2-Hydroxypropyl-β-Cyclodextrin Acts as a Novel Anticancer Agent

Masako Yokoo1, Yasushi Kubota1,2*, Keiichi Motoyama3, Taishi Higashi3, Masatoshi Taniyoshi3, Hiroko Tokumaru3, Rena Nishiyama3, Yoko Tabe4, Sakiko Mochinaga5, Akemi Sato1, Naoko Sueoka-Aragane1, Eisaburo Sueoka2,6, Hidetoshi Arima3,7, Tetsumi Irie3,7, Shinya Kimura1

1 Division of Hematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga, Japan, 2 Department of Transfusion Medicine, Saga University Hospital, Saga, Japan, 3 Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan, 4 Department of Clinical Laboratory Medicine, Juntendo University School of Medicine, Tokyo, Japan, 5 Department of Pharmacy, Saga University Hospital, Saga, Japan, 6 Department of Clinical Laboratory Medicine, Faculty of Medicine, Saga University, Saga, Japan, 7 Program for Leading Graduate Schools “HIGO (Health life science: Interdisciplinary and Global Oriented) Program”, Kumamoto University, Kumamoto, Japan

* kubotay@cc.saga-u.ac.jp

Abstract

2-Hydroxypropyl-β-cyclodextrin (HP-β-CyD) is a cyclic oligosaccharide that is widely used as an enabling excipient in pharmaceutical formulations, but also as a cholesterol modifier. HP-β-CyD has recently been approved for the treatment of Niemann-Pick Type C disease, a lysosomal lipid storage disorder, and is used in clinical practice. Since cholesterol accumulation and/or dysregulated cholesterol metabolism has been described in various malignancies, including leukemia, we hypothesized that HP-β-CyD itself might have anticancer effects. This study provides evidence that HP-β-CyD inhibits leukemic cell proliferation at physiologically available doses. First, we identified the potency of HP-β-CyD in vitro against various leukemic cell lines derived from acute myeloid leukemia (AML), acute lymphoblastic leukemia, and chronic myeloid leukemia (CML). HP-β-CyD treatment reduced intracellular cholesterol resulting in significant leukemic cell growth inhibition through G2/M cell-cycle arrest and apoptosis. Intraperitoneal injection of HP-β-CyD significantly improved survival in leukemia mouse models. Importantly, HP-β-CyD also showed anticancer effects against CML cells expressing a T315I BCR-ABL mutation (that confers resistance to most ABL tyrosine kinase inhibitors), and hypoxia-adapted CML cells that have characteristics of leukemic stem cells. In addition, colony forming ability of human primary AML and CML cells was inhibited by HP-β-CyD. Systemic administration of HP-β-CyD to mice had no significant adverse effects. These data suggest that HP-β-CyD is a promising anticancer agent regardless of disease or cellular characteristics.
Introduction

Advances in molecular targeting technologies have revolutionized cancer therapeutics, including imatinib mesylate for chronic myeloid leukemia (CML) and gefitinib for lung cancer [1,2]. Molecular-targeted drugs have superior anticancer effects compared to those of conventional chemotherapeutic agents, and have less adverse effects. However, resistance to chemotherapy, such as that resulting from point mutations or the existence of cancer stem cells, still hinders the treatment of cancer patients [3,4]. Thus, alternative therapeutic approaches that enhance neoplastic cell death are required for successful cancer treatment.

Cholesterol is one of the main components of lipid rafts, which provide signaling platforms capable of activating various cellular signaling pathways [5,6]. Cholesterol accumulation and/or dysregulated cholesterol metabolism is reported in various malignancies, including leukemia [7–9]. For example, breast and prostate cancer cell lines contain elevated levels of cholesterol, and are more sensitive to cholesterol depletion-induced cell death than their normal counterparts [10,11]. Breast cancer cells treated with mevalonate, a cholesterol precursor, demonstrate increased tumor cell growth in vivo and increased proliferation by accelerating cell-cycle progression [12]. Freshly isolated acute myeloid leukemia (AML) and CML cells show high rates of cholesterol import and/or synthesis [13,14]. Drug-resistant myeloid leukemia cell lines exhibit higher levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate limiting enzyme of the mevalonate pathway, suggesting that high cellular cholesterol may also improve leukemia cell survival and impart relative resistance to chemotherapy [14,15]. Taken together, modulation of cholesterol homeostasis might be a rational target for the development of anticancer agents.

Cyclodextrins (CyDs) are cyclic oligosaccharides, which have hydrophilic external faces and hydrophobic internal environments. The internal cavity of CyD has the ability to encapsulate lipophilic compounds and solubilize them in aqueous solutions (Fig 1A) [16]. CyDs are widely used in the pharmaceutical industry because of their ability to improve drug solubility and bioavailability [17,18]. CyDs interact with cell membrane components, such as cholesterol and phospholipids, resulting in the induction of hemolysis at high concentrations [19,20]. Amongst them, methyl-β-cyclodextrin (M-β-CyD) (Fig 1B) is used as a lipid raft disrupting agent through extraction of cholesterol and sphingolipids from these microdomains [21–24]. Several in vitro studies demonstrated that M-β-CyD induces apoptosis of various cancer cells [25–27]. However, systemic use of M-β-CyD is limited because of potential toxicities, such as its hemolytic effect on erythrocytes, and nephrotoxicity due to its high surface activity and inclusion ability [19,28,29].

2-Hydroxypropyl-β-cyclodextrin (HP-β-CyD) (Fig 1C) is used clinically as a pharmaceutical excipient for poorly water-soluble drugs. HP-β-CyD is available in registered oral, buccal, rectal, ophthalmic, and intravenous products [16]. Oral and intravenous solutions containing HP-β-CyD in complex with itraconazole, a broad-spectrum triazole antifungal agent, are widely used [30]. Furthermore, HP-β-CyD has recently been approved for the treatment of Niemann-Pick Type C disease (NPC), a lysosomal lipid storage disorder [31,32]. However, the potential anticancer activities of HP-β-CyD have not yet been noted. Given that cellular cholesterol accumulates in many malignancies and that HP-β-CyD is already used in clinical practice, we suspected that HP-β-CyD itself could be used as a novel anticancer agent by directly modulating cholesterol homeostasis in cancer cells. We therefore investigated anticancer activities of HP-β-CyD using various leukemic cell lines in vitro and leukemia mouse models in vivo.
Materials and Methods

Reagents and cell lines

HP-\(\beta\)-CyD was purchased from Tokyo Chemical Industry (Tokyo, Japan). M-\(\beta\)-CyD was obtained from Junsei Chemical (Tokyo, Japan). The average molecular weight of HP-\(\beta\)-CyD and M-\(\beta\)-CyD are 1391 and 1302, respectively. For in vitro use, compounds were dissolved in saline (Otsuka Pharmaceuticals, Tokyo, Japan) to a concentration of 500 mM. For in vivo use, HP-\(\beta\)-CyD and M-\(\beta\)-CyD were dissolved in saline to a concentration of 150 mM and 50 mM, respectively. These were stored at 4°C until use.
The human leukemic cell lines, BV173, K562, NALM6, HL60, Jurkat, and MOLT4, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). KBM-5 and KBM-5/STI cell lines were kindly provided by Dr M. Beran (The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA) [33]. An imatinib-sensitive cell line, MYL, and an imatinib-resistant Lyn-overexpressing cell line, MYL-R, were kindly provided by Dr H. Tanaka (Hiroshima City Asa Hospital, Hiroshima, Japan) [34]. Two hypoxia-adapted (HA)-CML cell lines, K562/HA and KCL22/HA were generated and cultured under 1.0% O₂ as described previously [35]. Ba/F3 BCR-ABLWT were generated as described previously [36]. Cells were maintained as suspension cultures in RPMI1640 (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Nichirei Bioscience Inc., Tokyo, Japan) at 37°C in a fully humidified chambers containing 5% CO₂ in air. Studies with human samples were approved by the Ethical Board of Saga University Hospital. Bone marrow (BM) or peripheral blood (PB) samples were obtained with written informed consent according to the Declaration of Helsinki from newly diagnosed CML and AML patients.

Mice

5- to 6-week-old Balb/cAJcl-nu/nu (nude) mice and 8-week-old NOD/ShiJic-scidJcl (NOD/SCID) mice were purchased from CLEA Japan, Inc. (Osaka, Japan), and used for in vivo transplantation experiments. All animal experiments were performed in accordance with the institutional guidelines of Saga University and with the approval of the Saga University Institutional Review Board.

