



## In vivo treatment of mutant *FLT3*-transformed murine leukemia with a tyrosine kinase inhibitor

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**Somatic mutation of the *FLT3* gene, in which the juxtamembrane domain has an internal tandem duplication, is found in 20% of human acute myeloid leukemias and causes constitutive tyrosine phosphorylation of the products. In this study, we observed that the transfection of mutant *FLT3* gene into an IL3-dependent murine cell line, 32D, abrogated the IL3-dependency. Subcutaneous injection of the transformed 32D cells caused leukemia in addition to subcutaneous tumors in C3H/HeJ mice. To develop a *FLT3*-targeted therapy, we examined tyrosine kinase inhibitors for *in vitro* growth suppression of the transformed 32D cells. A tyrosine kinase inhibitor, herbimycin A, remarkably inhibited the growth of the transformed 32D cells at 0.1  $\mu\text{M}$ , at which concentration it was ineffective in parental 32D cells. Herbimycin A suppressed the constitutive tyrosine phosphorylation of the mutant *FLT3* but not the phosphorylation of the ligand-stimulated wild-type *FLT3*. In mice transplanted with the transformed 32D cells, the administration of herbimycin A prolonged the latency of disease or completely prevented leukemia, depending on the number of cells inoculated and schedule of drug administration. These results suggest that mutant *FLT3* is a promising target for tyrosine kinase inhibitors in the treatment of leukemia. *Leukemia* (2000) 14, 374–378.**

**Keywords:** *FLT3*; leukemia; tyrosine kinase inhibitor; *in vivo* treatment; herbimycin A

### Introduction

Mutations of receptor tyrosine kinases (RTK), including c-KIT, PDGFR $\beta$  and *FLT3*, have been found in human leukemia.<sup>1–3</sup> An internal tandem duplication (ITD) of the juxtamembrane (JM) domain-coding sequence of the *FLT3* gene is the most frequent mutation among them.<sup>4–6</sup> In the mutated *FLT3* gene with ITD, the juxtamembrane (JM) domain-coding sequence, which primarily consists of exon 11 but sometimes includes intron 11 and exon 12, is arranged in a direct head-to-tail succession. Although its location and length vary from sample to sample, the mutated *FLT3* gene is always readable in frame, and the transcripts actually code mutant *FLT3* with a long JM domain without affecting other domains. The *FLT3* gene mutation is found in 20% of acute myeloid leukemia (AML) and in 3% of myelodysplastic syndrome (MDS) cases, whereas it is very rare in chronic myeloid leukemia and lymphoid malignancies.<sup>4</sup> The presence of the *FLT3* gene mutation was significantly related to high peripheral white blood cell (WBC) counts and a poor prognosis.<sup>5,6</sup> The ITD of the *FLT3* gene sometimes emerged during progression of MDS or at relapse of AML which had no ITD at the first diagnosis.<sup>7,8</sup> These findings strongly suggest that the *FLT3* gene mutation promotes leukemia progression.

*FLT3* products preferentially express on hematopoietic

progenitor cells and its ligand (FL) on bone marrow stroma, suggesting an important role in their survival, proliferation, and differentiation.<sup>9,10</sup> Clinical samples from AML frequently express functional *FLT3*, suggesting the *FLT3*-signal pathway is also important to proliferation and/or inhibition of apoptosis in leukemia cells.<sup>11,12</sup> The mutant *FLT3* is ligand-independently dimerized and phosphorylated in a dominant manner.<sup>13</sup> Similarly, in mast cell leukemia, the *c-KIT* gene reportedly has a mutation of the JM domain and the products are dimerized and phosphorylated.<sup>14</sup> The JM domain of RTK might be thus associated with regulation of RTK dimerization.

Recently the inhibition of the tyrosine kinase activities and the following signal pathways has been recognized as new therapeutic strategy for leukemia.<sup>15</sup> Using murine models, several experiments presented promising results the Bcr-Abl products-directed tyrosine kinase inhibitors could treat the Bcr-Abl<sup>+</sup> leukemia *in vivo*, as well as *in vitro*.<sup>16–19</sup> In this study, we transformed IL3-dependent murine cell lines with mutant. Furthermore, we examined tyrosine kinase inhibitors for *in vitro* and *in vivo* growth suppression of the transfectants to develop a model for *FLT3*-targeted therapy for leukemia.

