Ex Vivo Expansion of Human Hematopoietic Stem Cells by Garcinol, a Potent Inhibitor of Histone Acetyltransferase

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Abstract

Background: Human cord blood (hCB) is the main source of hematopoietic stem and progenitor cells (HSCs/PCs) for transplantation. Efforts to overcome relative shortages of HSCs/PCs have led to technologies to expand HSCs/PCs ex vivo. However, methods suitable for clinical practice have yet to be fully established.

Methodology/Principal Findings: In this study, we screened biologically active natural products for activity to promote expansion of hCB HSCs/PCs ex vivo, and identified Garcinol, a plant-derived histone acetyltransferase (HAT) inhibitor, as a novel stimulator of hCB HSC/PC expansion. During a 7-day culture of CD34⁺CD38⁻ HSCs supplemented with stem cell factor and thrombopoietin, Garcinol increased numbers of CD34⁺CD38⁻ HSCs/PCs more than 4.5-fold and Isogarcinol, a derivative of Garcinol, 7.4-fold. Furthermore, during a 7-day culture of CD34⁺HSCs/PCs, Garcinol expanded the number of SCID-repopulating cells (SRCs) 2.5-fold. We also demonstrated that the capacity of Garcinol and its derivatives to expand HSCs/PCs was closely correlated with their inhibitory effect on HAT. The Garcinol derivatives which expanded HSCs/PCs inhibited the HAT activity and acetylation of histones, while inactive derivatives did not.

Conclusions/Significance: Our findings identify Garcinol as the first natural product acting on HSCs/PCs and suggest the inhibition of HAT to be an alternative approach for manipulating HSCs/PCs.

Introduction

Hematopoietic stem cells (HSCs) have been applied to the treatment of a wide variety of blood disorders through HSC transplantations and gene therapy [1–3]. Related as well as unrelated human cord blood (hCB) has emerged as a source of HSCs and the use of these cells is increasing because of the low risk of graft-versus-host disease and facile procurement [4]. To date, over 10,000 hCB transplantations have been conducted [5]. However, the widespread application of hCB is limited by relatively low numbers of HSCs, resulting in a significant delay in hematopoietic recovery and increased transplant-related mortality. Thus approaches that can overcome low cell doses and delayed engraftment are of great interest. The cotransplantation of two CB units from different donors, which increases the available cell dose, has been used. Alternatively, numerous attempts have been made to expand hCB HSCs ex vivo [6–11]. The majority of cell culture systems have exploited protein-factor mixtures, including stem cell factor (SCF), thrombopoietin (TPO), fms-like tyrosine kinase 3 ligand (FL), a complex of interleukin 6 (IL-6) and soluble IL-6 receptor (IL-6/sIL-6R), the Notch ligand Delta1, Angiopoietin-like proteins, and Pleiotrophin. Notably, Delaney and colleagues reported that transplantation with Notch-mediated expansion ex vivo resulted in faster neutrophil engraftment compared to a control group receiving uncultured hCB [8]. However, additional clinical studies will be required to confirm the enhanced kinetics of engraftment in humans, and identification of the cell signaling that governs the self-renewal of HSCs is needed to improve existing methods of hCB HSC expansion ex vivo. It could also be pointed out that protein-factor combinations have proven to be neither cost-effective nor readily available.

Small-molecule compounds (SMCs), which comprise natural and chemically synthesized products, have played a pivotal role in molecular biology and pharmaceutical therapy. The use of SMCs has also facilitated elucidation of the signaling pathways that control stemness and been applied to HSC expansion ex vivo [12–16]. The method using tetraethylpentamine, a synthetic copper chelator, which expands hCB CD34⁺ cells and increases their...
potential for engraftment in immunodeficient mice, has shown feasibility in a Phase I/II study [13]. Boitano and colleagues reported that a chemically synthesized purine derivative induced hCB HSC expansion in culture by antagonizing the aryl hydrocarbon receptor [16]. We also reported that activation of the human thrombopoietin receptor by a small-molecule agonist promoted expansion of hCB HSCs [17]. Nonetheless, there is a need to identify more efficient SMCs and to design better compounds in terms of efficacy and safety for clinical use.