Effects of HP-β-CyD on in vitro cell growth

Cell viability was assessed using a trypan blue dye exclusion method and cell proliferation was evaluated using a modified methyl-thiazol-diphenyl- tetrazolium (MTT) assay with SF reagent (Nacalai Tesque, Kyoto, Japan) as described previously [37,38]. Cells, including human primary hepatocytes (Applied Cell Biology Research Institute, Kirkland, WA, USA) were seeded in flat-bottomed 96-well plates (Greiner Labortechnik, Hamburg, Germany) at a density of 1x10⁴ cells in 100 μL medium per well, and incubated with HP-β-CyD at various concentrations for 72 hours. The mean of three replicates was calculated for each concentration. Half-maximal inhibitory concentration values (IC₅₀) were determined by nonlinear regression using CalcuSyn software (Biosoft, Cambridge, UK).

Western blot analysis

Whole cell lysates of leukemic cells treated with or without HP-β-CyD were prepared from cells using lysis buffer, as reported previously, with minor modification [39]. Protein was separated using a 10% NuPAGE electrophoresis system (Novex®, Life Technologies Co., Carlsbad, CA, USA), transferred to a nitrocellulose membrane (Schleicher & Schuell, BioScience GmbH, Dassel, Germany), blocked with 5% bovine serum albumin at room temperature for 1 hour, and incubated with primary antibodies at 4°C overnight. Antibodies against Akt, phosphorylated-Akt (Thr308 or Ser473), phosphorylated-Erk1/2 (Thr202/Thr204), phosphorylated-Stat5, Lyn (Cell Signaling Technology, Danvers, MA, USA), Stat5, Erk1/2, Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and phosphorylated-Lyn (Abcam, Cambridge, UK) were used as primary antibodies. Horseradish peroxidase-coupled immunoglobulin IgG (GE Healthcare, Buckinghamshire, UK) was used as the secondary antibody. An enhanced chemiluminescence kit (GE Healthcare) was used for detection. The results are representative of at least two independent experiments. Intensity of the immunoblot signals after background subtraction was quantified using ImageJ software.
Cell-cycle analysis

Cell-cycle analyses of human leukemic cell lines were performed as described previously [39]. In brief, 1×10⁶ cells were treated with the indicated concentration of HP-β-CyD. Twelve or twenty-four hours after HP-β-CyD treatment, cells were collected and fixed with 70% ethanol. Cells were then incubated with 0.1% Triton X-100 and 0.5% RNase A at room temperature for 30 minutes and stained with 50 μg/ml propidium iodide (PI; BD Biosciences, San Jose, CA, USA). Cellular DNA content was analyzed by flow cytometry, and cell-cycle profiles were determined using a FACS Caliber flow cytometer with CellQuest software (BD Biosciences). Data are the mean ± SD of three independent experiments.

Apoptosis assays

Apoptosis assay was performed by staining cells with 7-amino-actinomycin D (7-AAD) and annexin V (BD Biosciences), according to the manufacturer’s instructions. Cells were cultured in 6-well plate at a density of 4×10⁵ cells, and incubated with various concentrations of HP-β-CyD for 12 or 24 hours. Then, cells were stained with 7-amino-actinomycin D (7-AAD) and Annexin V-APC (BD Biosciences), and analyzed using a FACS AriaII system with Diva software (BD Biosciences). Data are the mean ± SD of three independent experiments.

Hematopoietic colony-forming assays

HP-β-CyD toxicity in normal hematopoietic progenitors was investigated using a standard methylcellulose culture assay as described previously [40]. A total of 2×10⁴ mononuclear cells from the BM of 10-week-old C57BL/6N mice were exposed to 0, 5, 15, or 25 mM HP-β-CyD in 1 mL MethoCult M3434 (Stem Cell Technologies, Vancouver, Canada). After 8 days of culture, the number of colonies was counted using an inverted microscope. Data represent the mean number of colonies ± SD (n = 3).

Clinical samples were obtained with informed consent. Mononuclear cells from leukemia patients were cultured in semi-solid medium containing recombinant cytokines (MethoCult H4435; Stem Cell Technologies). Results are expressed as the percentage of colonies relative to the untreated control ± SD of three replicates.

Cholesterol assays

Leukemic cells (3×10⁶) were incubated with 5 or 10 mM HP-β-CyD in HBSS (pH 7.4) at 37°C for 1, 2, or 3 hours. Cell culture supernatants were recovered by centrifugation (3,000 rpm, 5 min). The concentration of total cholesterol in the supernatants was determined using a Cholesterol E-test Wako (Wako Pure Chemical Industries, Osaka, Japan). Data are the mean ± SD of three experiments. Cellular lipids were extracted with methanol:chloroform (1:2), and total cholesterol and free cholesterol were determined enzymatically. The amount of esterified cholesterol was calculated by subtracting free cholesterol from total cholesterol. Cellular protein concentration was determined by BCA assay. Data are the mean ± SD of three experiments.

For filipin staining, cells were incubated with β-CyDs (10 mM) for 1 hour. Thereafter, cellular cholesterol was detected using a Cholesterol Cell-Based Detection Assay Kit (Cayman Chemical, Ann Arbor, MI, USA).

Murine leukemia model

Two different experimental settings were used. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Saga University (Permit number: 25-028-0). First, nude mice were intravenously transplanted with 1×10⁶ EGFP⁺ Ba/F3 BCR-ABLWT cells. These
mice were intraperitoneally injected with 200 μL vehicle (saline, Otsuka Pharmaceuticals) or HP-β-CyD (50 or 150 mM) for 20 consecutive days 3 days after transplantation, and survival was monitored daily. Leukemic cell engraftment was confirmed by detection of GFP-positive cells in the recipient’s BM using flow cytometry.

The second experimental setting involved a human leukemia xenograft model. BV173 cells (1×10⁶) were intravenously injected into sublethally irradiated (2 Gy) NOD/SCID mice. After 72 hours, xenotransplanted mice were intraperitoneally injected with 200 μL vehicle or HP-β-CyD (50 or 150 mM) for 5 consecutive days every week for 13 weeks, and survival was monitored daily. The percentage of human leukemic cells in BM was determined by flow cytometry after double staining with FITC-conjugated anti-human CD19 (BD Biosciences) and PE/Cy7-conjugated anti-mouse CD45 (BioLegend, San Diego, CA, USA) antibodies. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Mice were euthanized with ether when they became moribund or unable to obtain food or water, as recommended by the institutional guidelines of Saga University. Survival data were analyzed by a log-rank nonparametric test and shown as Kaplan-Meier survival curves (n = 10 for each group).

**Lung histology**

HP-β-CyD was administered to NOD/SCID mice for 13 weeks as described in the above section entitled “Murine leukemia model”. Age-matched mice were used as a control. Lungs were perfused with 10% buffered formalin and excised. Tissues were fixed in 10% buffered formalin and embedded in paraffin. These blocks were then sectioned and stained with hematoxylin and eosin (H&E). Samples were reviewed by Dr C. Conti (Texas A&M Health Science Center). Representative images from at least two samples are shown.

**Statistical analysis**

Results are expressed as the mean ± standard deviation (SD). Data were analyzed using an unpaired two-tailed Student’s t-test. Values of P < 0.05 were considered significant. To analyze in vivo efficacy, survival curves were created using the Kaplan-Meier method and compared with a log-rank test.

**Results**

**HP-β-CyD inhibits the growth of various leukemic cell lines**

First, we evaluated the effect of HP-β-CyD on leukemic cell viability. HP-β-CyD significantly inhibited the growth of mouse Pro-B cell Ba/F3 cells expressing wild-type BCR-ABL (hereafter Ba/F3 BCR-ABLWT) (Fig 1D), and BV173 cells derived from a human Philadelphia chromosome-positive (Ph+) acute leukemia patient, in a dose- and time-dependent manner (Fig 1E). Notably, HP-β-CyD did not significantly interfere with growth of normal human primary hepatocytes at concentrations of up to 15 mM (Fig 1F).