### Materials and methods

#### Agents

Herbimycin A, CGP 52411, genistein, tyrphostin A9, and erbstatin were purchased from Sigma-Aldrich (St Louis, MO, USA). They were dissolved in DMSO at appropriate concentrations and stored at  $-20^{\circ}\text{C}$  until use. Recombinant murine IL3 was a generous gift of Kirin Brewery (Tokyo, Japan). Recombinant human *FLT3* ligand (FL) was purchased from R&D Systems (Minneapolis, MN, USA).

#### Transformation of 32D cells

Murine IL3-dependent myeloid cell line, 32D, was obtained from the RIKEN cell bank (Tsukuba, Japan) and maintained in RPMI1640 containing 10% fetal calf serum and 1 ng/ml murine IL3. Human full-length mutant *FLT3* cDNAs were cloned into the pMKIT-NEO vector and transfected into the cell lines by using TransFast (Promega, Madison, WI, USA) according to the manufacturer's instructions. Mt 4 clone<sup>13</sup> was used as the mutant *FLT3* cDNA. Two days after transfection, cells were selected by the culture with G418 (Gibco BRL, Gaithersburg, MD, USA) at a concentration of 800  $\mu\text{g}/\text{ml}$ . Expression of *FLT3* products was examined by flow cytometer (EPICS ELITE; Coulter, Hiialeah, FL, USA) using an anti-human *FLT3* monoclonal antibody (SF1.340; Immunotech, Marseille, France). The mutant *FLT3*-transfected cells were washed three times with the medium without IL3, thereafter maintained without IL3.

### In vitro screening assay of tyrosine kinase inhibitors

Each cell line ( $2 \times 10^5$ /ml) was seeded in 24-well culture dishes. After overnight culture, tyrosine kinase inhibitors were added at various concentrations in triplicate. Viable cells were counted using the trypan blue exclusion assay.  $IC_{50}$  was defined as the concentration of compound, which decreased the cell proliferation to 50%, compared with each untreated control.

### Immunoprecipitation and immunoblot analysis

Transfection of wild-type and mutant *FLT3* cDNA into Cos7 cells was described previously.<sup>13</sup> Transfected cells were treated with herbimycin A at 0.1, 0.5 and 1.0 nM in combination with or without FL at 50 ng/ml for 3 h. After the treatment of herbimycin A, cells were washed twice with ice-cold phosphate buffered saline (PBS), and analyzed for the phosphorylation status of FLT3 as described previously.<sup>13</sup>

### In vivo administration of herbimycin A

Eight-week-old female C3H/HeJ mice purchased from Japan SLC (Hamamatsu, Japan) were kept under standard laboratory conditions according to the guidelines of the Institute for Laboratory Animal Research, Nagoya University School of Medicine. This study was approved by the Institutional Ethics Committee for Laboratory Animals Used in Experimental Research. Mutant or wild-type *FLT3*-transfected 32D cells were subcutaneously inoculated in the right back. Treatment was started 1 day after the inoculation. A stock solution of herbimycin A (1 mM) prepared in DMSO was diluted with phosphate buffered saline (PBS) before each administration. Control mice were given an equal volume of PBS. Tumor size was monitored every day. Tumor weight was evaluated by the following formula:  $TW$  (mg) =  $(d^2 \times D)/2$ , where  $d$  (mm) and  $D$  (mm) are the shortest and longest diameters of the tumor, respectively.