Here, in a search for biologically active natural products that may activate signals required for HSC expansion, we screened natural products for effects on hCB CD34+CD38− cells, which are reported to be primitive hematopoietic stem and progenitor cells (HSCs/PCs) [18,19]. We found that Garcinol, a benzophenone derivative originally isolated from Garcinia indica [20,21], expands HSCs/PCs through an inhibitory effect on HAT. This is the first report of a small-molecule HAT inhibitor promoting HSC expansion ex vivo.

**Results**

Garcinol and its derivative expand human hematopoietic progenitors

To identify biologically-active natural products that act on HSCs/PCs ex vivo, we cultured hCB CD34+ HSCs/PCs with natural products in the presence of stem cell factor (SCF) and thrombopoietin (TPO) for 7 days and examined the number of CD34+CD38− HSCs in culture (Figure 1A). We screened 92 biologically-active natural products collected from commercially available compounds (Table S1), and identified Garcinol (GAR) as one of the most active compounds (Figure 1B and C). To evaluate the function of GAR in detail and estimate the structure-activity relationship, we synthesized its derivatives, Isogarcinol (ISO), O-monomethylisogarcinol (MMI), and O-dimethylisogarcinol (DMI) (Figure 1B). We then cultured hCB CD34+ cells in medium supplemented with SCF, TPO and the Garcinol derivatives for 7 days. GAR, ISO, and MMI facilitated the expansion of CD34+CD38− cells compared with the DMSO control (Figure 2A), but little affected the total cell numbers at their effective concentrations (10 μM of GAR: 109.7±10.3%, 5 μM of ISO: 71.5±23.7%, 2 μM of MMI: 91.1±2.5%, 0.5 μM of DMI: 95.0±2.1% relative to the blank control). We observed a more efficient effect by GAR, ISO, and MMI when hCB CD34+CD38− cells were used as the starting material (Figure 2B). During the 7-day culture, CD34+CD38− cells expanded in number 4.5, 7.4, 2.2, and 1.4-fold with GAR, ISO, MMI, and DMI, respectively, as compared with the blank culture. These results indicated that GAR derivatives other than DMI increased the number of primitive CD34+CD38− cells efficiently in culture.

To evaluate the number of functional HSCs/HPCs in cultures with GAR derivatives, we next performed colony assays. Cultured cells with GAR derivatives contained all types of myeloid progenitors, and there was no significant difference in total colony number between the control and GAR cultures, respectively, and the population of CD34+CD38− cells included increased numbers of SRCs or vehicle (DMSO)-treated cells (Fig. 3A). The frequency for SRCs was 1 in 10,921 (95% confidence interval of 1 of 14,109 to 1 of 8,453) among fresh CD34+ cells and 1 in 9,521 (95% confidence interval of 1 of 12,318 to 1 of 7,358) in the culture with vehicle (DMSO). In contrast, the frequency for SRCs in the culture with GAR was 1 in 4,328 (95% confidence interval of 1 of 5,844 to 1 of 3,206), which was 2.5-fold higher than for fresh CD34+ cells (p = 0.019) and 2.2-fold higher than for the vehicle cultures (p = 0.046) (Fig. 3B). These results demonstrated that GAR promoted the expansion of SRCs in culture.

