. Unexpectedly, Met inhibitors blocked cell growth, Met downstream signaling and peritoneal dissemination *in vivo* in only a subset of cell lines that exhibited remarkable overexpression of Met. Likewise, only cell lines with overexpression of fibroblast growth factor receptor 2 (FGFR2) or phosphorylation of FRS2 were sensitive to an FGFR2 inhibitor. A Src inhibitor saracatinib impaired growth in cell lines that are insensitive to both Met and FGFR2 inhibitors. Saracatinib also effectively impaired peritoneal dissemination of Met-independent and FGFR2-independent SGC cells. Moreover, DGC cell lines exhibited nearly mutually exclusive susceptibility to Met, FGFR and Src inhibitors. These results suggest that DGC have distinct sensitivities to molecular target drugs and that targeting Src is beneficial in the treatment of DGC insensitive to Met and FGFR inhibition.

Gastric adenocarcinomas are histologically classified into two major subtypes according to the Laurén classification: intestinal and diffuse.[1] Diffuse-type gastric carcinoma (DGC) is also called poorly differentiated gastric carcinoma and often exhibits aggressive progression.[2–4] Scirrhous gastric carcinoma (SGC) is a subtype of DGC characterized by rapid infiltrative growth accompanied by massive stromal fibrosis and frequent metastasis to lymph nodes and the peritoneum.[5] These aggressive characteristics contribute to the extremely poor prognosis of patients with SGC.[6,7] Several genetic alterations have been implicated in DGC, including gene amplification of *c-met* and *FGFR2/k-sam*.[8–10]

The oncogene *c-met* encodes Met receptor type tyrosine kinase whose ligand is hepatocyte growth factor (HGF). Met signaling regulates multiple aspects of cancer malignancies, including cell migration and invasion, cell proliferation and survival, and angiogenesis.[11] Amplification and germline and somatic mutations of *c-met* have been found in a wide spectrum of human cancers.[12] Therefore, Met is considered to be a promising therapeutic target, and dozens of Met inhibitors are being evaluated in clinical trials.[12–14] Met amplification is correlated with poor prognosis in gastric cancer patients.[10,15,16]

FGFR2/k-sam encodes fibroblast growth factor receptor (FGFR) type 2, a member of the FGFR receptor tyrosine kinase family, and its mutation and amplification have been detected and correlated with poor prognosis in several human cancers, including gastric cancers.[17] Similar to Met, FGFR2 signaling regulates many cellular functions that contribute to cancer progression, including cell proliferation, survival and migration.[17] Accordingly, FGFR inhibitors are being tested in clinical trials.[18]

Several studies have revealed that gastric cancer cell lines exhibiting Met amplification are sensitive to Met inhibitors.[16,19–24] Likewise, FGFR2 inhibitors have been shown to block cell growth and peritoneal dissemination of SGC cells with FGFR gene amplification.[25–27] However, amplification of *c-met* and *FGFR2* occurs only in a limited fraction: approximately 2–20% and 10% of all gastric cancers, respectively.[10,15,19,28–30] Therefore, a molecular target remains to be determined for the treatment of the fraction of DGC with neither *c-met* nor *FGFR2* amplification/activating mutation.

In this study, we performed a detailed analysis of tyrosine-phosphorylated proteins in a panel of gastric cancer cell lines to identify signaling pathways or molecules that could be molecular targets for DGC chemotherapy.

Materials and Methods

Cell culture. The human gastric cancer cell lines used, that is, HSC-39, HSC-43, HSC-59, HSC-60, HSC-64, HSC-44PE, 58As9, 58As1, 44As3 and 44As3Luc, have been described previously.[31–34] MKN1, MKN7, MKN74, NUGC-4, KATO-III, MKN45 and IM95 were obtained from the Health Science Research Resources Bank. AGS, NCI-N87 and SNU-5 were obtained from the American Type Culture Collection (ATCC). GCIY, ECC12, AZ521 and KE-97 were provided by the RIKEN Bio-Resource Center through the National Bio-Resource Project of the MEXT, Japan. These cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 10 units/mL of penicillin and 10 μg /mL of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Reagents and antibodies. Antibodies, including phospho-specific antibodies, against Met, Src, ERK, Akt, FRS2α and Stat3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Met and FRS2 were also purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against FGFR2α and phospho-FGFR1-4 were purchased from R&D Systems (Minneapolis, MN, USA). Anti-phosphotyrosine (4G10) antibody was obtained from Merck Millipore (Billerica, MA, USA). PHA-665752, crizotinib (PF-2341066), saracatinib (AZD0530) and JNJ-38877605 were purchased from Selleck Chemicals (Houston, TX, USA). Saracatinib was also obtained from Adooq BioScience (Irvine, CA, USA). PD-173074 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immunoblotting. Immunoblotting was carried out as described previously.[35] ImageJ software (version 1.41o; National Institute of Health, Bethesda, MD, USA) was used to quantify the band intensity from immunoblot data.

