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 P210

Herbimycin A, a benzoquinoid ansamycin antibiotic, was demonstrated to decrease intracellular phosphorylation by protein tyrosine kinase (PTK). In Philadelphia chromosome (Ph1)-positive leukemias such as chronic myelogenous leukemia (CML) and Ph1-positive acute lymphoblastic leukemia (ALL), both of which express bcr/abl fused gene products (P210bcr/abl or P190bcr/abl protein kinase) with augmented tyrosine kinase activities, herbimycin A markedly inhibited the in vitro growth of the Ph1-positive ALL cells and the leukemic cells derived from CML blast crisis. However, the same dose of herbimycin A did not inhibit in vitro growth of a broad spectrum of Ph1-negative human leukemia cells, and several other protein kinase antagonists also displayed no preferential inhibition. Furthermore, we demonstrated that herbimycin A has an antagonizing effect on the growth of transformed cells by a transfection of retroviral amphotrophic vector expressing P210bcr/abl into a murine interleukin (IL)-3-dependent myeloid FDC-P2 cell line. This inhibition was abrogated by the addition of sulfhydryl compounds, similar to the reaction previously described for Rous sarcoma virus transformation. The inhibitory effect of herbimycin A on the growth of Ph1-positive cells was associated with decreased bcr/abl tyrosine kinase activity, but no decrease of bcr/abl mRNA and protein, suggesting that the inactivation of bcr/abl tyrosine kinase activity by herbimycin A may be induced by its binding to the bcr/abl protein portion that is rich with sulfhydryl groups. The present study indicates that herbimycin A is a beneficial agent for the investigation of the role of the bcr/abl gene in Ph1-positive leukemias and further suggests that the development of agents inhibiting the bcr/abl gene product may offer a new therapeutic potential for Ph1-positive leukemias.

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MATERIALS AND METHODS

Chemicals. Herbimycin A was isolated as described previously and was used at a concentration of less than 1.0 μg/mL throughout the study. Dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical (Osaka, Japan). Genistin and staurosporine were purchased from Sigma (Munich, Germany). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazin dihydrochloride (H-7) and N-(6-aminoheptyl)-5-chloro-l-naphthalenesulfonamide hydrochloride (W-7) were purchased from Seikagaku Kogyo (Tokyo, Japan).

Transformation of retroviral vector expressing P210bcr/abl into interleukin 3-dependent FDC-P2 cells. Five million FDC-P2 cells were added to a mixture of 30 μL Lipofectin (GIBCO, Berlin, Germany) and 4 μg of pGD210 retroviral vectors expressing P210bcr/abl oncoprotein (Fig 1). The cells were cultured for 16 hours in serum-free Opti-MEM-I (GIBCO) in the presence of recombinant murine interleukin IL-3 (rIL-3) prepared from COS-1 cells.
transfected with pSV2neo containing mIL-3 cDNA (kindly provided by M. Kobayashi, Hokkaido University School of Medicine). The cells were selected in RPMI 1640 medium to which 10% fetal bovine serum (FBS) and 600 μg/mL Geneticin (G418 sulfate; Gibco) were added in the presence of rmIL-3 for 10 days. Geneticin-resistant cells were washed and plated in medium lacking rmIL-3 to select for IL-3-independent growth.

**Leukemic cell lines and patients.** Three Ph1-positive ALL cell lines, the TOM-1 cells, the ALL/MK cells, and the MR-87 cells, and the MC3 cells derived from CML of myeloid crisis have been described previously.13-16 MR-87 cells were kindly provided by Dr. J. Okamura (Kyushu National Cancer Institute, Kyushu, Japan). K562 cells (CML erythroid crisis)17 and NALM-1 cells (CML lymphoid crisis)18 were used as Ph1-positive CML cells in this study. All three Ph1-positive ALL cell lines and the CML cell lines expressed P190Ph1 and P210Ph1b', respectively. HL-60 cells (AML-M2), Reh cells (common ALL), Ball-1 cells (B-ALL), ARH cells and RPMI-8226 cells (myeloma) and MT-1 (adult T-cell leukemia) were used as control Ph1-negative leukemia cells. In addition, AML5q cells (AML-M2) and MO-91 cells (AML-M0) developed by Dr. J. Okamura (Kyushu National Cancer Institute, Kyushu, Japan). Geneticin-resistant cells were washed and plated in medium lacking rmIL-3 to select for IL-3-independent growth.