### Statistical analysis

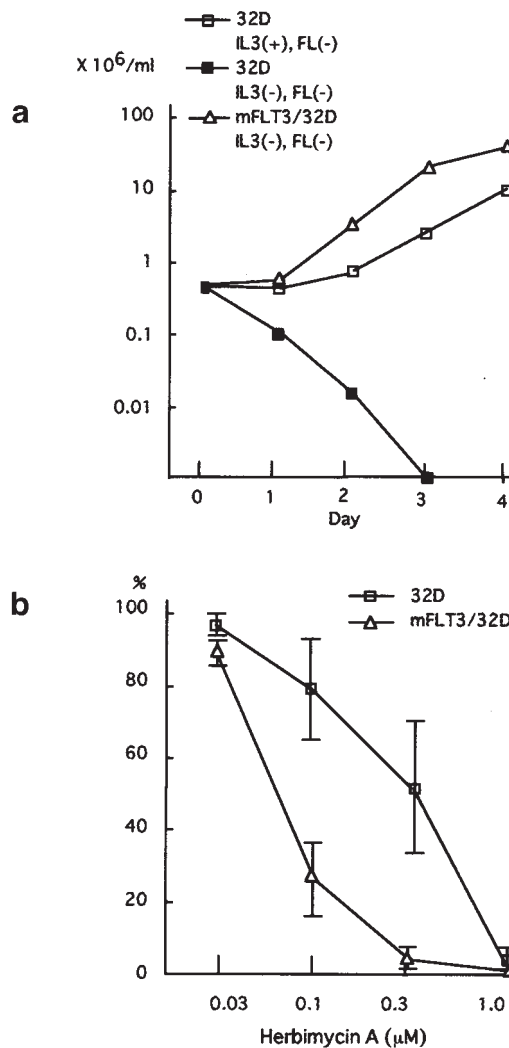
The statistical difference of tumor weight was calculated using Mann-Whitney *U* test. Survival time was analyzed by Kaplan-Meier curves and compared by the log-rank (Mantel-Cox) test. Statistical analyses were performed using the program Stat-View (SAS Institute, Cary, NC, USA).

## Results

### Transformation of 32D cells and effects of tyrosine kinase inhibitors

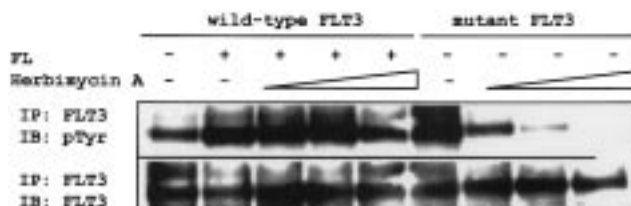
In the absence of IL3, 32D cells transfected with mutant *FLT3* cDNA (mFLT3/32D) proliferated without either IL3 or FL. The growth speed of mFLT3/32D was more rapid than that of parental 32D stimulated with IL3 (Figure 1a). The expression of FLT3 was confirmed by flow cytometer (data not shown). In an additional two independent experiments, IL3-independent clones were obtained by the transfection with mutant *FLT3*.

Several tyrosine kinase inhibitors were examined for growth inhibition using mFLT3/32D and parental 32D cells. Herbimycin A suppressed the proliferation of mFLT3/32D cells more

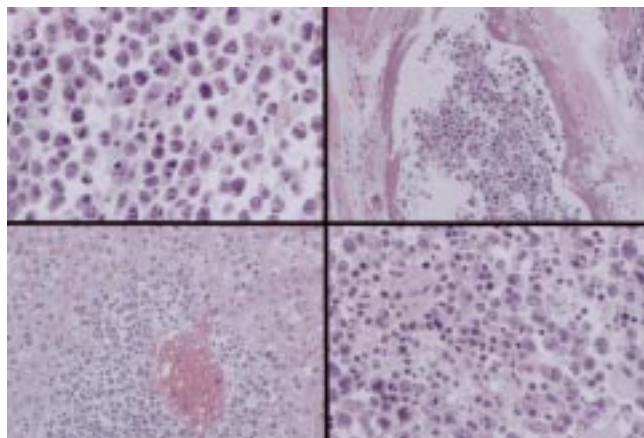


**Figure 1** (a) IL3-independent proliferation of 32D cells transfected with mutant *FLT3* gene. (b) An inhibitory effect of herbimycin A on the *in vitro* growth of parental 32D cells and mutant *FLT3*-transfected 32D (mFLT3/32D) cells. Herbimycin A was added to the culture at indicated concentrations, and viable cells were counted by trypan blue dye-exclusion assay after 72 h culture.