IC₅₀ values of HP-β-CyD were determined in 13 leukemia cell lines (Ph+ leukemia; BV173, K562, KBM-5, KBM-5/STI [33], KCL22, MYL and MYL-R [34], AML; HL-60, pre-B cell leukemia; NALM-6, T-cell leukemia; Jurkat and MOLT-4, adult T-cell leukemia; MT1, and MT2). The IC₅₀ values for HP-β-CyD after 72 hours exposure were in the range of 3.86–10.09 mM (Table 1). Interestingly, the HP-β-CyD-induced cell growth inhibition of imatinib-resistant cell lines, such as KBM-5/STI and MYL-R, was equivalent to that of the respective parental cells. The IC₅₀ value of HP-β-CyD in hepatocytes (18 mM) was approximately three-fold higher than those of the leukemic cell lines examined.
To determine the therapeutic window of HP-β-CyD, the susceptibility of normal hematopoietic progenitors to HP-β-CyD was examined using a colony formation assay. Under control conditions, 2×10^4 C57BL/6N BM cells formed 41 ± 3.5 colonies after 8 days of culture. When cells were treated with 5, 15, or 25 mM HP-β-CyD, the percentage of colonies was 93 ± 8.6%, 84 ± 23.5%, and 52.4 ± 9.7% of control, respectively (Fig 1G). These results indicate that concentrations greater than 15 mM HP-β-CyD are required to suppress BM colony formation.

HP-β-CyD inhibits cell growth by inducing apoptosis and G2/M cell-cycle arrest

Cell apoptosis was determined by Annexin V and 7-AAD staining. HP-β-CyD increased apoptosis in both BV173 and K562 cells in a dose- and time-dependent manner (Fig 2).

Furthermore, both BV173 (Fig 3A and 3B) and K562 cells (Fig 3C and 3D) responded to HP-β-CyD with a dose-dependent increase in the percentage of cells in G2/M phase. As the percentage of cells in G2/M increased, the percentage of G1 phase decreased, whereas the proportion of S-phase cells was not significantly altered by HP-β-CyD treatment. Treatment with HP-β-CyD also induced G2/M cell-cycle arrest in another Ph+ cell line, KBM5, and Ph-negative cell lines such as NALM-6, Jurkat, and MOLT4 (S1 Fig). Next, the effects of HP-β-CyD treatment on the expression of G2/M cell-cycle regulators, such as cdc25C, cdc2, cyclin A, cyclin B1, and p21, were examined by western blot analysis. HP-β-CyD had no effect on p-cdc25C and cyclin B1 protein expression; however, levels of cdc2 slightly decreased in BV173 cells (Fig 3E and 3F). Cyclin A and p21 decreased in BV173 cells, while in K562 cells, p21 expression was induced after 6 hours of HP-β-CyD treatment and slowly declined thereafter (Fig 3E and 3F). p21 is a cell cycle regulatory protein that can cause cell cycle arrest [41]. However, the role of p21 is complex because it also possesses pro- and anti-apoptotic abilities, and the function of p21 in apoptosis is cell type and cellular context specific [42–46]. Therefore, different behavior of p21 in HP-β-CyD-treated BV173 and K562 cells may be explained in part by the multiple roles of p21. These data indicate that HP-β-CyD-induced cell growth inhibition could be, in part, ascribed to induction of apoptosis and cell-cycle arrest at the G2/M checkpoint.

Table 1. IC_{50} values of HP-β-CyD in various leukemic cell lines.

| cell line                  | IC_{50} (mM) | SD  |
|----------------------------|--------------|-----|
| Ph+ leukemia               |              |     |
| BV173                      | 4.68         | 0.98|
| K562                       | 7.02         | 0.32|
| KBM5                       | 6.99         | 2.02|
| KBM5/STI                   | 8.47         | 0.6 |
| MYL                        | 10.09        | 0.35|
| MYL-R                      | 7.34         | 1.8 |
| KCL22                      | 8.06         | 0.71|
| Acute myeloid leukemia     |              |     |
| HL60                       | 8.26         | 1.03|
| Pre-B cell leukemia        |              |     |
| NALM6                      | 5.75         | 0.51|
| Mouse proB harboring BCR-ABL |           |     |
| Ba/F3 BCR-ABL WT           | 6.01         | 1.04|
| ATL                        |              |     |
| MT1                        | 8.23         | 1.17|
| MT2                        | 6.18         | 0.52|
| T-cell leukemia            |              |     |
| Jurkat                     | 4.73         | 0.61|
| MOLT4                      | 8.62         | 0.23|
| normal control             |              |     |
| hepatocyte                 | 18.65        | 4.84|

Values represent the mean ± SD of at least three independent experiments.

Values represent the mean ± SD of at least three independent experiments.

To determine the therapeutic window of HP-β-CyD, the susceptibility of normal hematopoietic progenitors to HP-β-CyD was examined using a colony formation assay. Under control conditions, 2×10^4 C57BL/6N BM cells formed 41 ± 3.5 colonies after 8 days of culture. When cells were treated with 5, 15, or 25 mM HP-β-CyD, the percentage of colonies was 93 ± 8.6%, 84 ± 23.5%, and 52.4 ± 9.7% of control, respectively (Fig 1G). These results indicate that concentrations greater than 15 mM HP-β-CyD are required to suppress BM colony formation.
Fig 2. HP-β-CyD induces apoptosis in BV173 cells and K562 cells. (A–D) BV173 cells and K562 cells were treated with 0, 5 mM, 10 mM, 15 mM HP-β-CyD, respectively. After 24 hours of culture, cells were collected and stained with Annexin V and 7-AAD. (A) FACS plots, representative of three independent experiments using BV173 cells. (B) Percentage of Annexin V-positive BV173 cells after culture with HP-β-CyD for 24 hours. Data are the mean ± SD of three independent experiments. **P < 0.01. (C) FACS plots, representative of three independent experiments using K562 cells. (D) Percentage of Annexin V-positive K562 cells after culture with HP-β-CyD for 24 hours. Data are the mean ± SD of three independent experiments. *P < 0.05. **P < 0.01. doi:10.1371/journal.pone.0141946.g002

Fig 3. HP-β-CyD causes cell-cycle arrest in leukemic cells. (A–D) BV173 and K562 cells were treated with the indicated concentration of HP-β-CyD for 12 hours, then flow cytometric analysis of PI-stained nuclei was performed. (A) Representative flow cytometric histograms of PI-stained BV173 cells. (B) The percentage of cells in G0/G1, S, or G2/M phase was assessed in viable BV173 cells. White: G1-phase, gray: S-phase, black: G2/M-phase. (C) Representative flow cytometric histograms of PI-stained K562 cells. (D) The percentage of cells in G0/G1, S, or G2/M phase was assessed in viable K562 cells. White: G1-phase, gray: S-phase, black: G2/M-phase. (E and F) Effects of HP-β-CyD on the expression of G2/M cell-cycle-associated proteins. BV173 (E) and K562 cells (F) were treated with 10 mM HP-β-CyD for the indicated times. Cells were lysed and analyzed by Western blotting. Western blot images are representative results from at least two independent experiments. Detection of β-actin was used as a loading control. Intensity of the immunoblot signals after background subtraction was quantified using ImageJ software.

doi:10.1371/journal.pone.0141946.g003
HP-β-CyD disturbs leukemic cell cholesterol homeostasis

We recently reported that M-β-CyD induces apoptosis in some solid cancer cell lines, possibly due to cholesterol depletion from cell membranes [27]. Therefore, to clarify whether HP-β-CyD-induced apoptosis is also associated with cholesterol depletion, we evaluated the effect of CyDs on cholesterol efflux from leukemic cells. HP-β-CyD or M-β-CyD treatment both increased cholesterol release in a time- and dose-dependent manner. Greater concentrations of cholesterol were released from Ba/F3 BCR-ABLWT cells treated with M-β-CyD than from cells treated with HP-β-CyD (Fig 4A). Similar results were also observed in BV173 cells and human hepatocytes (Fig 4B and S2A Fig). Next, we determined intracellular concentrations of cholesterol in leukemic cells treated with β-CyDs. Treatment with β-CyDs decreased cholesterol content in a dose-dependent manner (Fig 4C and 4D). Contrary to the results of the cholesterol efflux experiment, the cholesterol content of cells incubated with M-β-CyD was lower than that of the HP-β-CyD-treated cells (Fig 4C and 4D). Surprisingly, hepatocyte cholesterol content was not affected by treatment with the same concentration of HP-β-CyD (Fig 4E).