**Cell cycle analysis by flow cytometry.** IL-3 stimulated FDC-P2 cells or autonomously growing transformant FDC-P2 cells were analyzed for nuclear DNA content using the method previously described by Laneuville et al.21 Briefly, 1 x 10^6 cells were suspended in a solution containing 0.1% sodium citrate, 50 μg/mL propidium iodide (PI), 50 μg/mL RNAase, and 0.1% Triton X-100. The cell cycle was estimated by the analysis of DNA content using Epics C flow cytometer (Couler Electrics, Hialeah, FL).

**Assay of cellular PTK activity.** The cells were cultured with or without herbimycin A for 16 hours, harvested, and washed with phosphate-buffered saline (PBS). PTK activity was assayed according to the modified method of Swarup et al.22 previously described in detail. Briefly 1 x 10^7 cells were suspended in 0.25 mol/L sucrose-Tris buffer (pH 7.5) added to 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 50 mmol/L 2-mercaptoethanol, and 1.5 mmol/L EGTA, followed by homogenization with a Dounce homogenizer. The homogenates were centrifuged at 1,000g for 10 minutes to remove the nuclear fraction and unbroken cells. The resultant supernatant fluid was then centrifuged at 105,000g for 60 minutes to prepare the soluble (cytosol) fraction and the particulate fraction. The particulate fraction was suspended in sucrose-Tris buffer by sonication. The 200-μL reaction volume contained 50 mmol/L Tris-Ci (pH 7.5), 50 mmol/L MgCl2, 10 μmol/L vanadate, 10 μmol/L ZnCl2, and sample (30 μg of protein derived from cells); synthetic random polymer (molecular weight, 46,000; ratio of glutamine to tyrosine, 4:1; Sigma) was used as the substrate. The assay was initiated by the addition of 50 μmol/L (γ-32P)adenosine triphosphate (ATP). After a 10-minute incubation at 30°C, the reaction was halted by the addition of 5% trichloroacetic acid, and bovine serum albumin was then added. The precipitated protein was removed by centrifugation and the supernatant fluid was stopped on phosphocellulose paper. The dried papers were counted for radioactivity in a scintillation counter. Appropriate reaction mixtures containing no synthetic random polymer served as control samples.

**Western blotting and immune complex kinase assay of bcr/abl.** For Western blotting, 1 x 10^7 cells were disrupted in 50 mmol/L sodium phosphate (pH 7.5), 1% Triton X-100, 1 mmol/L PMSF fluoride with sonication. The lysate was centrifuged at 10,000g for 20 minutes. Thirty microliters of the supernatant was then applied to 7.5% gel of sodium dodecyl sulfate (SDS)-polyacrylamide as described by Laemmli.23 After electrophoresis, the gel was electrophoretically transferred to Clear Blot Membrane-P (Atto, Tokyo, Japan). The membrane was prepared for blotting by washing in 2% skim milk for 1 hour at 37°C, and was then reacted with a 1:20 dilution of mouse anti c-abl monoclonal antibody, Ab-3 (Oncogene Science, Mineola, NY) overnight at 4°C. The membrane was washed with PBS with 0.05% Tween 20 four times, and was then reacted with peroxidase-conjugated rabbit anti-mouse IgG for 2 hours at room temperature. The band of antibody bound to c-abl protein was detected by a KONICA HR Immunostain Kit (Konica, Tokyo, Japan).

The immune complex kinase assay was performed using a modified method described by Kurzrock et al.24 Briefly, the cells (1 x 10^7) were collected and washed with PBS and then lysed at

![Figure 1](image-url)
4°C by sonication in 50 mmol/L Tris-HCl (pH 7.5), 0.15 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L PMSF buffer. The diluted sample was treated with mouse monoclonal antibody against c-abl, Ab-3, for 1 hour at room temperature and was incubated overnight at 4°C. Immune complex with anti-c-abl antibody was precipitated with mouse anti-IgG antibody-coated protein A (Zymed Laboratories, San Francisco, CA), and immunoprecipitates were then reacted with 5 μCi (γ-32P)ATP for 10 minutes on ice. Next, immune complexes were analyzed on 7.5% SDS-polyacrylamide gels, followed by autoradiography with an intensifying screen.

Analyses of expression of bcr-abl mRNA. RNA was extracted by the guanidinium, thiocyanate-cesium chloride method. In Northern blot analysis, 20 μg of total RNA was electrophoresed through 1.0% agarose containing 7% formaldehyde. After the gel was blotted onto a Biodyne membrane (Pall, Glen Cove, NY), the membrane was hybridized with a nick-translated abl-specific probe (1.5-kb BglII fragment of pAb3).