Expansion of HSCs by a HAT Inhibitor

GAR-treated CD34+ cells include increased numbers of SRCs

To evaluate the number of functional HSCs in cultures with GAR, we performed a NOD/SCID-repopulation assay [19,23] to estimate the capacity for reconstitution among the progeny of hCB CD34+ cells cultured with GAR in addition to SCF, TPO, and FL. We used limiting numbers of cells for the repopulation assay to estimate the SRC frequencies. Increasing numbers (3×10², 1×10³, 2×10⁴) of fresh CD34+ cells or 7-day cultured cells corresponding to the same number of input CD34+ cells were transplanted into NOD/SCID mice. The average repopulation levels by human hematopoietic CD34+ cells were higher in recipient mice infused with GAR-treated cells than in those infused with fresh CD34+ cells or vehicle (DMSO)-treated cells (Fig. 3A). The frequency for SRCs was 1 in 10,921 (95% confidence interval of 1 of 14,109 to 1 of 8,453) among fresh CD34+ cells and 1 in 9,521 (95% confidence interval of 1 of 12,318 to 1 of 7,358) in the culture with vehicle (DMSO). In contrast, the frequency for SRCs in the culture with GAR was 1 in 4,328 (95% confidence interval of 1 of 5,844 to 1 of 3,206), which was 2.5-fold higher than for fresh CD34+ cells (p = 0.019) and 2.2-fold higher than for the vehicle cultures (p = 0.046) (Fig. 3B). These results demonstrated that GAR promoted the expansion of SRCs in culture.

GAR inhibits HAT activity and protein acetylation

GAR has been characterized as a non-specific HAT inhibitor and its derivative, ISO, was demonstrated to inhibit both p300 and PCAF [21]. To confirm the reported effect of GAR derivatives on intracellular HAT activity, we observed HAT activity in cells treated with GAR derivatives. Indeed, treatment of cells with GAR, ISO, and MMI, but not DMI, inhibited cellular HAT activity in HL60 cells (Figure 4A). The inhibitory effect of GAR and its derivatives on HAT correlated well with their capacity to expand HSCs/PCs ex vivo. GAR also inhibited HAT activity in hCB CD34+ cells (Figure 4B). Next, we observed the level of protein acetylation, which is regulated by HATs, in cells treated with GAR derivatives. In accordance with the result of HAT inhibition, GAR, ISO, and MMI, but not DMI, reduced cellular histone acetylation levels in HeLa cells (Figure 4C). A reduced level of acetylation was also observed in hCB CD34+ cells treated with GAR (Figure 4D). As with histone acetylation, GAR reduced the level of acetylation of p53 at K382 in RPMI8226 cells (Figure 4E).
GAR-treated cells show unique profiles of gene expression

To understand the molecular mechanisms by which GAR promotes the expansion of HSCs, we examined global transcription levels of genes in cultured CD34+/CD38− cells with GAR using a DNA microarray. Treatment of HSCs/PCs with GAR for 7 days led to the up-regulation of 20 genes and down-regulation of 9 genes in CD34+/CD38− cells (Table 1). Among them, we validated the expression of 6 genes by using real-time quantitative PCR (Figure 5). Treatment of HSCs/PCs with GAR resulted in a 1.98, 1.67, and 3.44-fold increase in AMICA1, BTG2 and HLF expression, respectively, and a 0.61, 0.43, and 0.33-fold decrease in IL8, PF4, and PPBP expression, respectively, in the CD34+/CD38− cell fraction. In contrast, DMI did not significantly change the expression of these genes, except for IL8. The transcriptional levels of other genes reported to be implicated in the self-renewal of HSCs, for example, HOXB4, BMI1, GATA2, NOTCH1, p21, p27, c-MYC, EGR, and EVI-1 [25,26], were not changed by GAR (data not shown).

Discussion

A search for biologically active natural products that promote HSC expansion uncovered GAR, a benzophenone derivative...
originally isolated from Garcinia indica [20,21]. GAR is the first plant-derived natural product found to act on HSCs/PCs. Isogarcinol, a derivative of GAR, was even more active, further supporting the efficacy of GAR. As reported before, GAR exerted its activity as a HAT inhibitor in HSCs/PCs. GAR reduced cellular HAT activity and the levels of histone acetylation in hCB CD34+ cells. Importantly, the inhibitory effect of GAR and its derivatives on HAT correlated well with their capacity to expand HSCs/PCs ex vivo. Together, our findings provide the first evidence of the effectiveness of inhibiting HAT on the expansion of HSCs/PCs. GAR also reduced the level of acetylation of p53 at K382 in RPMI8226 cells, although its effect was not obvious in hCB CD34+ cells (data not shown). p53 restricts the self-renewal of HSCs: p53-deficient mice have been reported to contain twice as many HSCs and their HSCs exhibit greater repopulating capacity than do wild-type HSCs [27]. p300 HAT is reported to acetylate p53 at K382, and, by doing so, enhances the sequence-specific DNA binding of p53 [28]. Thus, GAR might expand HSCs/PCs, at least partly, through suppression of p53 activity.