The reduced intracellular cholesterol content of CyDs-treated cells was also confirmed by filipin staining, which is a polyene antibiotic that specifically binds to cholesterol [47]. Although control (untreated) cells were positive for filipin, both M-β-CyD and HP-β-CyD-treated leukemic cells exhibited lower levels of filipin labeling than control cells (Fig 4F and 4G). In particular, M-β-CyD-treated cells demonstrated negligible labeling of filipin (Fig 4F and 4G). By contrast, hepatocytes treated with HP-β-CyD showed similar levels of filipin labeling to control cells (S2B Fig). These data support the results of the cholesterol efflux and intracellular cholesterol assays. The ability of M-β-CyD to markedly reduce cholesterol levels may reflect the degree of its cellular toxicity.

Effect of HP-β-CyD on signal transduction pathways involved in leukemia cell proliferation and survival

Signal transduction pathways such as RAS/RAF/MAPK, PI3K-AKT, STAT5, and Lyn, are activated in leukemia cells [48–50]. Thus, we next conducted experiments to elucidate the molecular mechanism by which HP-β-CyD inhibits the growth of leukemic cells. BV173 and K562 cells were exposed to 10 mM HP-β-CyD for varying times up to 24 hours, then changes in the expression and phosphorylation status of Akt, Erk, Stat5, and Lyn were examined. In BV173 cells, HP-β-CyD treatment markedly inhibited Stat5 phosphorylation. Akt and Lyn were also dephosphorylated on the treatment with HP-β-CyD, whereas the levels of phosphorylated (p)-ERK1/2 slightly increased in a time-dependent manner (Fig 5A). In K562 cells, HP-β-CyD treatment reduced levels of p-Lyn, which is known to reside in lipid rafts [51] after 0.5 and 2 hours of treatment, but p-Lyn recovered after 8 and 24 hours of treatment. The levels of p-ERK1/2 increased, with no significant effect on p-Stat5 or p-Akt observed (Fig 5B). Though BV173 and K562 cell lines were established from patients with Ph+ chronic myeloid leukemia in blast crisis [52,53], these two cell lines have some different characteristics [53,54]. Concordant with reports concerning M-β-CyD [10,25,26], these results suggest that HP-β-CyD effects on signal transduction may be cell context dependent.

HP-β-CyD inhibits the proliferation of a tyrosine kinase inhibitor (TKI)-resistant cell line

Although first- and second-generation TKIs, such as imatinib, nilotinib, and dasatinib, demonstrate improved outcomes in patients with CML and Ph+ acute lymphoblastic leukemia, a subset of patients with BCR-ABL mutations, particularly the T315I mutation, exhibit resistance to
these TKIs [3]. Currently, the third-generation TKI, ponatinib, is the only TKI that demonstrates notable activity against the T315I mutation [55].

In light of the data showing the inhibitory effect of HP-β-CyD on the proliferation of CML cell lines, we were prompted to evaluate the potential of HP-β-CyD to overcome TKI resistance. The results of a modified MTT assay using TKIs and HP-β-CyD are shown in Table 2. Ba/F3 cells expressing the T315I mutant (Ba/F3 BCR-ABL<sup>T315I</sup>) were resistant to imatinib and dasatinib [56]. By contrast, Ba/F3 BCR-ABL<sup>T315I</sup> cell growth was inhibited by ponatinib (IC<sub>50</sub>: 6.3 nM). These results are consistent with previous reports [55,56]. Most interestingly, the IC<sub>50</sub> of HP-β-CyD against Ba/F3 BCR-ABL<sup>T315I</sup> cells was comparable to that of the Ba/F3 BCR-ABL<sup>WT</sup> cells. Similar results were obtained in cell proliferation assays that used trypan blue staining (Fig 1D and S2 Fig). These results suggest that HP-β-CyD can inhibit the proliferation of TKI-resistant cell lines and may be of use against TKI resistance.
Administration of HP-β-CyD prolongs survival in leukemia mouse models

The *in vitro* data presented above demonstrate that HP-β-CyD has a significant inhibitory effects on leukemic cell growth. Next, to investigate the *in vivo* efficacy of HP-β-CyD, we generated mouse models of BCR-ABL-induced leukemia, and administered HP-β-CyD. Two different experimental settings were used. First, nude mice transplanted with Ba/F3 BCR-ABLWT were treated with 200 μL of vehicle, 50 mM HP-β-CyD [695.5 mg/kg intraperitoneal (i.p.); b.i.d] or 150 mM HP-β-CyD (2086.5 mg/kg i.p.; b.i.d) for 20 days beginning 3 days after transplantation. Ba/F3 BCR-ABLWT cell engraftment was confirmed by flow cytometric detection of EGFP+ cells in the BM of control mice (S4A Fig). All vehicle-treated mice died within 28 days. HP-β-CyD-treated mice survived longer than vehicle-injected mice, and the overall survival of recipients that received 50 mM or 150 mM HP-β-CyD was significantly higher than that of vehicle-injected mice (Fig 6A). Surprisingly, the survival time of HP-β-CyD-injected mice was longer than that of imatinib-treated mice in the same experimental setting, as performed in a previous study in our laboratory [57].

Table 2. Comparison of the IC50 values of TKIs and HP-β-CyD in Ba/F3 BCR-ABLWT and Ba/F3 BCR-ABL T315I cells.

| cell line               | Imatinib, μM  | IC50 | Dasatinib, nM | Ponatinib, nM | HP-β-CyD, mM |
|-------------------------|---------------|------|--------------|---------------|--------------|
| Ba/F3/BCR-ABLWT         | 0.09 ± 0.09   | 0.58 ± 0.15 | 0.89 ± 0.06  | 6.01 ± 1.04   |
| Ba/F3/BCR-ABL T315I     | 8.90 ± 1.21   | 2755.09 ± 120.15 | 6.30 ± 0.26  | 6.87 ± 0.76   |
| ratio (T315I/WT)        | 102.85        | 4727.69 | 7.06         | 1.14          |

Values shown are the mean ± SD of at least three independent experiments.

doi:10.1371/journal.pone.0141946.t002

doi:10.1371/journal.pone.0141946.g005
The second setting utilized a human leukemia xenograft model. BV173 cells were intravenously transplanted into sublethally irradiated NOD/SCID mice. Intraperitoneal injections of 50 mM HP-β-CyD, 150 mM HP-β-CyD, or vehicle were performed for 5 consecutive days every week for 13 weeks, beginning 3 days after transplantation. BV173 cell engraftment into BM was confirmed by flow cytometry (S4B Fig). HP-β-CyD-treated mice survived longer than control mice, and the log-rank test for overall survival showed statistically significant differences between vehicle- and 150 mM HP-β-CyD-treated recipients (Fig 6B).

It has been reported that HP-β-CyD shows only limited toxicity, although toxicity profiles of CyDs may differ according to the route of administration [58]. HP-β-CyD-treated mice
exhibited no gross lesions upon macroscopic examination. No hemolysis or anemia was observed (S1 and S2 Tables). More recently, HP-β-CyD-induced lung toxicity has been paid attention in the treatment of patients with NPC disease [59,60]. However, no obvious changes in lung histology were observed in HP-β-CyD-treated mice (Fig 6C–6F). Notably, all 150 mM M-β-CyD-injected mice died of diffuse alveolar hemorrhage within 24 hours of intraperitoneal administration (data not shown). These results clearly demonstrate that in vivo administration of HP-β-CyD inhibits leukemic cell growth, resulting in improved survival times. Furthermore, HP-β-CyD was well tolerated in mice with low toxicity.

**HP-β-CyD inhibits proliferation of hypoxia-adapted leukemic cells and human primary leukemic cell colony formation**

We previously established two leukemic cell lines that survive and proliferate under 1.0% O₂ hypoxic conditions [35]. These hypoxia-adapted (HA)-leukemic cells, namely, K562/HA and KCL22/HA, exhibit properties similar to leukemic stem cells, including relative cell dormancy, increase of side population fraction, resistance to Abl TKIs, and good engraftment ability. To investigate the effect of HP-β-CyD on leukemic stem-like cells, K562/HA and KCL22/HA cells were treated with HP-β-CyD. The IC₅₀ values of HP-β-CyD in K562/HA and KCL22/HA cells were comparable to that of their parental cells (Table 3), suggesting that HP-β-CyD targets not only proliferating leukemia cells but also dormant leukemic stem cells. Apoptosis and G₂/M cell-cycle arrest were also induced in K562/HA and KCL22/HA cells by HP-β-CyD treatment (S5 Fig).