Expression of results. Unless otherwise indicated, mean values ± 1 SD for measurements from triplicate culture are presented. Significance of difference was assessed using Student’s t-test; results were assessed significantly different at P < .01.

RESULTS

Herbimycin A abrogates the in vitro growth of P210(bcr-abl) expressing FDC-P2 cells. IL-3–dependent FDC-P2 cells were transfected with pGD210 murine retroviral vector expressing P210(bcr-abl) or mock pZIPNeoSV(X) in the presence of 100 U/mL of rmIL-3 and subsequently selected with G418, followed by transfer to medium free of mIL-3. In the absence of mIL-3, parental or mock-transfected cells died within 72 hours. In contrast, G418-resistant pGD210(bcr-abl) transfected cells grew in the media free of mIL-3 (Fig 2A). A Northern blot of total RNA derived from pGD210(bcr-abl) transfected transformed cells showed transcripts of bcr-abl and neo genes (Fig 2B). The expression of bcr-abl was confirmed by the immune complex kinase assay of bcr-abl protein from these cells (Fig 2B).

We have previously demonstrated that herbimycin A causes reversion of the transformed Rous sarcoma virus–infected rat kidney cells with reduction of P60v-src protein kinase activity.25 We investigated whether herbimycin A antagonizes P60v-src oncprotein-associated autonomous growth of FDC-P2 cells transformed by the transfection of the bcr-abl gene. Herbimycin A showed no significant inhibition of the growth of parental FDC-P2 cells in the presence of mIL-3, but did show a profound inhibition of the growth of transformant FDC-P2 cells in a dose-dependent fashion (Fig 3). By cell cycle analysis, the transformant FDC-P2 cells showed an increased DNA content and a striking increase of the fraction of cells in S phase. The treatment of transformant cells with herbimycin A for 16 hours decreased the fraction of cells in G1 and S phases (Fig 4).

Effects of herbimycin A against in vitro growth of various human leukemia cell lines and fresh leukemia cells. The effects of herbimycin A on the in vitro growth of human leukemic cells were screened in the liquid culture. The results concerning tests on the effect of herbimycin A on the in vitro growth of various cell lines in the liquid culture for 5 days are shown in Fig 5. Herbimycin A in excess of 1 μg/mL resulted in growth inhibition of a broad range of leukemic cell lines. However, at a concentration of less than 0.5 μg/mL, herbimycin A displayed no significant growth inhibition of a variety of selected leukemic cells presenting no Ph1, except for one cell line, MO-91, which we established from an AML-M0 patient, where herbimycin A exhibited the preferential inhibitory effects on the in vitro growth seen in all Ph1-positive leukemic cell lines. Growth inhibition as a result of incorporation of herbimycin A was more remarkable in the Ph1-positive lymphoblast cells, Ph1-positive ALL cells (TOM-1 cells, ALL/M1K cells, and MR-87 cells), and NALM-1 cells derived from CML of lymphoid crisis, than was observed in those of Ph1-negative nonlymphoblast cells, the K562 cells and the MC3 cells. In Ph1-positive lymphoid cells, most cells died by or on the 5th day when cultured in the presence of less than 0.5 μg/mL of herbimycin A. Fifty percent inhibition of cell growth occurred at a concentration of less than 0.02 μg/mL against the Ph1-positive lymphoid cells, and at a concentration of approximately 0.2 μg/mL against the Ph1-positive nonlymphoid cells, K562 and MC3 cells. On the other hand, other antagonists, including Genistain (tyrosine kinase antagonist), staurosporine or H7 (antagonists of protein kinase C), and W7 (calmodulin antagonist), showed no specific inhibition of the in vitro growth of Ph1-positive cell lines at the nontoxic dose for a broad range of human cells in vitro (data not shown).

Furthermore, herbimycin A suppressed 3H-TdR incorporation of the leukemic cells obtained from all four Ph1-positive ALL cases and two myeloid crisis cases, whereas suppression of 3H-TdR incorporation was observed in only some of the leukemic cells presenting no Ph1 (Fig 6).

Inhibition of bcr-abl protein kinase by herbimycin A. To clarify whether the growth inhibition of Ph1-positive leukemic cells by herbimycin A is associated with the inhibition of bcr-abl tyrosine kinase, we performed an assay of cellular PTK activity. The cells were cultured with or without herbimycin A for 16 hours and then assayed for cellular PTK activity in the cytosole and particulate fractions. The PTK activities of both fractions were remarkably decreased in Ph1-positive leukemia cell lines when compared with those of an AML cell line, AMLS5q, presenting no Ph1 (Fig 7). The immune complex kinase assay of bcr-abl on the K562 cells showed that herbimycin A dramatically suppressed the autophosphorylation activity of P210(bcr-abl) tyrosine kinase, although it did not affect bcr-abl mRNA and bcr-abl protein expression (Fig 8).

