Regular Article

Comparative Study of the Anti-leukemic Effects of Imatinib Mesylate, Glivec™ Tablet and Its Generic Formulation, OHK9511

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Long-term treatment with imatinib mesylate (IM) allows patients with chronic myeloid leukemia (CML) to live a near-normal lifespan. However, the fact that tyrosine kinase inhibitors, including IM, are extremely expensive is a major cause of poor adherence, resulting in disease relapse or drug resistance. Therefore, physicians are encouraged to prescribe generic drugs to reduce the financial burden of medical expenses. In Japan, only generic drugs that have a basic chemical structure and pharmacokinetic data that are the same as those of the original drug are approved. However, it is not mandatory to demonstrate that generic drugs have adequate biological effects. This is one of the reasons why Japanese hematologists do not often use generic IM. The aim of the present study was to compare the anti-leukemic effects of Glivec™ (a commercial IM) and its generic formulation, OHK9511. The IC₅₀ values of OHK9511 and Glivec™ were comparable, and both induced similar levels of apoptosis in several CML cell lines. Furthermore, the overall survival of OHK9511-treated mice transplanted with BCR-ABL-positive cells was similar to that of mice treated with Glivec™. Although the experiments performed herein were basic, the results suggest that physicians should consider using generic IM.

Key words OHK9511; generic; imatinib mesylate; chronic myeloid leukemia; adherence

Advances in the field of molecular targeting therapies have revolutionized cancer treatment, and molecular-targeted drugs have superior anticancer effects than conventional chemotherapeutic agents. For example, ABL tyrosine kinase inhibitors (TKIs) such as imatinib mesylate (IM; Glivec™), nilotinib, and dasatinib are used to treat patients with chronic myeloid leukemia (CML).^{1,2} In the pre-IM era, the median survival time for patients with CML was 5 to 6 years; the introduction of IM has improved the estimated 10-year survival from 20% to 80%.³ Glivec™ was the first commercially available IM.⁴

The cost of new cancer agents has increased over the last decade.⁵ Poor adherence to TKIs results in treatment failure due to the emergence of drug-resistant CML clones; indeed, at least 30% of patients are non-adherent to TKIs,^{6,7} a finding that has been linked to the high cost of TKIs.⁸ Therefore, the use of generic drugs is encouraged due to the potential cost savings. If a generic formulation is equivalent to the original drug in terms of qualitative and quantitative composition, it can be marketed in Japan as a “generic” without the need to conduct expensive clinical trials (the drug only need undergo pharmacokinetic (PK) testing). However, questions about the efficacy and safety of generic drugs remain, preventing the wider use of these agents. The aim of the present study was to compare the biological effects of the first commercially available IM (Glivec™) with those of its generic formulation, OHK9511.

MATERIALS AND METHODS

Reagents Glivec™ was purchased from Novartis Pharma (Basel, Switzerland). Imatinib extracted from the formulation recrystallized from acetone. We confirmed that impurities

were not contained in those crystals by measuring the NMR spectrum. In addition, it was confirmed that the crystal had enough purity by measuring it using high performance liquid chromatography (HPLC). The InertSustain C18 column (150 mm×4.6 mm, 3 μm; GL Sciences, Tokyo) was used. The mobile phase consisted of water (dissolve 0.79 g of ammonium hydrogen carbonate in 1000 mL of water, adjust to pH 9.0 with ammonium hydroxide, (A)) and methanol (B), and the linear gradient was as follows: time after injection of sample (0–50 min), mobile phase A (50–10%), mobile phase B (50–90%). The detector recorded UV spectra, and the HPLC chromatogram was monitored at 254 nm. The active pharmaceutical ingredient (API) of Glivec™ was isolated from the tablets as a colorless powder (purity: ca. 95% by HPLC analysis). The API of the generic drug OHK9511 was isolated as a colorless powder (purity: ca. 97% by HPLC analysis) in a similar manner. These procedures were performed at Ohara Pharmaceutical Co., Ltd. (Shiga, Japan). Because bulk used for OHK9511 is manufactured from the Japanese manufacturer different from the original drug (Glivec™), the process of manufacture is also different. The APIs were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, U.S.A.) at a final concentration of 10 mM and aliquots were stored at –80°C until required. For the *in vivo* experiments, these tablets were grinded in a mortar and dissolved as 200 mg/kg mouse weight aliquots in 0.5% methylcellulose (5 μg/mL).