We also evaluated the efficacy of HP-β-CyD in colony formation assays using mononuclear cells from two AML patients and from one accelerated phase CML patient. HP-β-CyD inhibited the formation of colonies in a concentration-dependent manner regardless of disease differences (Fig 7).

**Discussion**

In the present study, we provide evidence that HP-β-CyD-mediated cholesterol efflux inhibits leukemic cell proliferation. M-β-CyD and HP-β-CyD have been widely used as cholesterol-depletion agents. We previously showed that M-β-CyD had potent cytotoxic activity, and the involvement of cholesterol depletion in cell-death by M-β-CyD [27]. We and others demonstrated that M-β-CyD have higher cholesterol extraction ability than HP-β-CyD, probably because of the increase in hydrophobic space of the CyD cavity [61–63]. Thus, M-β-CyD is highly toxic. Yan et al. reported that M-β-CyD induces programmed cell death in CML cells and has anti-leukemic effects when used in combination with imatinib against imatinib-resistant CML cells in vitro [26]. Our data revealed that single use of HP-β-CyD effectively inhibits not only the growth of various leukemia cells, such as AML, ALL and CML, but also imatinib-resistant CML cells.

| cell line         | IC₅₀ (mM) | SD  |
|-------------------|----------|-----|
| Ph⁺ leukemia      |          |     |
| K562              | 7.02     | 0.32|
| K562/HA           | 3.86     | 0.85|
| KCL22             | 8.06     | 0.71|
| KCL22/HA          | 5.61     | 0.3 |
| Normal control    |          |     |
| hepatocyte        | 18.65    | 4.84|

Values represent the mean ± SD of at least three independent experiments.

doi:10.1371/journal.pone.0141946.t003
resistant CML cell growth, including cells harboring the T315I mutation (which are resistant to first- and second-generation TKIs), and stem cell-like CML cells, indicating that HP-β-Cyd has an anti-leukemic potential irrespective of leukemia type or the different characteristics. In vivo, systemic administration of HP-β-Cyd in murine leukemia models further demonstrated that HP-β-Cyd suppresses leukemia progression, resulting in improved survival. These results suggest that HP-β-Cyd is a potential novel anticancer agent.

Rapidly proliferating cells may conceivably require cholesterol for new membrane synthesis. Hence, increased endogenous cholesterol biosynthesis could be a common property of cancer. Statins, inhibitors of HMG-CoA reductase, exert growth inhibitory responses in leukemia cells in vitro [7,64,65]. These promising in vitro data led researchers to investigate possible clinical applications of statins as a single chemotherapeutic agent. Although conflicting results have been reported regarding their efficacy against cancers, including leukemia [66,67], the discrepancy between preclinical and clinical data may be explained in part by the difference in statin concentrations used. To achieve plasma statin concentrations comparable to those that demonstrated in vitro anticancer effects, considerably higher doses that may cause adverse effects, such as myopathy, rhabdomyolysis, and hepatotoxicity, must be administered. Recently, a leukemia-stroma co-culture system was used to screen for inhibition of stem cell properties, and identified 155 small-molecule compounds, including an HMGCR inhibitor, lovastatin, which preferentially inhibited leukemia stem cells (LSC) in vitro while sparing normal hematopoietic stem and progenitor cells. However, in vivo anti-LSC effect of lovastatin remains to be determined [68].

HP-β-Cyd induced apoptosis and G2/M cell-cycle arrest in vitro, and prolonged survival (without severe toxicity) in murine leukemia models. The mechanisms by which HP-β-Cyd induced apoptosis of cancer cells remain unclear. Rosenbaum et al. showed that M-β-Cyd/dextran conjugates enter Niemann-Pick Type C1 (NPC1)-defective cells and localize to cholesterol-enriched late endosomes/lysosomes [69]. Plazzo et al. also demonstrated that fluorescein-tagged M-β-Cyd is internalized into HeLa cells via clathrin-dependent endocytosis [70]. However, Onodera et al. demonstrated that although tetramethylrhodamine isothiocyanate (TRITC)-M-β-Cyd is associated with apoptosis of various cancer cells, TRITC-M-β-Cyd is not internalized, suggesting that M-β-Cyd may be membrane impermeable [27]. These contradictory results could be caused by different concentrations of M-β-Cyd or different cell types; thus, further studies are required to better understand how HP-β-Cyd induces cancer cell apoptosis.
Accumulating evidence indicates that many different signaling proteins are affected by CyDs. For example, M-β-CyD treatment decreases Akt phosphorylation in a time-dependent manner in human epidermoid carcinoma cells [10], whereas M-β-CyD has no effect on the phosphorylation of Akt, PLCγ1, and ERK1/2 in human megakaryoblastic leukemia cells [71]. Yan et al. reported that treatment of K562 cells with M-β-CyD resulted in a significant decrease in ERK1/2 phosphorylation, and thus concluded that an ERK/SPK1 signaling pathway may be an important mediator of M-β-CyD effects [26]. However, in our system, we observed that HP-β-CyD treatment increased p-ERK1/2 in a time-dependent manner with decreased p-Lyn levels. These phenomena could be explained by the observation that Lyn negatively regulates ERK activation [72]. In erythroleukemic cell line, K562 cells, the levels of p-Lyn recovered after 8 and 24 hours of HP-β-CyD treatment. Because Lyn plays an essential role for erythropoiesis and erythroleukemia development, HP-β-CyD-treated K562 cells might restored the levels of p-Lyn to survive [73,74]. More detailed biochemical analyses are required to characterize the exact molecular mechanisms modulated by HP-β-CyD in leukemia cells.

In this study, the concentration of HP-β-CyD required to induce cell death in vivo is greater than 2g/kg in BV173-xenografted leukemic mice. Matsuo et al. showed the effectiveness of intravenous HP-β-CyD treatment in two patients with Niemann-Pick type C disease (NPC) [31]. One patient was treated with approximately 2,500 mg/kg of HP-β-CyD (40,000mg, intravenously administered for 8h), a concentration comparable to our in vivo experiment (Fig 6 150 mM HP-β-CyD). Tanaka et al measured serum HP-β-CyD concentrations in this patient [75]. Serum HP-β-CyD concentrations were 1,295, 1,756 (1.3 mM), and 90 μg/mL at 2, 8, 12h after the start of infusion. Because HP-β-CyD was administered slowly (8h) in this patient, higher serum HP-β-CyD concentration would be obtained when the dripping speed is increased. Thus, the dose of HP-β-CyD used in the present study would be promising dose in a future clinical setting.

HP-β-CyD toxicity was also extensively investigated in mice in our study. HP-β-CyD is well tolerated in most species and shows limited toxicity, depending on dose and route of administration [58]. A study using acute intraperitoneal HP-β-CyD administration revealed that, in mice, up to 10,000 mg/kg HP-β-CyD, is tolerable [76]. Although CyD-induced hemolysis of human and rabbit red blood cells is well known [19,20], in the present study, neither single nor repeated HP-β-CyD administration resulted in hemolysis or anemia in mice. Furthermore, HP-β-CyD-induced lung toxicity, such as foamy macrophage infiltration, alveolitis, and pulmonary edema has been reported in several animal species [58,60]. The lungs of mice treated with HP-β-CyD for 13 weeks did not exhibit any histologic changes compared with those of control mice. No severe adverse effects were reported in two patients with NPC who received infusions of HP-β-CyD over 2 years [31]. Future studies are required to clarify the potential side effects of HP-β-CyD.

In conclusion, we have demonstrated that HP-β-CyD disrupts cholesterol homeostasis and inhibits the proliferation of leukemic cells by induction of apoptosis and cell-cycle arrest. Systemic HP-β-CyD administration has limited toxicity where examined. HP-β-CyD is also effective against mutated TKI-resistant clones and hypoxia-adapted cells, suggesting that HP-β-CyD has a broad anticancer effect irrespective of the cellular characteristics by modulating cholesterol homeostasis. To our knowledge, this paper is the first report to describe the efficacy of HP-β-CyD in leukemia in vitro and in vivo. Early-phase clinical trials are planned to verify the efficacy and safety of HP-β-CyD for treatment of leukemia.