Abrogation of herbimycin A action on Ph1-positive cells by the addition with sulfhydryl compounds. We have previously reported the possibility that sulfhydryl groups of p60v-src are involved in the inactivation of v-src tyrosine kinase activity by herbimycin A because of abrogation of the ability of herbimycin A to inactivate p60v-src kinase, as well as the ability to reverse transformed cell morphology by the addition of a sulfhydryl compound.26 We investigated the effect of sulfhydryl compounds such as DTT or 2-ME on the herbimycin-induced growth inhibition of Ph1-positive leukemia cells. As shown in Fig 9, the addition of either
Fig 2. (A) Autonomous growth of transformed FDC-P2 cells by transfection of a retroviral vector expressing P210\textsuperscript{bcr/abl}. WEHI-3B cell conditioned medium was used as mIL-3 in this study. MTT proliferation assay was performed after 72 hours of cultivation. The proliferation of parental FDC-P2 cells is dependent on mIL-3, but the transformant FDC-P2 cells transfected with pGD210\textsuperscript{bcr/abl} are able to proliferate autonomously in the absence of mIL-3. Bars indicate 1 SD. (B) Expression of \textit{bcr}/abl in transformant FDC-P2 cells. Northern blot of total cellular RNA derived from transformant cells hybridized to \textit{bcx} and neo probes. The immune complex kinase assay of anti-abl immunoprecipitates showed the expression of P210\textsuperscript{bcr/abl} in transformant cells.

DISCUSSION

Recent progression of study on the role of \textit{bcx}/\textit{abl} genes in the leukemogenesis of Ph\textsuperscript{1}-leukemias offers opportunities for improvements in the diagnosis and analysis of pathophysiology. In addition, the oncogenic role of \textit{bcx}/\textit{abl} fused protein is supported by the oncogenic outcome of transgenic mice or bone marrow-transplanted mice using transduction of the \textit{bcx}/\textit{abl} gene.\textsuperscript{12,25} Therefore, several strategies of a specific therapy for Ph\textsuperscript{1}-positive leukemia such as exploiting antagonists against the expression or product of \textit{bcx}/\textit{abl} gene are warranted. For example, disturbance of \textit{bcx}/\textit{abl} function with antisense oligodeoxynucleotides might offer the possibility for a new category of leukemia therapy in the future.\textsuperscript{26} A recent and promising strategy for therapy may be selective inhibition of tyrosine kinase.\textsuperscript{27} Tryphostin, a derivative molecule of erbstain, which is a prototype tyrosine analogue, has been reported

2-ME or DTT abrogated the inhibitory effects of herbimycin A on the growth of Ph\textsuperscript{1}-positive cells.
Concentration of herbimycin A (μg/ml)

Fig 3. Abrogative effect of herbimycin A on transformant FDC-P2 cells. Effects of herbimycin A on IL-3-dependent growth of parental FDC-P2 cells and autonomous growth of transformant FDC-P2 cells were assayed by MTT proliferation assay after 72 hours of cultivation. Bars indicate 1 SD.