Affinity purification and identification of tyrosine-phosphorylated proteins. 58As9 cells were lysed in a buffer containing 50 mM Hepes-NaOH (pH 7.0), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4 and protease inhibitors. The lysates were incubated with 4G10 coupled with cyanogen bromide-activated Sepharose 4B beads (GE Healthcare, Little Chalfont, UK). The beads were washed with lysis buffer, and 4G10-associated proteins were eluted using 0.1 M phenylphosphate. The purified proteins were subjected to SDS-PAGE, stained using a Silver Stain MS kit (Wako, Osaka, Japan), excised, digested with trypsin and subjected to liquid chromatography-tandem mass spectrometry analysis. Proteins were identified using a Mascot search.

Cell proliferation assay. Cells were plated onto 96-well plates at 1–2 × 10^3 per well and cultured for 4 days in the presence or absence of inhibitors. Cell growth was determined using a Premix WST-1 Cell Proliferation Assay System (Takara, Shiga, Japan) according to the manufacturer's instructions. Absorbance at 450 nm was measured with an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), and the measurement was conducted in quadruplicate.

Peritoneal dissemination assay. 44As3 (5 × 10^5) or 58As9 cells (4 × 10^6) were inoculated intraperitoneally into BALB/c nude mice purchased from CLEA Japan (Tokyo, Japan). Inhibitors (PHA-665752, 10 mg/kg; saracatinib, 50 mg/kg) were administrated via intraperitoneal injection, thrice per week, starting 1 day after inoculation. In the case of 44As3Luc, the progression of metastasis was monitored via bioluminescence analysis using an IVIS system (Xenogen, Alameda, CA, USA) as described previously.[32] At 9–11 days for 44As3 and 16–18 days for 58As9 after inoculation, the mice were killed and dissected to evaluate peritoneal dissemination, liver metastasis, omental tumor weight and ascites formation. These experiments were approved by the Committee for Ethics of Animal Experimentation of the National Cancer Center and conducted in accordance with the guidelines for animal experiments of the National Cancer Center.

Statistical analysis. The data are representative of at least three independent experiments. Statistical analysis and calculation of median inhibitory concentration (IC50) values were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

Results

Diffuse-type gastric carcinoma cells exhibit different patterns of protein tyrosine phosphorylation. We first examined protein tyrosine phosphorylation in a panel of gastric cancer cell lines (Table S1) by immunoblotting with an anti-phosphotyrosine antibody 4G10 (Fig. 1a). The amount of phosphotyrosine-containing proteins mainly distributed from 37 to 250 kDa was greatly elevated in several DGC cell lines, including MKN45, KATO-III, 58As1, 58As9 and SNU-5. Other cell lines exhibited moderate or only a modest level of tyrosine-phosphorylated proteins. Notably, the band pattern of phosphotyrosine-containing proteins in DGC cell lines, which apparently varied among lines, was significantly distinct from those in differentiated gastric cancer cells. These observations indicate that DGC display specific activation of some intracellular signaling pathways involving tyrosine phosphorylation.

Met is overexpressed and highly phosphorylated in diffuse-type gastric carcinoma cells. To identify molecules that account for increased tyrosine phosphorylation in DGC cell lines, phosphotyrosine proteins were affinity purified from 58As9 cells and subjected to mass spectrometry. As a result, the most prominent band at approximately 140 kDa was identified as Met, a receptor tyrosine kinase for HGF ligand (Fig. S1). Many of the DGC cell lines showed significant increases in both expression and phosphorylation of Met compared with those in other gastric cancer cell lines (Fig. 1a,b). Notably, MKN45, 58As1, 58As9 and SNU-5 cells have higher amounts of Met than those in other DGC cell lines. This observation is consistent with previous studies showing that these cell lines display amplification of *c-met*.[23,24]

Different sensitivities to Met inhibitors in diffuse-type gastric carcinoma cell lines. The effects of Met inhibitors (PHA-665752, JNJ-38877605 and PF-2341066 [crizotinib]) on the growth of DGC cell lines were determined (Fig. 1c). These Met inhibitors effectively suppressed the growth of MKN45, 58As1, 58As9 and SNU-5 cells that showed the highest expression of Met. By contrast, Met inhibitors had no effect on the growth of other cell lines. These results are consistent with those of other studies that have used similar but different sets of gastric cancer cell lines and Met inhibitors.[20,23,24] Met inhibitor-insensitive 44As3 and Met inhibitor-sensitive 58As9 cells were chosen for further analyses, as both are highly metastatic in a mouse peritoneal dissemination model.[33]

The IC50 values of Met inhibitors in 44As3 and 58As9 cells were approximately 1500 and 19 nM for PHA-665752, >10 000 and 6.9 nM for JNJ-38877605, and 2600 and 21 nM