*Figure 2. Garcinol efficiently expands numbers of human CD34+CD38– cells and colony-forming cells. A. The effects of Garcinol derivatives on hCB CD34+ HSCs/PCs. hCB CD34+ cells were cultured in StemSpan SFEM medium with Garcinol derivatives or the same volume of DMSO (blank) in the presence of 20 ng/mL of hTPO and 100 ng/mL of hSCF for 7 days. Cultured cells were counted with a hemocytometer (trypan blue exclusion) and analyzed by FACS for CD34 and CD38 expression. The numbers of CD34+CD38– cells relative to the blank are indicated (mean±SEM, n = 3). *p<0.05. B. The effects of Garcinol derivatives on hCB CD34+CD38– HSCs. CD34+CD38– cells were isolated from hCB cells by FACS Aria and cultured as in A. Bars represent the fold-increase in the number of CD34+CD38– cells in the 7-day cultures compared with the initial number of CD34+CD38– cells (mean±SEM, n = 3). *p<0.05. C. The effects of Garcinol derivatives on colony-forming cells. The 7-day cultures of hCB CD34+ cells treated with Garcinol derivatives or the same volume of DMSO (blank) were plated in Methocult GF H4435 methylcellulose medium (500 cells/plate), and colonies were counted after 12 days. Bars represent the number of colony-forming units in culture (CFU-C) and HPP-CFCs per 500 cultured cells (mean±SEM, n = 3). *p<0.05. D. The effects of Garcinol derivatives on HSCs/PCs in the presence of multiple cytokines. hCB CD34+ cells were cultured with Garcinol (10 μM) or the same volume of DMSO (blank) in the presence of SCF (100 ng/mL) alone, SCF and TPO (20 ng/mL), or SCF, TPO, and FL (50 ng/mL) for 7 days. The numbers of CD34+CD38– cells relative to that in the control culture supplemented with SCF only are indicated (mean±SEM, n = 3). The number of CD34+CD38– cells in the control culture was arbitrarily set to 1. *p<0.05.

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Several SMCs have also been reported to be effective in the manipulation of HSCs/PCs by inhibiting components of the epigenetic machinery. Araki et al. proposed that the treatment of hCB HSCs with a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, followed by a histone deacetylase (HDAC) inhibitor, trichostatin A, efficiently expands HSCs [29,30]. However, inhibitors for DNA methyltransferases or HDACs are also effective against cancer cells and restrict their tumorigenic activity by releasing the transcriptional repression of tumor suppressor genes [31]. So, their efficacy in normal HSCs/PCs might need further evaluation. By contrast, we previously reported that forced expression of \textit{Bmi1}, one of the polycomb-group genes, augments stem cell activity [32]. The polycomb-group proteins transcriptionally repress tumor suppressor genes by adding repressive histone modifications, the trimethylation of histone H3 at lysine 27 and monoubiquitylation of histone H2A at lysine 119, to their promoters [33,34]. Forced expression of \textit{Bmi1} reinforces the transcriptionally repressive state of tumor suppressor genes, such as \textit{Ink4a} and \textit{Arf}. We speculate that GAR acts in a similar fashion to \textit{Bmi1} by inhibiting the activity of HATs. Given the broad effects of GAR on cellular acetylation levels, the limited effect on gene expression was unexpected, but may suggest a specific function of GAR in maintenance of the transcriptionally repressive state of tumor suppressor genes. Nevertheless, the up-regulated gene expression of hepatic leukemia factor (HLF), a member of the proline and acidic-rich protein family, is intriguing because HLF has been implicated in the control of human HSC function: Forced expression of HLF in human HSCs reportedly protects against apoptosis and enhances in vivo reconstitution [35].