significantly than 32D cells stimulated with IL3 at low concentrations from 0.1 to 0.3  $\mu$ M (Figure 1b). The viable cell number after 3-day culture was reduced by 50% ( $IC_{50}$ ) at 0.06 and 0.2  $\mu$ M in mFLT3/32D cells and 32D cells, respectively (Figure 1b). In the other tyrosine kinase inhibitors (CGP52411,



**Figure 2** Inhibition of the autophosphorylation of mutant FLT3 by herbimycin A. Cos7 cells were transfected with wild-type and mutant *FLT3* cDNA. After 72 h culture, the cells were treated with herbimycin A at 0.1, 0.5 and 1.0 nM in combination with or without FL at 50 ng/ml for 3 h. Cell lysates were immunoprecipitated by anti-FLT3 antibody and immunoblotted by anti-phosphotyrosine antibody.



**Figure 3** Histopathology of C3H/HeJ mouse transplanted with mFLT3/32D cells. Left upper, subcutaneous tumor ( $\times 400$ ); right upper, bone marrow ( $\times 100$ ); left lower, liver ( $\times 100$ ); right lower spleen ( $\times 400$ ).

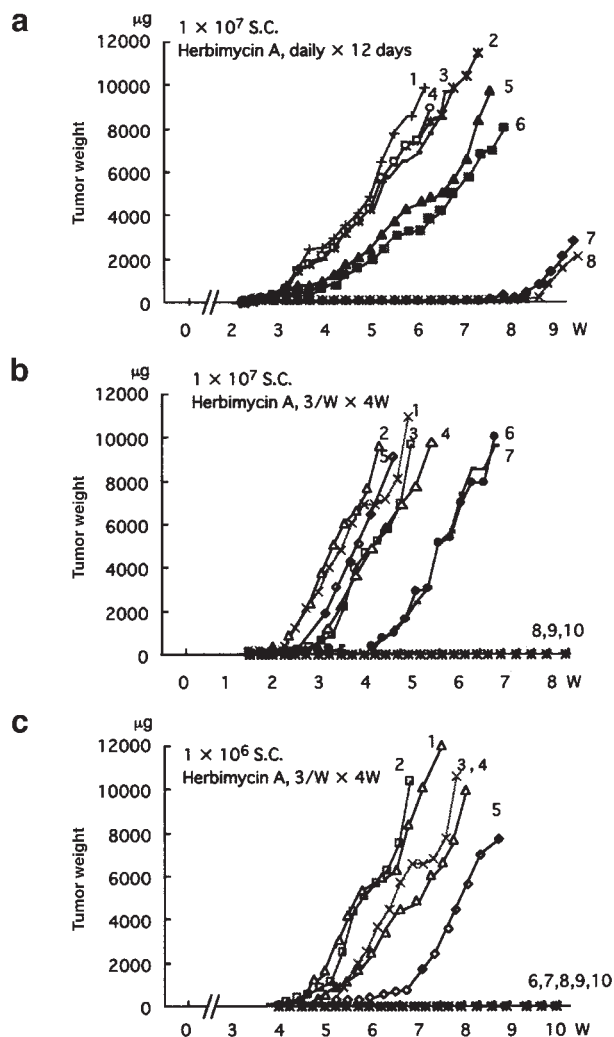
genistein, tyrphostin A9, and erbstatin), the  $IC_{50}$  to mFLT3/32D cells were 8, 3, 0.5 and 13  $\mu M$ , respectively. However, the selective cytotoxicity as in herbimycin A was not observed. Accordingly, mFLT3/32D cells and herbimycin A were used in the murine model below.