Cell Lines and Culture Conditions The CML-derived cell lines, K562, BV173, KCL22, KBM-5, and MYL, were used in this study. KBM-5/STIR, a subclone of KBM-5 harboring a mutation in BCR-ABL (T315I), and MYL-R, a subclone of MYL overexpressing LYN, were also used.^{9,10} Mouse pro-B cell (Ba/F3) lines expressing p210 BCR-ABL (wild-type

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Fig. 1. Growth Inhibition by OHK9511 or Glivec™ in CML Cells

K562, BV173, KCL22, KBM-5, KBM-5/STIR, MYL, MYL-R, Ba/F3 BCR-ABL^{WT}, and Ba/F3 BCR-ABL^{T315I} cells were cultured in the presence of the indicated concentrations of OHK9511 or Glivec™. After 48 h, the effects on cell growth were examined in an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and the IC₅₀ (inhibitory concentration, 50%) was calculated. Graphs show the mean ± S.E.M. of three independent experiments.

[WT]) or T315I were established as previously described.¹¹⁾ The sources of all the cell lines used are listed in Supplemental Table 1. All cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO₂.

Mice Mice (5–6-week-old Balb/cAJcl-nu/nu [nude] mice and 8-week-old NOD/ShiJic-scid Jcl [NOD/SCID] mice) were purchased from CLEA Japan, Inc. (Osaka, Japan) and used for the *in vivo* transplantation experiments. All animal experiments were approved by the Saga University Institutional Review Board.

Cell Viability and Measurement of Apoptosis Cell proliferation was determined using the trypan blue dye exclusion method or the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, U.S.A.) according to the manufacturer's protocol. IC₅₀ values (inhibitory concentration, 50%) were assessed using CalcuSyn software (BioSoft, Cambridge, U.K.) as previously described.¹²⁾ Apoptotic cell death was analyzed in an Annexin V-propidium iodide

(PI) binding assay as previously described.¹³⁾ Briefly, fresh cells were washed twice with binding buffer (10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 140 mM NaCl, and 5 mM CaCl₂, pH 7.4) and then stained with fluorescein isothiocyanate-conjugated Annexin V (Roche Diagnostics, Indianapolis, IN, U.S.A.) and PI. Annexin V fluorescence was measured in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). Flow cytometric data were analyzed using CellQuest software (BD Biosciences, San Diego, CA, U.S.A.).

Western Blot Analysis Western blot analysis was performed as previously described.¹⁴⁾ Cells were solubilized in lysis buffer (phosphate buffered saline (PBS), 1× cell lysis buffer [Cell Signaling Technology, Beverly, MA, U.S.A.], 1× protease inhibitor [Roche], 1× phosphatase inhibitor cocktails I and II [Calbiochem, San Diego, CA, U.S.A.]) and incubated for 30 min on ice. Subsequently, the lysates were centrifuged for 10 min at 10000×g at 4°C and the supernatants were analyzed. The protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Her-

Fig. 2. Apoptosis Induced by OHK9511 or Glivec™ in CML Cells

The percentage of apoptotic cells was quantified by Annexin V/PI staining of K562 and BV173 cells at 24 and 48 h post-treatment. Four independent experiments were performed. Data are expressed as the mean $\pm$ S.E.M. * $p < 0.05$.

cules, CA, U.S.A.) according to the manufacturer's protocol. Total protein (30 μ g) was separated in sodium dodecyl sulfate polyacrylamide gels (Bio-Rad Laboratories) and transferred to polyvinylidene-fluoride membranes (0.45 μ m; GE Healthcare, Buckinghamshire, U.K.). The blots were then probed with appropriate primary and secondary antibodies according to the manufacturer's protocols. The following primary antibodies were used: anti-c-Abl (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-c-Kit (DAKO, Atlanta, GA, U.S.A.), anti-platelet-derived growth factor receptor (PDGFR) Type A/B (Upstate Biotechnology, Lake Placid, NY, U.S.A.), anti-phosphotyrosine-PY20 (BD Transduction Laboratories, San Jose, CA, U.S.A.), anti- α -tubulin (Sigma-Aldrich, St. Louis, MO, U.S.A.), anti-Stat5, and anti-p-Stat5Tyr694. Horseradish peroxidase-linked anti-mouse and anti-rabbit immunoglobulin G (IgG) (all from Cell Signaling Technology) were used as secondary antibodies.

Murine Leukemia Model Two different experimental protocols were used. First, nude mice were intravenously injected with 1×10^4 enhanced green fluorescent protein (EGFP)⁺ Ba/F3 BCR-ABL^{WT} cells, as previously described.¹⁵ These mice were then orally administered OHK9511 (200 mg/kg/dose), Glivec™ (200 mg/kg/dose), or vehicle alone (0.5% methylcellulose) twice daily from Day 2 through to Day 11 post-injection.