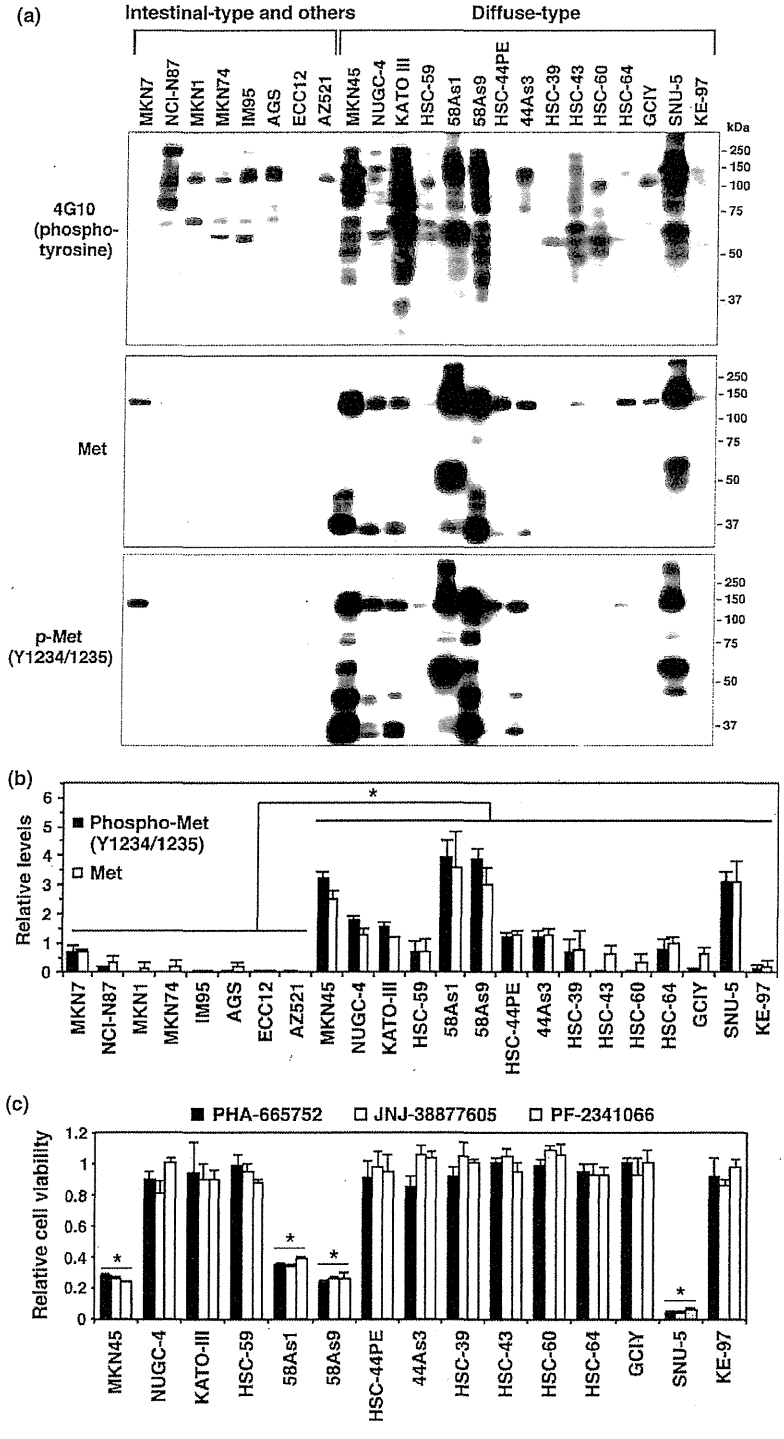
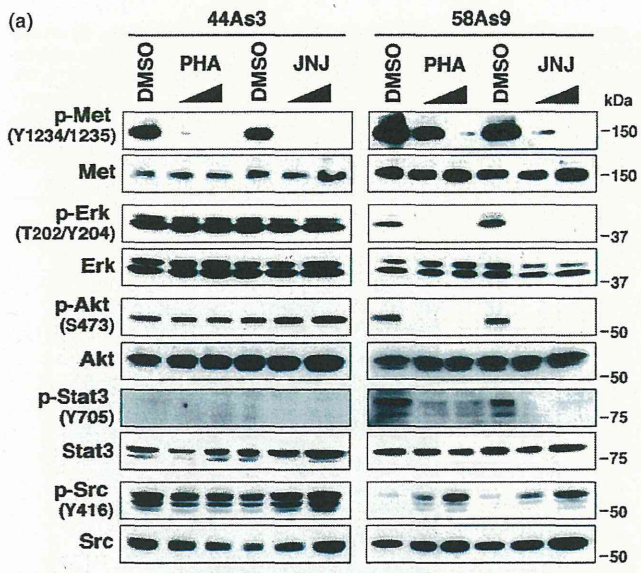
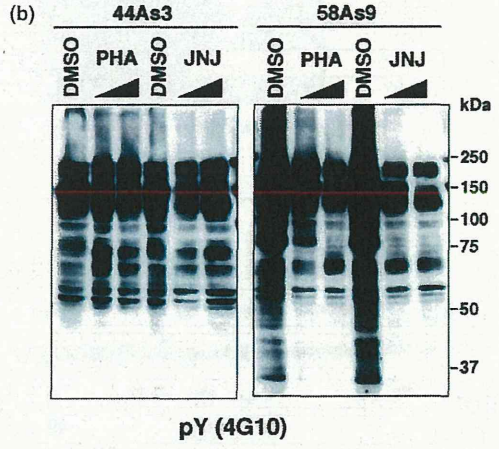
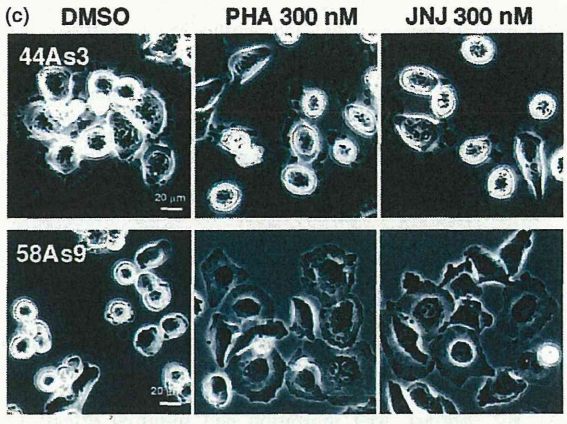