In summary, GAR and its derivatives modulate the acetylation of not only histones but also key HSC regulators such as p53 to establish a gene expression profile and molecular functions...
favorable for HSC/PC expansion. Thus, the inhibition of HAT represents a new approach to HSC manipulation and therapy. It is important to find out more potent, specific, and less toxic HAT inhibitors to precisely revalidate their effectiveness on HSCs/PCs and apply them to the manipulation of HSCs/PCs ex vivo.

Commercially available HAT inhibitors, curcumin [36] and anacardic acid [37], were toxic and less active on HSCs/PCs compared with GAR (data not shown). Additional screening of HAT inhibitors might be required to obtain suitable compounds for the expansion of HSCs/PCs ex vivo. Of interest, GAR did not have any antagonistic function against aryl hydrocarbon, and thus exerted an additive effect on the expansion of HSCs/PCs in combination with SR-1, an antagonist of aryl hydrocarbon receptor [16] (data not shown). These findings suggest that the combination of SMCs with different molecular targets would improve the efficacy of HSC/PC expansion ex vivo.

**Materials and Methods**

**Ethics Statement**

All experiments using the mice were performed in accordance with our institutional guidelines for the use of laboratory animals.
and approved by the review board for animal experiments of Chiba University (approval ID: 21–150). The study using hCB cells was approved by the institutional ethics committees of Chiba University (approval ID: 692).

Reagents

Natural products and chemical derivatives for screening were collected from the library LOPAC 1280 (Sigma-Aldrich, Missouri) or purchased from Cosmo Bio (Tokyo, Japan). GAR was obtained from Enzo Life Sciences (New York, USA) and its derivatives were chemically synthesized as reported previously [21]. Briefly, GAR was stirred for 10 hours in toluene and hydrochloric acid at room temperature, and left for 17 hours at 4°C. The reaction mixture was then filtered, and the residue was washed with distilled water and acetonitrile. Isogarcinol (ISO) was finally obtained by recrystallization in acetonitrile. ISO was then methylated by addition of potassium carbonate and methyl iodide for 17 hours at 25°C. The product was extracted with ethyl acetate and concentrated. O-monomethylisogarcinol (MMI) and O-dimethylisogarcinol (DMI) were separated by silica gel column chroma-

| Symbol | Gene Description | Fold-change |
|--------|------------------|-------------|
| ADRB1  | Adrenergic, beta-1, receptor | 2.6 |
| AMICA1 | Adhesion molecule, interacts with CXADR antigen1 | 1.5 |
| BTG2   | BTG family, member2 | 1.8 |
| CNTNAP5| Contactin associated protein-like5 | 7.3 |
| COL5A1 | Collagen, type V, alpha 1 | 1.6 |
| CYP1B1 | Cytochrome P450, family 1, subfamily B, polypeptide 1 | 1.9 |
| FAM55C | Family with sequence similarity 55, member C | 2.2 |
| FBXL17 | F-box and leucine-rich repeat protein 17 | 1.7 |
| HLF    | Hepatic leukemia factor | 3.1 |
| IGF    | Immunoglobulin J polypeptide | 3.0 |
| JAG2   | Jagged 2 | 1.9 |
| LRBA   | LPS-responsive vesicle trafficking, beach and anchor containing | 2.7 |
| NUP1L1 | Nucleoporin like 1 | 3.2 |
| PTK2   | PTK2 protein tyrosine kinase 2 | 2.2 |
| SEMA3C | Sema domain, immunoglobulin domain (Ig) | 2.3 |
| SLC41A2| Solute carrier family 41, member 2 | 3.2 |
| THAP9  | THAP domain containing 9 | 2.0 |
| TRIM59 | Tripartite motif-containing 59 | 1.6 |
| ZN407  | Zinc finger protein 407 | 6.3 |
| ZN623  | Zinc finger protein 623 | 2.1 |
| CST7   | Cystatin F (leukocystatin) | 0.7 |
| HBD    | Hemoglobin, delta | 0.6 |
| IL8    | Interleukin 8 | 0.7 |
| INPP5F | Inositol polyphosphate-5-phosphatase F | 0.7 |
| PF4    | Platelet factor 4 | 0.6 |
| PPBP   | Pro-platelet basic protein | 0.6 |
| RPS2   | Ribosomal protein S2 | 0.6 |
| TP73   | Tumor protein p73 | 0.6 |
| TPS3111| Tumor protein p53 inducible protein 11 | 0.4 |