The second protocol utilized a previously described human leukemia xenograft model.¹⁶ Briefly, BV173 cells (1×10^6) were intravenously injected into sublethally irradiated (2 Gy) NOD/SCID mice. After 8 d, xenotransplanted mice were orally administered OHK9511 (200 mg/kg/dose), Glivec™ (200 mg/kg/dose), or vehicle alone (0.5% methylcellulose) twice daily for 10 d, beginning 8 d after transplantation. Survival was monitored daily, and mice were sacrificed when they became moribund or were unable to take food or water (as recommended by the institutional guidelines of Saga University).

Statistical Analysis Data are expressed as the mean $\pm$ S.E.M. A Student's paired *t*-test was used to compare data between groups. The *p*-values < 0.05 were considered statistically significant. To analyze *in vivo* efficacy, survival curves were created using the Kaplan–Meier method and compared using the log-rank test.

RESULTS

Growth Inhibitory Effects and Apoptosis Induction by OHK9511 First, we compared the effects of OHK9511 and Glivec™ on the viability of leukemia cell lines. OHK9511 suppressed the growth of IM-sensitive BCR-ABL-positive cell lines (K562, BV173, KCL22, KBM5, MYL, MYL-R, and Ba/F3 BCR-ABL^{WT}) (Fig. 1). The *in vitro* growth inhibitory

Fig. 3. OHK9511 Blocks the Autophosphorylation of Wild-Type BCR-ABL and Other Tyrosine Kinases

The expression of Stat5, phosphorylated (p-)Stat5, c-Abl, p-c-Abl, c-Kit, p-c-Kit, PDGFR, and p-PDGFR was examined in K562 and BV173 cells at 24 h post-treatment with the indicated concentrations of OHK9511 or Glivec™. The results are representative of three independent experiments. Intensity of the immunoblot signals after background subtraction was quantified using ImageJ software, and the relative intensities of p-Stat5, p-c-Abl, p-c-Kit, and p-PDGFR compared to those of Stat5, c-Abl, c-Kit, and PDGFR were respectively calculated.

A. Ba/F3 BCR-ABL^{WT} to Balb/cA nu/nu

B. BV173 to NOD/SCID

Fig. 4. *In Vivo* Activity of OHK9511 or Glivec™ in Murine BCR-ABL-Induced Leukemia Models

(A) Survival of mice transplanted with EGFP⁺ Ba/F3 BCR-ABL^{WT} cells. EGFP⁺ Ba/F3 BCR-ABL^{WT} cells (1×10^4) were transplanted into 8-week-old nude mice. Two days after transplantation, 200 μL of vehicle, 200 mg/kg/dose OHK9511, or 200 mg/kg original IM were administered orally twice a day for 10 d. Survival was then monitored daily. Kaplan-Meier survival curves were constructed and survival was analyzed using a nonparametric log-rank test. * $p=0.017$, ** $p=0.0102$. (B) Survival of human leukemia-xenografted mice transplanted with BV173 cells (1×10^6) were intravenously injected into irradiated (2 Gy) NOD/SCID mice. Eight days after transplantation, mice received 200 μL of vehicle, 200 mg/kg/dose OHK9511, or 200 mg/kg Glivec™ orally twice a day for 10 d. Survival was monitored daily. Kaplan-Meier survival curves were constructed and survival data were analyzed using a nonparametric log-rank test. * $p=0.035$, ** $p=0.006$.

effects of OHK9511 were not significantly different from those of Glivec™. Furthermore, OHK9511 was not effective against KBM-5/STIR or Ba/F3 BCR-ABL^{T3151}, which are IM-resistant cell lines (Fig. 1). The IC₅₀ values for OHK9511 and Glivec™ against IM-sensitive cell lines ranged from 0.11–0.6 μM and 0.07–0.84 μM, respectively (Fig. 1).

We also examined apoptosis induction in OHK9511- and Glivec™-treated cells by Annexin V and PI staining. The percentages of apoptotic cells increased in a time-dependent manner after treatment with OHK9511 or Glivec™ (Fig. 2).

OHK9511 Blocks Autophosphorylation of BCR-ABL

We next compared the ability of OHK9511 and Glivec™ to inhibit the tyrosine kinase activity of WT BCR-ABL. BCR-ABL-positive K562 and BV173 cells were treated with both drugs and the autophosphorylation of BCR-ABL was examined by Western blotting. As shown in Fig. 3, both OHK9511 and Glivec™ blocked BCR-ABL autophosphorylation. Next, we compared the ability of OHK9511 and Glivec™ to inhibit the tyrosine kinase activity of native BCR-ABL by examining the inhibitory effects of both drugs on the tyrosine kinase activity of PDGFR and c-Kit. OHK9511 suppressed the phosphorylation of PDGFR and c-Kit at levels comparable with

those of Glivec™ (Fig. 3).