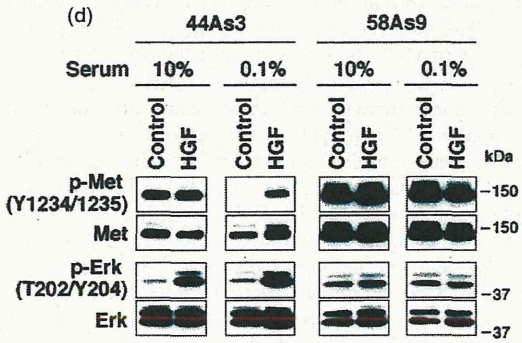
Fig. 1. Diffuse-type gastric carcinoma (DGC) cell lines with robust overexpression of Met are sensitive to Met inhibitors. (a) DGC and other gastric carcinoma cell lines were subjected to immunoblot analysis with anti-phosphotyrosine (4G10) and anti-Met antibodies. (b) The relative levels of phospho-Met and total Met were quantified from immunoblot data as described in the Materials and Methods. Bars, SD ($n = 4$ for phospho-Met and 3 for total Met). *$P < 0.01$, by Student's t-test. (c) DGC cell lines were treated with 300 nM of Met inhibitors for 4 days, and cell viability was assessed. Bars, SD ($n = 4$). *$P < 0.0001$ versus other cell lines, by ANOVA with Tukey's test.

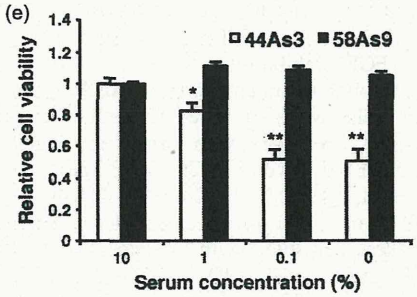
for PF-2341066, respectively (Fig. S2a). Met inhibitors significantly blocked phosphorylation of Met in both 44As3 and 58As9 cells (Fig. 2a). Nonetheless, overall protein tyrosine phosphorylation and phosphorylation of ERK, Akt and Stat3 were significantly decreased only in 58As9 cells (Fig. 2a,b). Interestingly, Src phosphorylation was rather increased in 58As9 cells upon Met inhibition, whereas it was unchanged in 44As3 cells (Fig. 2a). Similar results were obtained with PF-2341066 (Fig. S2b). Met inhibitor treatment also induced marked changes in cell morphology in 58As9 but not in 44As3 cells: rounded morphology was converted to flattened and adherent phenotypes (Fig. 2c). Serum starvation reduced and following HGF stimulation increased Met phosphorylation in 44As3 cells, whereas Met was constitutively phosphorylated in 58As9 cells (Fig. 2d). Consequently, growth of 44As3 but not of 58As9 cells was sensitive to serum starvation (Fig. 2e). These results indicate that overexpressed Met proteins confer the capability for serum-independent growth to 58As9 cells via