The gene expression analysis was performed using total RNA of CD34<sup>+</sup>CD38<sup>-</sup> cells sorted from 7-day cultures of hCB CD34<sup>+</sup> cells with 10 μM of GAR, 0.5 μM of DMI, or the same volume of DMSO (blank). The DNA microarray assay was conducted with a GeneChip 3 expression array (Kurabo, Osaka, Japan). The genes whose expression was up-regulated greater than 1.5-fold or down-regulated less than 0.7-fold upon treatment with GAR compared to the blank control are shown.

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Figure 5. Treatment of GAR modifies the gene expression in CD34<sup>+</sup>CD38<sup>-</sup> cells. hCB CD34<sup>+</sup> cells were cultured with 10 μM of GAR or 0.5 mM of DMI in the presence of rhSCF, rhTPO and rhFL for 7 days. Bars represent the mean of fold-changes in gene expression relative to the blank (n = 3–5) detected by quantitative real-time PCR. The peptidylprolyl isomerase A or beta-2-microglobulin gene was used as an internal control.

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were isolated by fluorescence-activated cell sorting using a BD The purity of hCB CD34 cell sorting CD34 progenitor kit (Miltenyi Biotech, California). were immunomagnetically enriched using a magnetic-activated cell sorter CD34 (clone DREG-56, BD Pharmingen) and analyzed on a JSAN desktop cell sorter.

Cell culture
Human cord blood (hCB) CD34+ cells were purchased from Lonza (Basel, Switzerland) or purified from hCB obtained from the Tokyo Cord Blood Bank (Tokyo, Japan). Mononuclear cells were separated by density gradient centrifugation. CD34+ cells were immunomagnetically enriched using a magnetic-activated cell sorting CD34 progenitor kit (Miltenyi Biotech, California). The purity of hCB CD34+ cells was over 95%. CD34+CD38– cells were isolated by fluorescence-activated cell sorting using a BD FACSAria (BD Bioscience, California). Purified CD34+ and CD34+CD38– cells were plated at 1×10^6 cells/well in a 24-well plate precoated with 25 μg/mL of fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) [17] and cultured in serum-free medium, StemSpan SFEM (Stem Cell Technologies, Vancouver, Canada) supplemented with a 1% penicillin-streptomycin mixture (Sigma) at 37 °C in a humidified atmosphere flushed with 5% CO2 in air. Recombinant human (rh)SCF (Wako Pure Chemical Industries, Osaka, Japan) was added at 100 ng/mL, rhFL (PeproTech, New Jersey) at 50 ng/mL, and rhTPO (PeproTech) at 20 ng/mL. Garcinol derivatives were added in the indicated amounts. Human leukemia cell lines, HL60 [38] and RPMI8226 [39], were purchased from DS Pharma Biomedical (Osaka, Japan) and cultured in RPMI 1640 medium (Invitrogen, California) containing 10% fetal bovine serum and a 1% penicillin-streptomycin mixture (Sigma) at 37 °C under 5% CO2 in a humidified incubator and passed every three days. The human epithelial carcinoma cell line HeLa was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and a 1% penicillin-streptomycin mixture (Sigma).