to block phosphorylation of tyrosine residue and inhibit epidermal growth factor (EGF)-dependent cell proliferation in vitro at concentrations showing little toxicity,28 thus providing an insight into development of therapy against various types of cancers associated with amplifications of erb B-1 and -2 oncogenes. In the present study, we analyzed the effect against bcr/abl oncoprotein-associated cell proliferation of herbimycin A, a protein kinase inhibitor that we isolated on the basis of its ability to cause rat kidney cells transformed by v-src to revert from the rounded, transformed morphology to the normal, more flattened morphology with a loss of PTK activity of the transforming protein of p60v-src.9 Until now, it was believed that the antitumor effect of herbimycin A was relatively selective for cells transformed by oncogenes coding PTK such as src, yes, fes, ros, abl, and erb B.10 In this study, we demonstrated that herbimycin A antagonizes the transformation of IL-3-dependent murine hematopoietic cells by transfection of bcr/abl genes. Further, we showed that it effectively inhibits the in vitro growth of Ph1-positive leukemia cells in less than 0.2 μg/mL concentrations exhibiting little toxicity, whereas Genistain (another inhibitor of tyrosine kinase), as well as other antagonists of protein kinase including staurosporine, H7, and W7, showed no preferential inhibition on Ph1-positive leukemic cells. As these results suggest the likelihood that herbimycin A-induced inhibition of Ph1-positive leukemia cells results from the inhibition on bcr-abl kinase, we investigated the effect of herbimycin A on the cellular PTK activity. The present study showed that herbimycin A treatment reduced the activity of cellular PTK associated with inhibition of bcr/abl kinase activity in Ph1-positive leukemia cells. The inhibitory activity by herbimycin A on the in vitro growth of Ph1-positive cells was abrogated by sulphydryl compounds such as 2-ME or DTT. Similarly, we previously reported that the reversal action on Rous sarcoma virus transformation by herbimycin A was abrogated by the addition of various sulphydryl compounds.
Fig 5. The effects of herbimycin A on the in vitro growth of human leukemia cell lines in liquid culture. Cells were cultured with or without various concentrations of herbimycin A in 96-well culture plates, and MTT proliferation assay was performed 5 days later. As the optical measurements were verified to be correlated to viable cell numbers, the percentage of mean value of OD in wells cultured in the presence of herbimycin A to those without herbimycin A was calculated as the percentage of cell number.

Fig 6. The inhibitory effect of herbimycin A on the in vitro growth of leukemic cells obtained from patients with Ph'-positive or -negative leukemias. \(^{3}H\)-TdR incorporation was inhibited significantly with herbimycin A (0.2 \(\mu\)g/mL) treatment in all cases of Ph'-positive leukemia cells from four Ph'-positive ALL cases presenting either M-bcr rearrangement or its nonrearrangement, and two CML cases in myeloid crisis phase \((P < .01)\).

Fig 7. Suppression of cellular PTK activity in Ph'-positive cells by herbimycin A. PTK activity of the cytosol (○) and particulate (□) fraction was assayed in the leukemic cell lines treated with 0.2 \(\mu\)g/mL of herbimycin for 16 hours and in nontreated cell lines. As a control sample of Ph'-negative leukemia, the AML5q cell line (AML M2, unpublished) was used in this study. % Suppression of PTK was calculated according to the following formula: % Suppression of PTK = \((1 - \text{PTK activity (pmol/min/\(\mu\)g protein) of treated cells})/\text{PTK activity (pmol/min/\(\mu\)g protein) of nontreated cells}) \times 100\%\).

due to the formation of an adduct with a thiol compound, possibly through conjugation between the highly polarized double bonds (position 17 or 19) of the benzoquinone moiety of herbimycin A and the highly reactive sulfhydryl group of thiols29 (Fig 10). On the other hand, herbimycin A exhibited no obvious inhibition on the expression of bcr-abl mRNA and the amount of bcr-abl oncoprotein. In addition, we have previously shown the specific inhibition of cytoplasmic PTK of P210bcr-abl by herbimycin A in vitro.30 These results suggest that the antibiotic is likely to bind reactive groups of bcr-abl or bcr-abl protein kinase (p210 or p190) through the same mechanism as described above because of a high homology in src homology regions, including kinase domain, between both gene products. Determining the binding site of herbimycin A may offer new insights into the structural and regulatory mechanisms of the bcr-abl tyrosine kinase in expressing kinase activity.

(continued)
Herbimycin A showed more profound inhibition on the growth of Ph'-positive lymphoblast cell lines, irrespective of whether they present P190(Kr-ABL) or P210(Kr-ABL), than it did on nonlymphoid crisis cell lines. At present, it is uncertain whether this finding may indicate an alternative sensitivity to herbimycin A between lymphoid cells and nonlymphoid cells, because of an involvement of other oncogenes and/or anti-oncogenes other than bcr-abl gene in the proliferation of nonlymphoid crisis cell lines used in this study. In fact, we have found the alteration of p53 anti-oncogene in the MC3 cells and the K562 cells, which was not found in 4 Ph'-positive lymphoid cells.

Finally, herbimycin A promises to be an effective ex vivo...
Fig 10. The structure of herbimycin A and presumable mechanism of herbimycin A binding to thiol groups of PTK.

bone marrow-purging agent in autologous bone marrow transplantation of Ph1-positive leukemias, and, in the future, might offer promising therapeutic potential. Investigations of these potential uses for herbimycin A, including its in vivo antitumor effect in a mouse model, are in progress. Furthermore, this study suggests the possibility that antagonists against oncogene products such as herbimycin A may provide important insights into the study of leukemogenesis and into the development of a new, promising therapy in leukemias.

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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

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