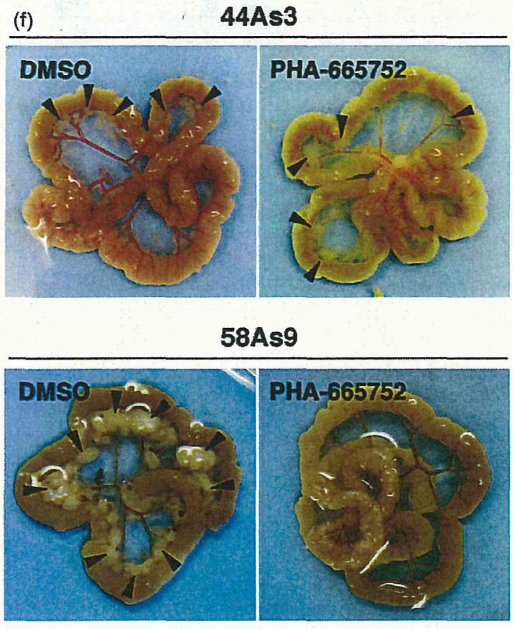












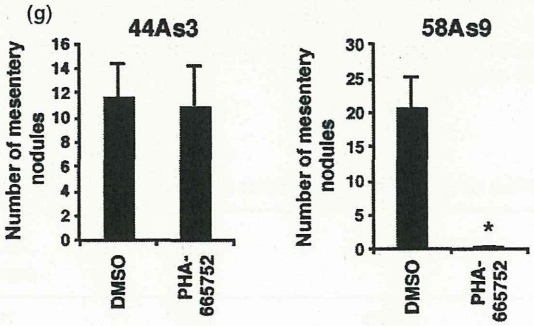


Fig. 2. (On previous page) 44As3 and 58As9 scirrhous gastric carcinoma (SGC) cells have different sensitivities to Met inhibitors. (a, b) 44As3 and 58As9 cells were treated with 100 or 300 nM of Met inhibitors PHA-665752 (PHA) or JNJ-3887760 (JNJ) for 2 h and subjected to immunoblot analyses. (c) Phase contrast micrographs of cells treated with Met inhibitors for 1 day. (d) Cells were cultured in the presence of 10 or 0.1% serum for 1 day and then stimulated with 100 ng/mL hepatocyte growth factor (HGF) for 10 min. The cells were then analyzed with immunoblotting. (e) Viability of cells cultured in the presence of various concentrations of serum for 3 days. Bars, SD ($n = 4$). *$P < 0.005$ and **$P < 0.0005$ by Student's *t*-test. (f) 44As3 and 58As9 cells were inoculated intraperitoneally into nude mice, and DMSO (vehicle) or the Met inhibitor PHA was administered. Representative macroscopic views of mesentery tumor nodules are shown. (g) The number of mesentery tumor nodules (>1 mm in diameter). Bars, SEM ($n = 11$ for DMSO and 13 for PHA in 44As3; $n = 10$ for DMSO and PHA in 58As9). *$P < 0.001$ by the Mann–Whitney test.

activation of downstream signaling pathways, and, therefore, that 58As9 cells are susceptible to Met inhibitors.

Next, the effect of Met inhibition on peritoneal dissemination of 44As3 and 58As9 cells was examined. Met inhibition remarkably reduced the formation of ascites and peritoneal dissemination in mice injected with 58As9 cells (Fig. 2f,g; Table 1). By contrast, Met inhibition had minimal effects on peritoneal dissemination of 44As3 cells; only a modest decrease in ascites formation was observed (Table 1). Taken together, Met inhibition seems to be extraordinarily effective against a portion of DGC that express high levels of Met proteins, although it is ineffective against other DGC.

Src inhibitor blocks growth of Met/fibroblast growth factor receptor-independent diffuse-type gastric carcinoma cell lines. The effects of several inhibitors targeted to signaling molecules other than Met were then examined. PD-173074, a pan-FGFR inhibitor, treatment strongly inhibited the growth of KATO-III and HSC-39 and moderately inhibited HSC-43 and HSC-64 (Fig. 3a). These sensitive cell lines displayed increased phosphorylation of FRS2α, an adaptor protein for FGFR (Fig. 3b). KATO-III and HSC-43 cells also showed robust overexpression and phosphorylation of FGFR2α, which is consistent with *k-sam* amplification in those cell lines.[36] It is unclear why FGFR2α expression was not detected in HSC-39 cells, which also have *k-sam* amplification.[36] Taken together, phosphorylation of FRS2α and overexpression of FGFR2α seem to be correlated with sensitivity to FGFR inhibition.

Because Met inhibitor treatment upregulated Src phosphorylation in 58As9 cells, we next tested the effect of the Src inhibitor saracatinib. Treatment with saracatinib significantly blocked the growth of several DGC cell lines, including NUGC-4, KATO-III, HSC-59, HSC-44PE, 44As3, HSC-60, GCIY and KE-97, but it had no effect on Met-dependent cell lines (Fig. 3a). These cell lines, with the exception of KATO-III, also lacked sensitivity to PD-173074. We also tested whether the combination of Met inhibitor and saracatinib has a synergistic effect on the proliferation of 58As9 cells. However, simultaneous addition of the both inhibitors had a minimal effect (Fig. S3). These observations clearly suggest that DGC have different, and nearly mutually exclusive, sensitivities to Met, FGFR and Src inhibitors.