In Vivo Effects of OHK9511 in BCR-ABL-Related Leukemia Models To investigate the effects of OHK9511, we generated mouse models of BCR-ABL-induced leukemia and then treated them with OHK9511 or Glivec™ (see Materials and Methods for details of the two experimental protocols used). Engraftment of Ba/F3 BCR-ABL^{WT} cells was confirmed by detecting EGFP⁺ cells in the bone marrow of control mice by flow cytometry (data not shown). The overall survival of mice treated with OHK9511 was significantly higher than that of vehicle-injected mice, and was comparable with that of mice treated with Glivec™ (Fig. 4A).

In the human leukemia xenograft model, mice treated with OHK9511 or Glivec™ survived longer than control mice. There was a statistically significant difference between the overall survival of vehicle- and OHK9511-treated mice, and the overall survival of mice treated with Glivec™ was comparable with that of mice treated with OHK9511 (Fig. 4B).

DISCUSSION

This study compared the anti-leukemic effects of Glivec™ with those of its generic formulation, OHK9511. The results showed that OHK9511 was just as effective as Glivec™ both *in vitro* and *in vivo*. OHK9511 suppressed the growth of BCR-ABL-positive cell lines but not that of KBM-5/STIR or Ba/F3 BCR-ABL^{T3151} cells at concentrations similar to those of Glivec™. The apoptosis-inducing and BCR-ABL-blocking activities of the two drugs were not significantly different. In addition, OHK9511 prolonged the survival of mice engrafted with BCR-ABL-positive cells (Ba/F3 BCR-ABL^{WT} or BV173).

The introduction of Glivec™ led to a marked improvement in the quality of life of CML patients due to lower levels of toxicity; indeed, the overall survival of patients treated with Glivec™ is not different from that of the general population.^{3,17,18} However, TKIs are extremely expensive and patients must take them every day for the remainder of their lives. Poor adherence to Glivec™ results in CML progression and treatment failure due to the emergence of drug-resistant CML clones.^{19,20} Dusetzina *et al.* reported that 17% of CML patients with higher co-payments discontinued Glivec™ therapy within the first 180 d, and that 30% of patients with higher co-payments were non-adherent.⁸ Kodama *et al.* conducted a retrospective survey of the household incomes of Japanese CML patients, taking into account out-of-pocket medical expenses, final co-payment after refunds, and the perceived financial burden of medical expenses. They found that age, household income, and final co-payments for medical expenses were predictors of Glivec™ discontinuation.²¹ These results suggest a need to reduce the costs of CML treatment incurred by the patient.

A generic formulation must be bioequivalent to the original brand name drug. In Japan, a drug can be marketed as “generic” without undergoing comprehensive preclinical studies or expensive clinical trials (the only data required are those from PK studies of healthy volunteers). However, inter-individual pharmacokinetic variability is affected not only by genetic heterogeneity of drug targets, but also by the pharmacogenetic background of the patient (*e.g.* cytochrome P450 and ABC transporter polymorphisms), adherence to treatment, and environmental factors such as drug–drug interaction.²² Several

case reports demonstrate hematologic relapse after switching from Glivec™ to a generic IM.^{23–26} By contrast, recent reports show that the efficacy and tolerability of generics were comparable with those of Glivec™ after switching.^{27–29} In the former reports,^{23–26} the measurement of plasma concentration of imatinib was not performed at before and after changing to generic drugs. Accumulating evidence indicates that trough imatinib plasma level is correlated to response in the treatment of patients with CML.^{30–32} Therefore, physicians should carry out the therapeutic drug monitoring (TDM) at before and after changing to generic drugs, because the clinical bioequivalence of generic products has not been clearly established.

In summary, the present study showed that the anti-leukemic effects of OHK9511 were comparable to those of Glivec™ both *in vitro* and *in vivo*. Ideally, prospective randomized trials should be performed to confirm the efficacy of generic formulations; however, such trials are too expensive. In this regard, our data ensure the efficacy of OHK9511 to BCR-ABL-positive cells, and may help to select OHK9511 among many generics to treat CML patients. The basic *in vitro* and *in vivo* experiments described herein are another option and could easily be performed to address the efficacy of generic drugs, which may give physicians more confidence to use them.

Conflict of Interest This study was funded by a research grant from Ohara Pharmaceutical Co., Ltd. S.K. is a consultant for and has received research funding from Novartis, and has received honoraria from Bristol-Myers Squibb.

Supplementary Materials The online version of this article contains supplementary materials.

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