Flow cytometry
Cultured hCB CD34+ cells were stained with allophycocyanin (APC)-conjugated anti-human CD34 and phycoerythrin (PE)-conjugated anti-human CD38 antibodies (BD Pharmingen, California). Then 1 μg/mL of propidium iodide (Sigma) was added to exclude nonviable cells. Cells were analyzed on a BD FACSAria II (BD Bioscience) or a JSAN desktop cell sorter (Bay Bioscience, Kobe, Japan). For analyzing hematopoietic engraftment in NOD/SCID mice, BM cells were stained with APC-conjugated CD45 antibody (BD Pharmingen). To measure the acetylation levels of histones and pS3 protein, cultured cells were fixed and permeabilized with a Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) and then stained with Alexa Fluor 488-conjugated anti-acetylated histone H3 antibody (clone C5B11, Cell Signaling) and Alexa Fluor 647-conjugated mouse anti-p53/akK382 antibody (clone L82-51, BD Biosciences), respectively. To measure the expression levels of homing receptors on cell surface, cultured hCB CD34+ cells were stained with APC-conjugated anti-human CD34, PE-conjugated anti-human CD18 (clone 12G5, BD Pharmingen), and fluorescein isothiocyanate-conjugated anti-human CD62L antibodies (clone DREG-56, BD Pharmingen) and analyzed on a JSAN desktop cell sorter.

Colonies forming assay
hCB CD34+ cells, which were cultured with Garcinol for 7 days, were plated in Methocult GF H4435 methylcellulose medium containing 30 ng/mL human SCF, 10 ng/mL human granulo- cyte-macrophage colony-stimulating factor, 10 ng/mL human IL-3, and 3 U/mL human Epo (StemCell Technologies). After 12 to 14 days of culture, the colonies were counted under a microscope.

Histone acetylation assay
The histone acetylation activity in nuclear extracts and the levels of acetylated lysine in test cells were evaluated using an EpiQuik HAT activity/inhibition assay kit (EpigenTech, Brooklyn, NY) and Cellular histone acetylation assay kit (CycLex, Nagoya, Japan), respectively.

Gene expression analysis
hCB CD34+ cells were cultured in the presence of Garcinol derivatives or DMSO for 7 days, and then CD34+CD38– cells were isolated by cell sorting with a FACSAria (BD Bioscience). Total RNA of the isolated cells was extracted using an RNaseasy Mini kit (Qiagen, California). Gene expression was analyzed with a GeneChip 3’ expression array (Kurabo, Otsuka, Japan). All data is MIAME compliant and that the raw data was deposited in Gene Expression Omnibus (accession number GSE29459). The total RNA (1 μg) was reverse-transcribed with a SuperScript one-step RT-PCR kit (Invitrogen). Real-time PCR was carried out for 40 to 45 cycles of 1 minute at 60 °C and 15 seconds at 95 °C in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, CA). All Taqman primers and probes were obtained from Applied Biosystems.

Transplantation of hematopoietic cells into NOD/SCID mice
NOD/SCID mice at 8–10 weeks of age were sublethally irradiated with a dose of 2.75 Gy. Fresh and cultured hCB CD34+ cells were injected intravenously. At 8 weeks post-transplantation, the mice were sacrificed and bone marrow (BM) cells were analyzed with a JSAN desktop cell sorter (Bay Bioscience) for the presence of human CD45+ cells. To obtain the frequency of SCRs, assays were performed using limiting doses of test cells and the data were analyzed using L-Calc software (StemCell Technologies).

Statistical analysis
All results are presented as the mean±standard error of the mean (SEM). Statistical significance was analyzed with Student’s t-test or Williams’ test. The level of significance was set at 0.05.

Supporting Information

Table S1 List of natural products screened.

(DOC)

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Author Contributions
Conceived and designed the experiments: TN AI. Performed the experiments: TN CW KK MO AI. Analyzed the data: TN CW KK MO AI. Contributed reagents/materials/analysis tools: TN HN AI. Wrote the paper: TN HN AI.
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