(a)

	PF-2341066	PD-173074	Saracatinib
MKN45	0.240	0.969	0.909
NUGC-4	1.003	1.055	0.186
KATO-III	0.894	0.279	0.259
HSC-59	0.880	0.990	0.381
58As1	0.387	1.064	0.986
58As9	0.265	0.982	0.993
HSC-44PE	0.946	0.935	0.611
44As3	1.041	1.065	0.523
HSC-39	1.011	0.228	0.861
HSC-43	0.948	0.704	0.905
HSC-60	1.060	0.984	0.308
HSC-64	0.923	0.851	1.147
GCIY	1.009	0.985	0.345
SNU-5	0.063	0.956	0.913
KE-97	0.981	1.093	0.771

Relative cell viability 0 1

(b)

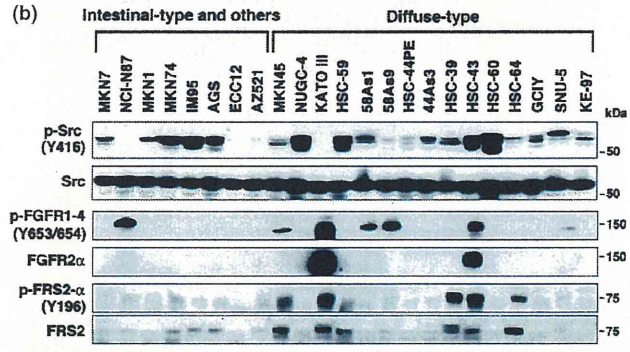


Fig. 3. Diffuse-type gastric carcinoma (DGC) cell lines have different sensitivities to Met, fibroblast growth factor receptor (FGFR) and Src inhibitors. (a) Viability of DGC cell lines cultured in the presence of Met inhibitor PF-2341066 (300 nM), FGFR inhibitor PD-173074 (300 nM), or Src inhibitor saracatinib (3 μM) for 4 days. Numbers shown are mean values of relative cell viability ($n = 4$). The sensitivity of each cell line to each inhibitor is indicated by the increasing intensity of the red signal on a blue background. (b) Immunoblot analysis for expression and phosphorylation of Src, FGFR and FRS2.

Table 1. Effect of Met inhibition on ascites formation and dissemination of 44As3 and 58As9 cells

Cell line	Treatment	Ascites	Metastasis			
			Omentum	Mesentery	Diaphragm	Liver
44As3	DMSO	6/11	11/11	11/11	5/11	5/11
	PHA	3/13	12/13	12/13	4/13	6/13
58As9	DMSO	8/10	8/10	8/10	8/10	8/10
	PHA	0/10	6/10	1/10	0/10	0/10

Number of mice bearing ascites or tumors at the indicated site per total number of mice bearing tumors.

Immunoblot analysis showed that Src is equally expressed in all gastric cancer cell lines tested, and its phosphorylation also occurred in several DGC and other cell lines (Fig. 3b). Notably, however, the amount of phosphorylated Src was inversely correlated with that of phosphorylated Met in some DGC cell lines: cell lines with Met overexpression (MKN45, 58As1, 58As9 and SNU-5) have lower amounts of phosphorylated Src, whereas those with low expression of Met (NUGC-4, HSC-59 and HSC-60) have relatively high amounts of phosphorylated Src. As expected, saracatinib, but not Met and FGFR inhibitors, decreased overall tyrosine phosphorylation and ERK and Akt phosphorylation in saracatinib-responsive cell lines GCIY and HSC-60 (Fig. S4). Similarly, Met inhibitors, but not saracatinib or PD-173074, blocked overall tyrosine phosphorylation, as well as ERK, and Akt phosphorylation in MKN45 and 58As1. In HSC-39 and KATO-III cells, FGFR inhibition significantly suppressed phosphorylation of these signaling molecules.