Supporting Information

S1 Fig. HP-β-CyD induces cell-cycle arrest in leukemic cells. (A–D) Leukemic cells were treated with the indicated concentration of HP-β-CyD for 12 hours, then flow cytometric analysis of PI-stained nuclei was performed. The percentage of cells in G0/G1, S, or G2/M phase was assessed in
viable leukemic cells. White: G₁-phase, gray: S-phase, black: G₂/M-phase. (A) NALM-6, (B) KBM5, (C) Jurkat, (D) MOLT-4 cells. Data are the mean ± SD of three independent experiments. (PPTX)

S2 Fig. Effects of β-CyDs on efflux of cholesterol from hepatocytes. (A) Hepatocytes (1 × 10⁷ cells) were incubated in HBSS (pH 7.4) with or without HP-β-CyD or M-β-CyD (0, 5, 10 mM) for 1 hour. The concentration of cholesterol in HBSS were determined by Cholesterol E-test Wako®. (B) Image of filipin staining for hepatocytes. Primary hepatocytes were incubated with M-β-CyD (10 mM) or HP-β-CyD (10 mM) for 1 hour. Then, cells were treated with Filipin solution, and were scanned with a fluorescence microscope. (TIF)

S3 Fig. Effect of HP-β-CyD on the growth of Ba/F3 BCR-ABL<sup>T315I</sup> cells. Ba/F3 BCR-ABL<sup>T315I</sup> cells were exposed to 0 mM (■), 5 mM (▲), 7.5 mM (●), 10 mM (○), 15 mM (△), and 20 mM (○) HP-β-CyD. Viable cells were counted by a trypan blue dye exclusion method. Data are the mean ± SD of three independent experiments. (TIF)

S4 Fig. Leukemic cell engraftment into bone marrow in the BCR-ABL-induced leukemic mouse models. (A) Flow cytometric histogram of EGFP-positive BM cells from untreated nude mice that received EGFP<sup>+</sup> Ba/F3 BCR-ABL<sup>WT</sup> cells. (B) Representative FACS plot of BV173 cell-transplanted NOD/SCID mice. BM cells of NOD/SCID mice were analyzed by FACS 4 weeks after BV173 cell transplantation using an anti-human CD19 antibody and anti-mouse CD45 antibody. (TIF)

S5 Fig. HP-β-CyD inhibits hypoxia-adapted cell growth by inducing apoptosis and G₂/M cell-cycle arrest. (A-B) K562/HA cells and KCL22/HA cells were treated with 0, 5 mM, 10 mM, 15 mM HP-β-CyD, respectively. After 24 hours of culture, cells were collected and stained with Annexin V and 7-AAD. (A) Percentage of Annexin V-positive K562/HA cells after culture with HP-β-CyD for 24 hours. Data are the mean ± SD of three independent experiments. (B) Percentage of Annexin V-positive KCL22 cells after culture with HP-β-CyD for 24 hours. Data are the mean ± SD of three independent experiments. **P < 0.01. (C-D) HP-β-CyD causes cell-cycle arrest in hypoxia-adapted leukemic cells. K562/HA and KCL22/HA cells were treated with the indicated concentration of HP-β-CyD for 12 hours, then flow cytometric analysis of PI-stained nuclei was performed. (C) The percentage of cells in G<sub>0</sub>/G<sub>1</sub>, S, or G<sub>2</sub>/M phase was assessed in viable K562/HA cells. White: G<sub>1</sub>-phase, gray: S-phase, black: G<sub>2</sub>/M-phase. (D) The percentage of cells in G<sub>0</sub>/G<sub>1</sub>, S, or G<sub>2</sub>/M phase was assessed in viable KCL22/HA cells. White: G<sub>1</sub>-phase, gray: S-phase, black: G<sub>2</sub>/M-phase. Data are the mean ± SD of three independent experiments. (PPTX)

S1 Table. Red blood cell count in HP-β-CyD-injected nude mice. Data from CBC counts of peripheral blood collected by retro-orbital bleeding of vehicle-, and HP-β-CyD-injected nude mice. Data are mean ± SD of three mice. (DOCX)

S2 Table. Red blood cell count in HP-β-CyD-injected NOD/SCID mice. Data from CBC counts of peripheral blood collected by retro-orbital bleeding of vehicle-injected, and NOD/SCID mice that received 50 mM HP-β-CyD administration for 7 weeks. Data are average of two mice. (DOCX)
Acknowledgments

We thank C. Conti for the histological review of HP-β-CyD-treated mouse lung tissue; K. Kojima and T. Shindo for helpful discussions. This work was supported in part by a Grant-in-Aid for Challenging Exploratory Research 25670452 (Y.K.) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.

Author Contributions

Conceived and designed the experiments: YK SK. Performed the experiments: MY YK KM TH MT HT RN YT SM AS NSA ES. Analyzed the data: MY YK KM YT HA TI SK. Wrote the paper: YK SK.

References

1. Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res. 2002; 62: 5749–5754. PMID:12384534
2. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001; 344: 1031–1037. PMID:11287972
3. O'Hare T, Zabriskie MS, Eiring AM, Deininger MW. Pushing the limits of targeted therapy in chronic myeloid leukaemia. Nat Rev Cancer. 2012; 12: 513–526. doi:10.1038/nrc3317 PMID: 22825216
4. Perl A, Carroll M. BCR-ABL kinase is dead; long live the CML stem cell. J Clin Invest. 2011; 121: 22–25. doi: 10.1172/JCI43605 PMID: 21157035
5. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature. 1990; 343: 425–430. PMID: 1967820
6. Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. Nature. 2005; 438: 612–621. PMID: 16319881
7. Li HY, Appelbaum FR, Willman CL, Zager RA, Banker DE. Cholesterol-modulating agents kill acute myeloid leukemia cells and sensitize them to therapeutics by blocking adaptive cholesterol responses. Blood. 2003; 101: 3628–3634. PMID: 12506040
8. Ghalaut VS, Pahwa MB, Sunita, Ghalaut PS. Alteration in lipid profile in patients of chronic myeloid leukaemia before and after chemotherapy. Clin Chim Acta. 2006; 366: 239–242. PMID: 16386722
9. Mulas MF, Abete C, Pulisci D, Pani A, Massidda B, Dessi S, et al. Cholesterol esters as growth regulators of lymphocytic leukaemia cells. Cell Prolif. 2011; 44: 360–371. doi: 10.1111/j.1365-2184.2011.00758.x PMID: 21645151
10. Li YC, Park MJ, Ye SK, Kim CW, Kim YN. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. Am J Pathol. 2006; 168: 1107–1118; quiz 1104–1105. PMID: 16565487
11. Freeman MR, Solomon KR. Cholesterol and prostate cancer. J Cell Biochem. 2004; 91: 54–69. PMID: 14689582
12. Duncan RE, El-Sohemy A, Archer MC. Mevalonate promotes the growth of tumors derived from human cancer cells in vivo and stimulates proliferation in vitro with enhanced cyclin-dependent kinase-2 activity. J Biol Chem. 2004; 279: 33079–33084. PMID: 15155733
13. Rudling M, Gafvels M, Parini P, Gaarson T, Angelin B. Lipoprotein receptors in acute myelogenous leukemia: failure to detect increased low-density lipoprotein (LDL) receptor numbers in cell membranes despite increased cellular LDL degradation. Am J Pathol. 1998; 153: 1923–1935. PMID: 9846982
14. Vitale S, Norgren S, Juliusson G, Tatidis L, Luthman H. Multilevel regulation of low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in normal and leukemic cells. Blood. 1994; 84: 2669–2698. PMID: 7919382
15. Maksumova L, Ohnishi K, Murathkodjaev F, Zhang W, Pan L, Takeshita A, et al. Increased sensitivity of multidrug-resistant myeloid leukemia cell lines to lovastatin. Leukemia. 2000; 14: 1444–1450. PMID: 10942241
16. Davis ME, Brewster ME. Cyclodextrin-based pharmaceutics: past, present and future. Nat Rev Drug Discov. 2004; 3: 1023–1035. PMID: 15573101
17. Uekama K, Pirayama F, Irie T. (1998) Cyclodextrin Drug Carrier Systems. Chem Rev. 1998; 98: 2045–2076. PMID: 11849599
18. Szente L, Szejti J. Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development. Adv Drug Deliv Rev. 1999; 36: 17–28. PMID: 10837706