#### Inhibition of tyrosine phosphorylation of FLT3 by herbimycin A

To study whether at the above concentrations, herbimycin A directly suppresses the phosphorylation of FLT3, FLT3 cDNA was transfected into Cos7 cells and the phosphorylation status of FLT3 was analyzed (Figure 2). Phosphorylation of FLT3 was augmented by the addition of the FLT3 ligand (FL), which was poorly inhibited by the treatment with herbimycin A. In contrast, mutant FLT3 was significantly phosphorylated in a FL-independent manner. Herbimycin A remarkably suppressed the phosphorylation of mutant FLT3. The expressed level of FLT3 did not change during the treatment with herbimycin A. These results indicated that herbimycin A inhibits the auto-phosphorylation of mutant FLT3 more significantly than the ligand-dependent phosphorylation of wild-type FLT3.

#### Establishment of an *in vivo* model

Inoculation of mFLT3/32D at  $1 \times 10^6$  cells or more, produced subcutaneous tumors in non-irradiated C3H/HeJ mice with 100% efficiency. The latency time was from 2 to 4 weeks, depending on the inoculated cell counts. However, parental 32D cells did not produce tumors even when inoculated at  $2 \times 10^7$  cells. After observation of the subcutaneous tumors, mice died within 5 weeks. Histopathologic examination of the subcutaneous tumors revealed features of myeloid leukemia, including intermediate-sized myeloblasts partly differentiated. All lymph nodes were affected, as was the periportal region of the liver, with diffuse leukemic infiltration of the lung, spleen and bone marrow (Figure 3).



**Figure 4** Effects of herbimycin A on the *in vivo* growth of mFLT3/32D cells in C3H/HeJ mice. (a)  $1 \times 10^7$  cells of 32D/mFLT were injected subcutaneously into 8-week-old female C3H/HeJ mice. A5 to A8 were administered by i.p. injection of 2.5  $\mu g$  herbimycin A from day 1 to 12. A1 to A4 were control mice. All mice died of leukemia within 10 weeks. (b) Mice were inoculated with  $1 \times 10^7$  cells and treated with i.p. injection of 2.5  $\mu g$  herbimycin A on 3 days per week for 4 weeks. B1 to B5 were control mice. (c) The inoculated cell number was reduced to  $1 \times 10^6$ . All of the non-treated mice (C1 to C5) developed subcutaneous tumors within 6 weeks after injection, but the treated mice (C6 to C10) did not develop tumors and survived for more than 16 weeks.

#### *In vivo* treatment with herbimycin A

The effect of herbimycin A was examined in this murine model  $1 \times 10^7$  cells of 32D/mFLT were injected subcutaneously into eight mice (Figure 4a). Half of the mice (A5 to A8) were administered by intraperitoneal (i.p.) injection of 2.5  $\mu g$  herbimycin A from days 1 to 12. Tumor growth inhibition was statistically significant from day 22 ( $P = 0.02$ , by Mann-Whitney  $U$  test), which continued during the observation. On day 28, tumor weight was  $475 \pm 561$  and  $2125 \pm 122$   $\mu g$  in treated and non-treated groups, respectively. However, two of four treated mice (A5 and A6) gradually developed subcutaneous tumors. Another two mice (A7 and A8) had a long latency of tumors and died in the 10th week. Thus survival time was elongated by the treatment ( $P = 0.006$ , by the log-rank test).

One of the treated mice (A5) showed oral bleeding, whereas the other mice displayed no obvious sign of toxicity. This experiment indicates that the treatment for 12 consecutive days could not eradicate leukemia but caused some side-effects.

In the second experiment, we tried a longer treatment schedule, in which mice were administered by i.p. injection 3 days per week for 4 weeks (Figure 4b). In two of five mice (B6 and B7) treated with this schedule, tumor latency was prolonged by approximately the same duration as herbimycin A therapy. In the other three mice (B8 to B10), tumors did not develop. No sign of side-effects was observed. This experiment suggests that the longer schedule with intermittent administration might be better than the short one with daily administration.

In the third experiment, the inoculated cell number was reduced to  $1 \times 10^6$  (Figure 4c). All of the non-treated mice developed subcutaneous tumors within 6 weeks after injection, and tumor weight was  $3380 \pm 2550 \mu\text{g}$  on day 42. However, none of the treated mice developed tumors. Histopathologic examination of the survivors did not reveal any signs of leukemia infiltration. These results suggested that herbimycin A inhibited the growth of tumor or prevented tumor progression, but complete eradication depended on the initial tumor cell number.