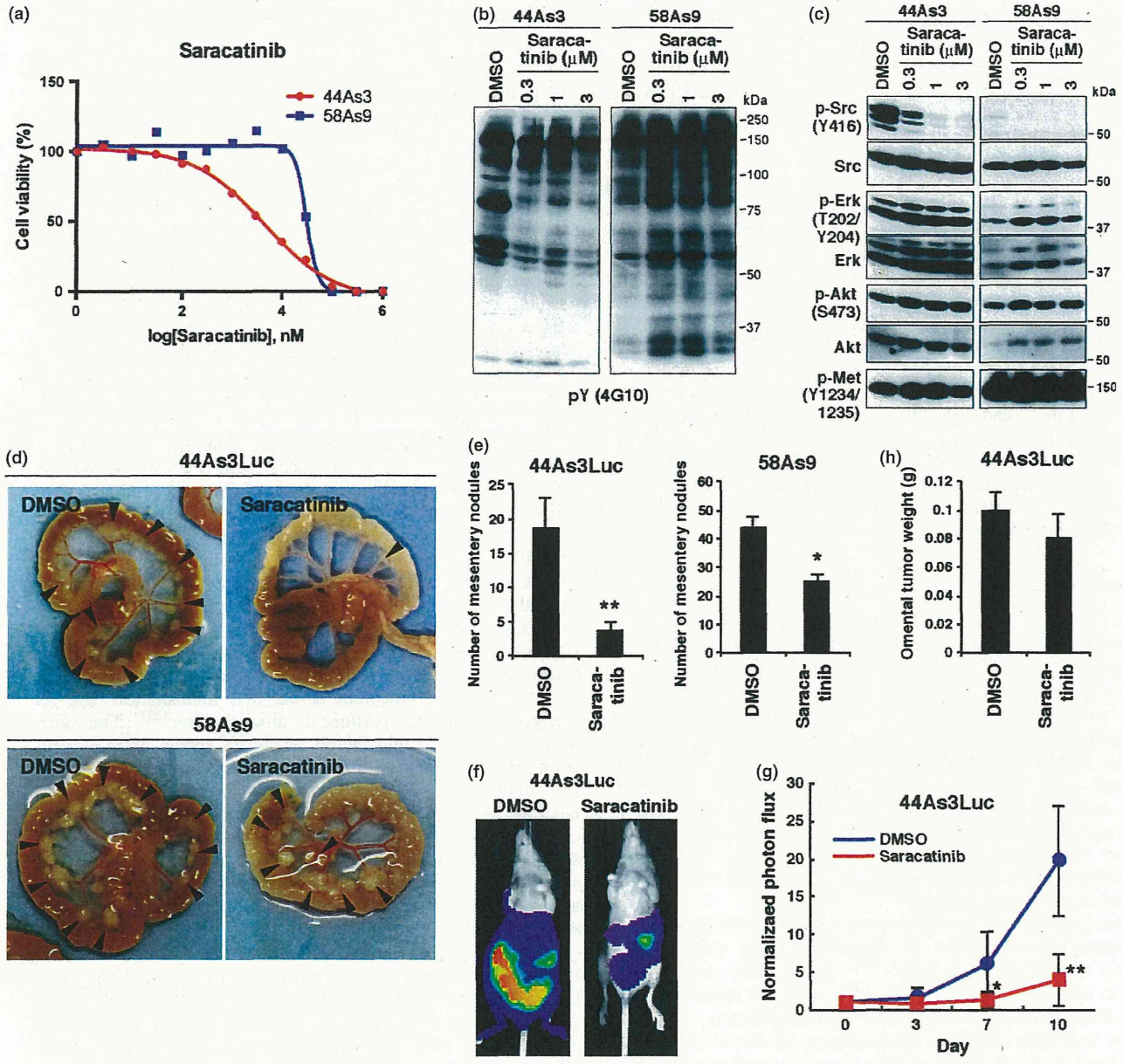


Fig. 4. Saracatinib impairs peritoneal dissemination of 44As3 cells. (a) Viability of 44As3 and 58As9 cells treated with various concentrations of saracatinib for 4 days. (b, c) Cells were treated with saracatinib for 2 h and subjected to immunoblot analyses with indicated antibodies. (d) 44As3Luc and 58As9 cells were intraperitoneally injected into nude mice and DMSO (vehicle) or saracatinib was administered. Representative macroscopic views of mesentery tumor nodules are shown. (e) The number of mesentery tumor nodules. Bars, SEM (n = 16 for DMSO and 18 for saracatinib in 44As3Luc; n = 8 for DMSO and 7 for saracatinib in 58As9). $P < 0.001$; and $^{**}P < 0.0001$ by the Mann–Whitney test. (f) Representative images of mice obtained via *in vivo* imaging. (g) Quantitative analysis of luminescence photon counts. Bars, SD (n = 8 for DMSO and 9 for saracatinib). $^{*}P < 0.05$; and $^{**}P < 0.0005$ by the Mann–Whitney test. (h) Omental tumor weight in mice inoculated with 44As3Luc cells. Bars, SEM (n = 10 for DMSO and 9 for saracatinib). $P = 0.3451$ by the Mann–Whitney test.

Table 2. Effect of saracatinib on ascites formation and dissemination of 44As3Luc and 58As9 cells

Cell line	Treatment	Ascites	Metastasis			
			Omentum	Mesentery	Diaphragm	Liver
44As3Luc	DMSO	14/16	14/16	16/16	4/16	9/16
	Saracatinib	3/18	16/18	15/18	0/18	4/18
58As9	DMSO	8/8	8/8	8/8	8/8	6/8
	Saracatinib	3/7	7/7	7/7	5/7	5/7

Number of mice bearing ascites or tumors at the indicated site per total number of mice bearing tumors.