19. Motoyama K, Toyodome H, Onodera R, Irie T, Hirayama F, Uekama K, et al. Involvement of lipid rafts of rabbit red blood cells in morphological changes induced by methylated β-cyclodextrins. Biol Pharm Bull. 2009; 32: 700–705. PMID: 19336908

20. Ohtani Y, Irie T, Uekama K, Fukunaga K, Pitha J. Differential effects of α-, β-, and γ-cyclodextrins on human erythrocytes. Eur J Biochem. 1989; 186: 17–22. PMID: 2598927

21. George KS, Wu S. Lipid raft: A floating island of death or survival. Toxicol Appl Pharmacol. 2012; 259: 311–319. doi: 10.1016/j.taap.2012.01.007 PMID: 22289360

22. Simons K, Ehehalt R. Cholesterol, lipid rafts, and disease. J Clin Invest. 2002; 110: 597–603. PMID: 12208858

23. Galbiati F, Razani B, Lisanti MP. Emerging themes in lipid rafts and caveolae. Cell. 2001; 106: 403–411. PMID: 11525727

24. Anderson RG, Jacobson K. A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. Science. 2002; 296: 1821–1825. PMID: 12052946

25. Park EK, Park MJ, Lee SH, Li YC, Kim J, Lee JS, et al. Cholesterol depletion induces anoikis-like apoptosis via FAK down-regulation and caveolae internalization. J Pathol. 2009; 218: 337–349. doi: 10.1002/jpath.2531 PMID: 19288501

26. Yan J, Li QF, Wang LS, Wang H, Xiao FJ, Yang YF, et al. Methyl-β-cyclodextrin induces programmed cell death in chronic myeloid leukemia cells and, combined with imatinib, produces a synergistic down-regulation of ERK/SKP1 signaling. Ant-cancer drugs. 2012; 23: 22–31. PMID: 2187502

27. Onodera R, Motoyama K, Okamatsu A, Higashi T, Kariya R, Okada S, et al. Involvement of cholesterol depletion from lipid rafts in apoptosis induced by methyl-β-cyclodextrin. Int J Pharm. 2013; 452: 116–123. doi: 10.1016/j.ijpharm.2013.04.071 PMID: 23884659

28. Onodera R, Motoyama K, Okamatsu A, Higashi T, Anima H. Potential use of folate-appended methyl-β-cyclodextrin as an anticancer agent. Sci Rep. 2013; 3: 1104. doi: 10.1038/srep01104 PMID: 23346361

29. Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins: basic science and product development. J Pharm Pharmacol. 2010; 62: 1607–1621. doi: 10.1111/j.2042-7158.2010.01030.x PMID: 21039545

30. De Beule K, Van Gestel J. Pharmacology of itraconazole. Drugs. 2001; 61 Suppl 1: 27–37. PMID: 11219548

31. Matsuo M, Togawa M, Hirabaru K, Mochinaga S, Narita A, Adachi M, et al. Effects of cyclodextrin in two patients with Niemann-Pick Type C disease. Mol Genet Metab. 2013; 108: 76–79. doi: 10.1016/j.ijpmg.2013.07.011 PMID: 23807315

32. Anderson RG, Jacobson K. A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. Science. 2002; 296: 1821–1825. PMID: 12052946

33. Matsuo M, Togawa M, Hirabaru K, Mochinaga S, Narita A, Adachi M, et al. Effects of cyclodextrin in two patients with Niemann-Pick Type C disease. Mol Genet Metab. 2013; 108: 76–81. PMID: 23218948

34. Rosenbaum AI, Maxfield FR. Niemann-Pick type C disease: molecular mechanisms and potential therapeutic approaches. J Neurochem. 2011; 116: 789–795. doi: 10.1111/j.1471-4159.2010.06976.x PMID: 20807315

35. Ricci C, Scappini B, Divoky V, Gatto S, Onida F, Verstovsek S, et al. Mutation in the ATP-binding pocket of the ABL kinase domain in an STI571-resistant BCR/ABL cell line. Cancer Res. 2002; 62: 5995–5998. PMID: 12414617

36. Ito T, Tanaka H, Kimura A. Establishment and characterization of a novel imatinib-sensitive chronic myeloid leukemia cell line MYL, and an imatinib-resistant subline MYL-R showing overexpression of Lyn. Eur J Haematol. 2007; 78: 417–431. PMID: 17432977

37. Takeuchi M, Kimura S, Kuroda J, Ashihara E, Kawatani M, Osada H, et al. Glyoxalase-I is a novel target of the third-generation bisphosphonate zoledronate synergistically augments the anti-Ph+ leukemia activity of imatinib mesylate. Blood. 2003; 102: 2229–2235. PMID: 12763930

38. Yokoo M, Kubota Y, Tabe Y, Kimura S. Comparative Study of the Anti-leukemic Effects of Imatinib Mesylate, Glivec Tablet and Its Generic Formulation, OHH9511. Biol Pharm Bull. 2015; 38: 411–416. doi: 10.1248/bpb.b14-00652 PMID: 25757922

39. Hisatomi T, Sueoka-Aragane N, Sato A, Tomimasu R, Ide M, Kunimasa A, et al. NK314 potentiates antitumor activity with adult T-cell leukemia-lymphoma cells by inhibition of dual targets on topoisomerase IIα and DNA-dependent protein kinase. Blood. 2011; 117: 3575–3584. doi: 10.1182/blood-2010-02-270439 PMID: 21245486
40. Kubota Y, Osawa M, Jakt LM, Yoshikawa K, Nishikawa S. Necdin restricts proliferation of hematopoietic stem cells during hematopoietic regeneration. Blood. 2009; 114: 4383–4392. doi: 10.1182/blood-2009-07-230292 PMID: 19770359

41. Roninson IB. Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. Cancer Lett. 2002; 179: 1–14. PMID: 11880176

42. Bearss DJ, Lee RJ, Troyer DA, Pestell RG, Windle JJ. Differential effects of p21(WAF1/CIP1) deficiency on MMTV-ras and MMTV- myc mammary tumor properties. Cancer Res. 2002; 62: 2077–2084. PMID: 11929828

43. Wu Q, Kirschmeier P, Hockenberry T, Yang TY, Brassard DL, Wang L, et al. Transcriptional regulation during p21WAF1/CIP1-induced apoptosis in human ovarian cancer cells. J Biol Chem. 2002; 277: 36329–36337. PMID: 12138103

44. Orlandi L, Bearzatto A, Abolafio G, De Marco C, Daidone MG, Zaffaroni N. Involvement of bcl-2 and p21 proteins in response of human breast cancer cell clones to Tomudex. Br J Cancer. 1999; 81: 252–260. PMID: 10496350

45. Martin-Caballero J, Flores JM, Garcia-Palencia P, Serrano M. Tumor susceptibility of p21(Waf1/Cip1)-deficient mice. Cancer Res. 2001; 61: 6234–6238. PMID: 11507077

46. Yang WC, Mathew J, Velich A, Edelman W, Kucherlapati R, Lipkin M, et al. Targeted inactivation of the p21(WAF1/cip1) gene enhances Apc-initiated tumor formation and the tumor-promoting activity of a Western-style high-risk diet by altering cell maturation in the intestinal mucosal. Cancer Res. 2001; 61: 556–569. PMID: 11212250

47. de Kruijf B, Gerritsen WJ, Oerlemans A, van Dijck PW, Demel RA, van Deenen LL. Polyene antibiotic-sterol interactions in membranes of Acholesplasma laidlawii cells and lecithin liposomes. II. Temperature dependence of the polyene antibiotic-sterol complex formation. Biochim Biophys Acta. 1974; 339: 44–56. PMID: 4277610

48. Cortez D, Reuther G, Pendergast AM. The Bcr-Abl tyrosine kinase activates mitogenic signaling pathways and stimulates G1-to-S phase transition in hematopoietic cells. Oncogene. 1997; 15: 2333–2342. PMID: 9393877

49. Kawauski K, Ogasawara T, Yasuyama M, Ohkawa S. Involvement of Akt kinase in the action of STI571 on chronic myelogenous leukemia cells. Blood Cells Mol Dis. 2003; 31: 11–17. PMID: 12850478