## Discussion

In this study, we showed that (1) herbimycin A inhibited the *in vitro* growth of mutant FLT3-transformed 32D cells but not IL-3 dependent 32D cells at the concentration of  $0.1 \mu\text{M}$ ; (2) herbimycin A inhibited the phosphorylation of mutant FLT3 but not that of ligand-stimulated wild-type FLT3; and (3) *in vivo* administration of herbimycin A prolonged the latency of disease or prevented the tumor progression, depending on the number of cells inoculated and the schedule of drug administration.

The first finding indicates that 32D and mFLT/32D cells are useful for screening compounds for mutant FLT3-target therapy. Since the specific inhibitor of FLT3 kinase is unknown, herbimycin A, CGP 52411, genistein, tyrphostin A9, and erbstatin were examined in this study. Erbstatin, genistein and CGP 52411 were isolated or synthesized by the screening for the inhibition of EGFR kinase.<sup>20,21</sup> Tyrphostin A9 is an inhibitor of the kinase activity of the platelet-derived growth factor (PDGF) receptor.<sup>22</sup> Herbimycin A was isolated from the culture broth of a strain of *Streptomyces* on the basis of its ability to revert rat kidney cells transformed by v-src to a normal morphology.<sup>23</sup> Further study of this compound revealed that it inhibited the kinase activity of p60v-src.<sup>24</sup> Now it is known that herbimycin A inactivates various non-receptor tyrosine kinases and reverts cultured cells transformed by oncogenes including src, yes, fps, ros and abl.<sup>25</sup> Direct binding to the Cys residue of the kinase reportedly causes inhibition of the kinase activity. Herbimycin A was also reported to selectively down-regulate RTK,<sup>26</sup> although the expression level of FLT3 did not change in this study. Preferential growth suppression of Ph1-positive human leukemia cell lines has been achieved using low concentrations of herbimycin A.<sup>17,18</sup>

The second finding is particularly notable, in that the ITD of FLT3 does not affect the kinase domain. Research into signal transduction has revealed that several SH2-containing proteins, such as PLC $\gamma$ , SHC, SHP-2 and Grb2, recognize the phosphorylated Tyr residues of FLT3.<sup>27,28</sup> Although the func-

tional role of the JM domain of FLT3 remains unclear, it is possible that like PDGFR, src-family kinase(s) is physically associated with the JM domain.<sup>29</sup> In general, herbimycin A suppresses non-receptor kinases rather than RTK.<sup>25</sup> Accordingly, one reason why herbimycin A inhibited mutant FLT3 more than the wild-type is that since the elongated JM domain might aberrantly recruit some tyrosine kinase(s) which augments phosphorylation of mutant FLT3, herbimycin A inhibits the tyrosine kinase(s) in addition to FLT3 kinase. A cell-free kinase assay is necessary to clarify this issue.

The third finding is important to the development of tyrosine kinase inhibitors as targeted therapy for cancer. In murine models of Bcr-Abl<sup>+</sup> leukemia, administration of herbimycin A prolonged the latency periods of leukemia and/or suppressed the tumor growth, although it could not eradicate leukemia.<sup>18</sup> We showed that the treatment schedule was significantly associated with the therapeutic response. The longer schedule (three times a week for 4 weeks) was more effective than consecutive 12-day administration, even if the total dose was the same. Moreover, the inoculated cell number was a critical factor for *in vivo* response, as pointed out previously.<sup>17</sup> Accordingly, tyrosine kinase inhibitors should not be used solely *in vivo*. Since herbimycin A reportedly accelerates the apoptosis of Bcr-Abl<sup>+</sup> leukemia cells following etoposide treatment or  $\gamma$ -irradiation,<sup>30</sup> the treatment using tyrosine kinase inhibitors might be further improved by the combination with other cytotoxic drugs.