Saracatinib impairs peritoneal dissemination of Met/fibroblast growth factor receptor-independent scirrhous gastric carcinoma cells. The IC50 values of saracatinib for the cell growth of 44As3 and 58As9 cells were 8.2 and 30 μM, respectively (Fig. 4a). Saracatinib treatment significantly decreased overall protein tyrosine phosphorylation in 44As3 but not in 58As9 cells (Fig. 4b). Interestingly, saracatinib did not affect the phosphorylation of Met, ERK and Akt (Fig. 4c). Saracatinib administration reduced the incidence of ascites formation and tumor dissemination to diaphragm and liver in 44As3 cells expressing luciferase (44As3Luc; Table 2). Although the incidence of dissemination to mesentery was not affected much by saracatinib, a marked reduction in the number of mesentery nodules was observed (Fig. 4d,e). *In vivo* imaging analysis confirmed that saracatinib treatment significantly reduces the growth and dissemination of 44As3Luc cells (Fig. 4f,g). In contrast, neither the incidence of omental metastasis nor the growth of omental tumors was affected by saracatinib treatment (Table 2 and Fig. 4h). Although saracatinib tended to suppress the formation of ascites and peritoneal dissemination by 58As9 cells, the effects were more moderate (Table 2 and Fig. 4d,e). These data indicate that saracatinib can suppress peritoneal dissemination of SGC cells that are insensitive to Met and FGFR inhibitors.

Discussion

This study showed that significant increases and changes in protein tyrosine phosphorylation occur in DGC cell lines. In particular, several cell lines showed remarkable increases in the levels of protein tyrosine phosphorylation mainly due to Met overexpression. Although Met was overexpressed and phosphorylated in a large portion of DGC cell lines, only a subset of cell lines with remarkable amounts of Met protein through gene amplification was sensitive to Met inhibitors. One such cell line, 58As9, exhibited constitutive activation of Met and resistance to serum starvation. These results are consistent with those of previous studies showing that genetic alteration of Met drives addiction to Met activity and predicts effective treatment outcome.[12,14,15,19,24,37]

Two previous studies have reported that Met inhibitors block peritoneal dissemination of MKN45 poorly differentiated gastric adenocarcinoma cells.[16,21] The present study is the first to show that Met inhibition also markedly reduces peritoneal dissemination of 58As9 SGC cells. Importantly, Met inhibition had little effect on dissemination of 44As3 SGC cells that have moderate levels of Met expression and phosphorylation. Likewise, FGFR inhibition had growth-inhibitory effects only in cell lines with a high FGFR or FRS2 activation status. Therefore, the activation status of the receptor signaling pathway may need to be considered in the clinical use of these molecular targeted drugs. We demonstrated that the amount of Met and FRS2 phosphorylation are closely correlated with sensitivity to Met and FGFR2 inhibitors, respectively. At least in the case of Met, immunohistochemical studies have demonstrated that Met is expressed and phosphorylated in human DGC with high Met gene copy number.[15,37] Thus, our results imply that the phosphorylation status of Met and FRS2 may be a good marker for predicting the therapeutic efficacy of Met and FGFR inhibitors.

To our knowledge, this study is the first to show that saracatinib, an Src inhibitor, suppresses peritoneal dissemination of SGC cells. More importantly, saracatinib reduced cell growth of DGC that are insensitive to both Met and FGFR inhibitors. We also found that sensitivities to Met, FGFR and Src inhibitors are almost mutually exclusive in DGC cell lines. Similar results regarding different sensitivities to Met and Src inhibitors *in vitro* have been reported in a different set of gastric carcinoma cell lines.[23] Our data show that SGC cell lines lacking amplification of Met and FGFR but displaying relatively high levels of Src phosphorylation tend to be responsive to saracatinib. Immunohistochemical study has shown that DGC cells display activation of Src that increases with the depth of invasion and correlates with the progression of DGC.[38] Thus, the activation status of Src may predict a good outcome for anti-Src therapy in DGC. Saracatinib treatment also effectively suppressed the growth of MKN74 and IM95 intestinal-type gastric carcinoma cells with high levels of Src phosphorylation (Figs S5 and 3b). Therefore, Src inhibitors may also be applicable to intestinal-type gastric carcinomas.

Administration of saracatinib did not affect the growth of omental tumors in mice inoculated with 44As3 cells, while it effectively blocked the formation of mesentery nodules. It is reported that the omentum is the first implantation site for malignant cells in peritoneal dissemination.[39] Therefore, saracatinib may mainly inhibit secondary spread of SGC cells from omental tumors. Alternatively, saracatinib may inhibit cell functions that are more important for colonization in mesenterium than in omentum. Src is a well-established regulator of cell migration and invasion, contributing to metastatic spread of malignant tumors.[40] This raises the possibility that saracatinib targets phosphoproteins involved in cell migration and invasion to exert such inhibitory effects on peritoneal dissemination of SGC cells.

In summary, our results demonstrate that Met and FGFR inhibitors are effective in subsets of DGC but not in other DGC. We found that the Src inhibitor saracatinib blocks cell growth and peritoneal dissemination of SGC cells that are insensitive to both Met and FGFR inhibitors. These results may provide a novel approach for molecular targeted therapy in DGC patients.

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