50. Steelman LS, Pohnter SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia. 2004; 18: 189–218. PMID: 14737178

51. Tabe Y, Jin L, Iwabuchi K, Wang RY, Ichikawa N, Miida T, et al. Role of stromal microenvironment in the p21WAF1/CIP1-induced apoptosis. Cancer Res. 1999; 59: 2173–2178. PMID: 10716642

52. Anderson LC, Nilsson K, Gahmberg CG. K562—a human erythroleukemic cell line with positive Philadelphia chromosome. Blood. 1975; 45: 321–334. PMID: 163658

53. Pegoraro L, Matera L, Ritz J, Pulombu A, Biagini G. Establishment of a Ph1-positive human cell line (BV173). J Natl Cancer Inst. 1983; 70: 447–453. PMID: 6572735

54. Andersson LC, Nilsson K, Gahmberg CG. K562—a human erythroleukemic cell line. Int J Cancer. 1979; 23: 143–147. PMID: 367973

55. O’Hare T, Shakespeare WC, Zhu X, Eide CA, Rivera VM, Wang F, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell. 2009; 16: 401–412. doi: 10.1016/j.ccr.2009.09.028 PMID: 19878872

56. Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell. 2005; 7: 129–141. PMID: 15710326

57. Naito H, Kimura S, Nakaya Y, Naruoka H, Kimura S, Ito S, et al. In vivo antiproliferative effect of NS-187, a dual Bcr-Abl/Lyn tyrosine kinase inhibitor, on leukemic cells harbouring Abl kinase domain mutations. Leuk Res. 2006; 30: 1443–1446. PMID: 16546254

58. Gould S, Scott RC. (2005) 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD): a toxicology review. Food Chem Toxicol. 2005; 43: 1451–1459. PMID: 16018907

59. Matsuo M, Arima H, Irie T. Reply: lung toxicity of hydroxypropyl-β-cyclodextrin infusion. Mol Genet Metab. 2012; 109: 233. doi: 10.1016/j.ymgme.2013.04.010 PMID: 23660393

60. Chien YH, Shieh YD, Yang CY, Lee NC, Hwu WL. Lung toxicity of hydroxypropyl-β-cyclodextrin infusion. Mol Genet Metab. 2012; 109: 231–232. doi: 10.1016/j.ymgme.2013.04.003 PMID: 23611577

61. Irie T, Uekama K. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J Pharm Sci. 1997; 86: 147–162. PMID: 9040088

62. Gould S, Scott RC. (2005) 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD): a toxicology review. Food Chem Toxicol. 2005; 43: 1451–1459. PMID: 16018907

63. Matsuo M, Arima H, Irie T. Reply: lung toxicity of hydroxypropyl-β-cyclodextrin infusion. Mol Genet Metab. 2012; 109: 233. doi: 10.1016/j.ymgme.2013.04.010 PMID: 23660393

64. Chien YH, Shieh YD, Yang CY, Lee NC, Hwu WL. Lung toxicity of hydroxypropyl-β-cyclodextrin infusion. Mol Genet Metab. 2012; 109: 231–232. doi: 10.1016/j.ymgme.2013.04.003 PMID: 23611577

65. Irie T, Uekama K. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J Pharm Sci. 1997; 86: 147–162. PMID: 9040088

66. Matsuo M, Arima H, Irie T. Reply: lung toxicity of hydroxypropyl-β-cyclodextrin infusion. Mol Genet Metab. 2012; 109: 233. doi: 10.1016/j.ymgme.2013.04.010 PMID: 23660393

67. Chien YH, Shieh YD, Yang CY, Lee NC, Hwu WL. Lung toxicity of hydroxypropyl-β-cyclodextrin infusion. Mol Genet Metab. 2012; 109: 231–232. doi: 10.1016/j.ymgme.2013.04.003 PMID: 23611577

68. Irie T, Uekama K. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J Pharm Sci. 1997; 86: 147–162. PMID: 9040088
62. Yunomae K, Arima H, Hirayama F, Uekama K. Involvement of cholesterol in the inhibitory effect of dimethyl-β-cyclodextrin on P-glycoprotein and MRP2 function in Caco-2 cells. FEBS Lett. 2003; 536: 225–231. PMID: 12586368

63. Hinzey AH, Kline MA, Kotha SR, Sliman SM, Butler ES, Shelton AB, et al. Choice of cyclodextrin for cellular cholesterol depletion for vascular endothelial cell lipid raft studies: cell membrane alterations, cytoskeletal reorganization and cytotoxicity. Indian J Biochem Biophys. 2012; 49: 329–341. PMID: 23259319

64. Dimitroulakos J, Nohynek D, Backway KL, Hedley DW, Yeger H, Freedman MH, et al. Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. Blood. 1999; 93: 1308–1318. PMID: 9949174

65. Banker DE, Mayer SJ, Li HY, Willman CL, Appelbaum FR, Zager RA. Cholesterol synthesis and import contribute to protective cholesterol increments in acute myeloid leukemia cells. Blood. 2004; 104: 1816–1824. PMID: 15161671

66. Minden MD, Dimitroulakos J, Nohynek D, Penn LZ. Lovastatin induced control of blast cell growth in an elderly patient with acute myeloblastic leukemia. Leuk Lymphoma. 2001; 40: 659–662. PMID: 11426537

67. Vitols S, Angelin B, Julisson G. Simvastatin impairs mitogen-induced proliferation of malignant B-lymphocytes from humans—in vitro and in vivo studies. Lipids. 1997; 32: 255–262. PMID: 9076662

68. Hartwell KA, Miller PG, Mukherjee S, Kahn AR, Stewart AL, Logan DJ, et al. Niche-based screening identifies small-molecule inhibitors of leukemia stem cells. Nat Chem Biol. 2013; 9: 840–848. doi: 10.1038/nchembio.1367 PMID: 24161946

69. Rosenbaum AI, Zhang G, Warren JD, Maxfield FR. Endocytosis of β-cyclodextrins is responsible for cholesterol reduction in Niemann-Pick type C mutant cells. Proc Natl Acad Sci U S A. 2010; 107: 5477–5482. doi: 10.1073/pnas.0914309107 PMID: 20212119

70. Piazzo AP, Hofer CT, Jicsinszky L, Fenyvesi E, Szente L, Schiller J, et al. Uptake of a fluorescent methyl-β-cyclodextrin via clathrin-dependent endocytosis. Chem Phys Lipids. 2012; 165: 505–511. doi: 10.1016/j.chemphyslip.2012.03.007 PMID: 22503802

71. Caliceti C, Zambonin L, Prata C, Vieceli Dalla Sega F, Hakim G, Hrelia S, et al. Effect of plasma membrane cholesterol depletion on glucose transport regulation in leukemia cells. PloS one. 2012; 7: e41246. doi: 10.1371/journal.pone.0041246 PMID: 22859971

72. Kitaura J, Kawakami Y, Maeda-Yamamoto M, Horejsi V, Kawakami T. Dysregulation of Src family kinases in mast cells from epilepsy-resistant ASK versus epilepsy-prone EL mice. J Immunol. 2007; 178: 455–462. PMID: 17182584

73. Tilbrook PA, Palmer GA, Bittorf T, McCarthy DJ, Wright MJ, Sama MK, et al. Maturation of erythroid cells and erythroleukemia development are affected by the kinase activity of Lyn. Cancer Res. 2001; 61: 2453–2458. PMID: 11289114

74. Slavova-Azmanova NS, Kucera N, Louw A, Satiaputra J, Handoko A, Singer P, et al. Lyn kinase plays important roles in erythroid expansion, maturation and erythropoietin receptor signalling by regulating inhibitory signalling pathways that control survival. Biochem J. 2014; 459: 455–466. doi: 10.1042/BJ20130903 PMID: 24552351

75. Tanaka Y, Yamada Y, Ishitsuka Y, Matsuo M, Shiraishi K, Wada K, et al. Efficacy of 2-Hydroxypropyl-β-cyclodextrin in Niemann-Pick Disease Type C Model Mice and Its Pharmacokinetic Analysis in a Patient with the Disease. Biol Pharm Bull. 2015; 38: 844–851. doi: 10.1248/bpb.b14-00726 PMID: 26027824

76. Fromming KH, Szejtl J. Pharmacokinetics and toxicology of cyclodextrins. In: Szejlt J, Szent L, editors; 1996; Budapest. Springer. pp. 33–45.