The importance of tyrosine kinase signaling as a potent target for novel cancer treatment has recently been noticed. A synthetic tyrosine kinase inhibitor, CGP57148B, inhibited the phosphorylation of Bcr-Abl and eradicated a Bcr-Abl<sup>+</sup> human leukemia cell line transplanted in nude mice.<sup>19</sup> Although CGP57148B is not specific for Bcr-Abl, *in vivo* the use of the compound produced a significant therapeutic response. The investigators noticed that not a single but a continuous block of Bcr-Abl kinase was needed to produce important biological effects *in vivo*. Further analysis of tyrosine kinase inhibitors in the animal model should identify novel therapies to treat human leukemias. Herbimycin A might serve as a starting compound for the development of derivatives.

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## References

- 1 Furitsu T, Tsujimura T, Tono T, Ikeda H, Kitayama H, Koshimizu U, Sugahara H, Butterfield JH, Ashman LK, Kanayama Y, Matsuzawa Y, Kitamura Y, Kanakura Y. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest* 1993; **92**: 1736–1744.
- 2 Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor beta to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell* 1994; **77**: 307–316.
- 3 Nakao H, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, Sonoda Y, Fujimoto T, Misawa S. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia* 1996; **10**: 1911–1918.
- 4 Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, Sonoda Y, Abe T, Kashima K, Matsuo Y, Naoe T. Internal tandem dupli-

- cation of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. *Leukemia* 1997; **11**: 1605–1609.
- 5 Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, Kuriyama K, Takeshita A, Saito K, Hasegawa S, Shimodaira S, Tamura J, Shimazaki C, Matsue K, Kobayashi H, Arima N, Suzuki R, Morishita H, Saito H, Ueda R, Ohno R, the Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). Internal tandem duplication of *FLT3* associated with leukocytosis in acute promyelocytic leukemia. *Leukemia* 1997; **11**: 1447–1452.
  - 6 Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood* 1999; **93**: 3074–3080.
  - 7 Horiike S, Yokota S, Nakao M, Iwai T, Sasai Y, Kaneko H, Taniguchi M, Kashima K, Fujii H, Abe T, Misawa S. Tandem duplications of the *FLT3* receptor gene are associated with leukemic transformation of myelodysplasia. *Leukemia* 1997; **11**: 1442–1446.
  - 8 Nakano Y, Kiyoi H, Miyawaki S, Asou N, Ohno R, Saito H, Naoe T. Molecular evolution of acute myeloid leukaemia in relapse: unstable N-ras and FLT3 genes compared with p53 gene. *Br J Haematol* 1999; **104**: 659–664.
  - 9 Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, Toiron Y, Birg F, Birnbaum D. Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. *Blood* 1993; **82**: 1110–1119.
  - 10 Lyman S, Jacobsen S. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood* 1998; **91**: 1101–1134.
  - 11 Drexler HG. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. *Leukemia* 1996; **10**: 588–599.
  - 12 Lisovsky M, Estrov Z, Zhang X, Consoli U, Sanchez-Williams G, Snell V, Munker R, Goodacre A, Savchenko V, Andreeff M. Flt3 ligand stimulates proliferation and inhibits apoptosis of acute myeloid leukemia cells: regulation of Bcl-2 and Bax. *Blood* 1996; **88**: 3987–3997.
  - 13 Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, Saito H, Naoe T. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. *Leukemia* 1998; **12**: 1333–1337.
  - 14 Tsujimura T, Morimoto M, Hashimoto K, Moriyama Y, Kitayama H, Matsuzawa Y, Kitamura Y, Kanakura Y. Constitutive activation of *c-kit* in FMA3 murine mastocytoma cells caused by deletion of seven amino acids at the juxtamembrane domain. *Blood* 1996; **87**: 273–283.
  - 15 Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science* 1995; **267**: 1782–1788.
  - 16 Okabe M, Uehara Y, Miyagishima T, Itaya T, Tanaka M, Kuni-Eda Y, Kurosawa M, Miyazaki T. Effect of herbimycin A, an antagonist of tyrosine kinase, on bcr/abl oncoprotein-associated cell proliferations: abrogative effect on the transformation of murine hematopoietic cells by transfection of a retroviral vector expressing oncoprotein P210bcr/abl and preferential inhibition on Ph1-positive leukemia cell growth. *Blood* 1992; **80**: 1330–1338.
  - 17 Okabe M, Uehara Y, Noshima T, Itaya T, Kunieda Y, Kurosawa M. *In vivo* antitumor activity of herbimycin A, a tyrosine kinase inhibitor, targeted against BCR/ABL oncoprotein in mice bearing BCR/ABL-transfected cells. *Leukemia Res* 1994; **18**: 867–873.
  - 18 Honma Y, Matsuo Y, Hayashi Y, Omura S. Treatment of Philadelphia-chromosome-positive human leukemia in SCID mouse model with herbimycin A, bcr-abl tyrosine kinase activity inhibitor. *Int J Cancer* 1995; **60**: 685–688.
  - 19 le Coutre P, Mologni L, Cleris L, Marchesi E, Buchdunger E, Giardini R, Formelli F, Gambacorti-Passerini C. *In vivo* eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. *J Natl Cancer Inst* 1999; **91**: 163–168.
  - 20 Umezawa H, Imoto M, Sawa T, Isshiki K, Matsuda N, Uchida T, Iinuma H, Hamada M, Takeuchi T. Studies on a new epidermal growth factor-receptor kinase inhibitor, erbstatin, produced by MH435-hF3. *J Antibiot* 1986; **39**: 170–173.
  - 21 Ogawara H, Akiyama T, Ishida J, Watanabe S, Suzuki K. A specific inhibitor for tyrosine protein kinase from *Pseudomonas*. *J Antibiot* 1986; **39**: 606–608.
  - 22 Levitzki A, Gilon C. Tyrphostins as molecular tools and potential antiproliferative drugs. *Trends Pharmacol Sci* 1991; **12**: 171–174.
  - 23 Uehara Y, Hori M, Takeuchi T, Umezawa H. Screening of agents which convert ‘transformed morphology’ of Rous sarcoma virus-infected rat kidney cells to ‘normal morphology’: identification of an active agent as herbimycin and its inhibition of intracellular src kinase. *Jpn J Cancer Res* 1985; **76**: 672–675.
  - 24 Uehara Y, Murakami Y, Sugimoto Y, Mizuno S. Mechanism of reversion of Rous sarcoma virus transformation by herbimycin A: reduction of total phosphotyrosine levels due to reduced kinase activity and increased turnover of p60v-src1. *Cancer Res* 1989; **49**: 780–785.
  - 25 Uehara Y, Murakami Y, Mizuno S, Kawai S. Inhibition of transforming activity of tyrosine kinase oncogenes by herbimycin A. *Virology* 1988; **164**: 294–298.
  - 26 Murakami Y, Fukazawa H, Mizuno S, Uehara Y. Effect of herbimycin A on tyrosine kinase receptors and platelet derived growth factor (PDGF)-induced signal transduction. *Biol Pharm Bull* 1998; **21**: 1030–1035.
  - 27 Dosit M, Wang S, Lemischka IR. Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. *Mol Cell Biol* 1993; **13**: 6572–6585.
  - 28 Zhang S, Mantel C, Broxmeyer HE. Flt3 signaling involves tyrosyl-phosphorylation of SHP-2 and SHIP and their association with Grb2 and Shc in Baf3/Flt3 cells. *J Leuk Biol* 1999; **65**: 372–380.
  - 29 Mori S, Ronnstrand L, Yokote K, Engstrom A, Courtneidge SA, Claesson-Welsh L, Heldin CH. Identification of two juxtamembrane autophosphorylation sites in the PDGF beta-receptor; involvement in the interaction with Src family tyrosine kinases. *EMBO J* 1993; **12**: 2257–2264.
  - 30 Riordan FA, Bravery CA, Mengubas K, Ray N, Borthwick NJ, Akbar AN, Hart SM, Hoffbrand AV, Mehta AB, Wickremasinghe RG. Herbimycin A accelerates the induction of apoptosis following etoposide treatment or gamma-irradiation of bcr/abl-positive leukaemia cells. *Oncogene* 1998; **16**: 1533–1542.