

Table 1 IC₅₀ scores of tyrosine kinase inhibitors, alkylating agents and Glo-I inhibitors for human CML cell lines and their hypoxia-adapted (HA) subclones

	K562	K562/HA	KCL22	KCL22/HA
<i>ABL TKIs</i>				
Imatinib (nM)	900	7400	—	—
Dasatinib (nM)	3.6	8.9	46.2	2264
INNO-406 (nM)	10.7	142.6	—	—
<i>Alkylating agents</i>				
Daunorubicin (nM)	—	—	105	954.3
Busulfan (mM)	2.4	4.5	2.2	3.0
<i>Glo-I inhibitor</i>				
BBGC (μM)	21.6	5.7	40.7	12.6
COTC (μM)	45.9	16.8	29.6	17.9
m-GFN (μM)	> 300	230.2	> 300	174.2

BBGC, S-p-bromobenzyl glutathione cyclopentyl diester; CML, chronic myeloid leukemia; COTC, 2-crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone; Glo-I, glyoxalase-I; TKI, tyrosine kinase inhibitor

The levels of phosphorylation of Bcr-Abl and its downstream effector Erk were reduced in K562/HA and KCL22/HA cells under hypoxic conditions (1.0% O₂), compared with levels in the parental cells cultured in normoxic conditions. The levels of phosphorylated Akt (p-Akt) and Stat5 (p-Stat5) were similar in both HA and parental cells (Figure 2f). We examined the levels of apoptosis-related proteins such as Bcl-2, Bcl-xL, Bim, Bad, Mcl-1L and Mcl-1S. The levels of Bcl-2 were lower in K562/HA cells than in K562 cells, whereas the levels of Bcl-xL were higher. Bim and Bid interact with Bcl-2 and Bcl-xL to induce apoptosis. The levels of Bim and Bid in K562/HA cells were not different from those in the parental cells. The level of the antiapoptotic Mcl-1L was the same in K562/HA and parental cells, whereas the level of the proapoptotic Mcl-1S decreased in K562/HA cells (Figure 2g). In contrast to K562 and K562/HA cells, there were no differences between KCL22 and KCL22/HA cells (Figure 2g). These findings suggest that adaptation to hypoxia may have some impact on the mechanisms for executing apoptosis, although further investigation will be required to confirm this hypothesis.

Glo-I in HA-CML cells. We examined the ATP levels in parental and HA-CML cells. The amounts of ATP in K562/HA and KCL22/HA cells were 73.0 and 93.2%, respectively, of the levels in their respective parental cell lines under normoxia (Figure 3a). In addition, both HA cell lines exhibited high levels of glucose consumption and lactate production compared with their respective parental cells (Supplementary Figure 4). Normally, one molecule of glucose produces approximately 34–36 ATPs by aerobic respiration, but only 2 ATPs by glycolysis (Figure 3b). Our results suggest that anaerobic glycolysis generates sufficient ATP for survival of HA-CML cells in hypoxic conditions.

When cells preferentially use glycolysis for energy production, glycolysis-specific cytotoxic by-products, such as methylglyoxal, accumulate intracellularly. As glyoxalase-I (Glo-I) protects cells and promotes cell survival by detoxifying methylglyoxal (Figure 3b), we examined Glo-I in HA-CML cells. Both K562/HA and KCL22/HA cells had higher Glo-I

protein levels (Figure 3c) and enzymatic activity (Figure 3d) than the parental cells. Furthermore, the Glo-I protein level increased markedly in the parental K562 cells after cultivation in 1.0% O₂ for 28 days (Figure 3e). In addition, we examined the Glo-I protein levels in primary Bcr-Abl⁺ cells from CML and Ph⁺ ALL patients. Both samples of primary Bcr-Abl⁺ cells possessed higher Glo-I protein levels than normal BM or PB cells (Supplementary Figure 5). These findings indicate that Glo-I expression was induced not only in the artificially generated HA-CML cell lines but also in primary Bcr-Abl⁺ cells, suggesting that primary leukemic cells may be adapted to hypoxia *in vivo*.

High Glo-I expression is sustained in HA-CML cells after 6 months in normoxia. The HA cells returned to normoxic culture conditions revert to the parental cell proliferation rate after 5 days (Supplementary Figure 6a), and the fraction of cells in G₀ decreases within 48 h after return to normoxic conditions (Supplementary Figure 6b). Interestingly, the high level of Glo-1 expression developed by HA-CML cells was sustained after 6 months in culture under normoxic conditions (Supplementary Figure 6c).

Engraftment of HA-CML cells in NOG mice. We established stable subclones of K562 and K562/HA cells (K562^{Luc-EGFP} and K562/HA^{Luc-EGFP}, respectively) that coexpressed luciferase and enhanced green fluorescent protein (EGFP). When we examined the bioluminescence of the cells, K562^{Luc-EGFP} cells produced approximately 10 times more bioluminescence than did K562/HA^{Luc-EGFP} cells (Figure 4a). As the strength of luciferase bioluminescence depends on the availability of ATP and oxygen, we hypothesized that these factors may have a role in the reduced bioluminescence of K562/HA^{Luc-EGFP} cells. Engraftment and proliferation of K562^{Luc-EGFP} and K562/HA^{Luc-EGFP} cells in NOG mice were monitored using *in vivo* imaging (Figure 4a). Despite the lower level of bioluminescence in K562/HA^{Luc-EGFP} cells in culture, there were no significant differences in total photon emission between K562^{Luc-EGFP}- and K562/HA^{Luc-EGFP}-engrafted mice before day 34. After day 34, the total photon emission of K562/HA^{Luc-EGFP}-engrafted mice increased much more sharply than that of K562^{Luc-EGFP} mice (Figure 4b). In addition, K562/HA^{Luc-EGFP}-transplanted mice died significantly earlier than did K562^{Luc-EGFP} mice (Figure 4c). These results indicate that K562/HA^{Luc-EGFP} cells engrafted more efficiently in NOG mice than did the parental K562^{Luc-EGFP} cells.

To investigate why K562/HA^{Luc-EGFP} cells engrafted more efficiently, we examined the cell cycle distribution of K562 and K562/HA cells. The percentages of cells in G₀ in K562 and K562/HA cells were 0.87 ± 0.58 and 4.9 ± 2.1%, respectively, indicating that the K562/HA grafts included more quiescent cells than did the parental line (Figures 4d and e). Next, we examined the expression of c-kit, Tie-2, CXCR4, Notch, N-cadherin, VLA-4, LFA-1 and CD44, all of which have been reported to indicate stemness.²³ There was no difference in the expression levels of these proteins in K562/HA or KCL22/HA cells and the respective parental cell lines (data not shown). Interestingly, both HA cell lines expressed higher levels of β-catenin, which is thought to be important for

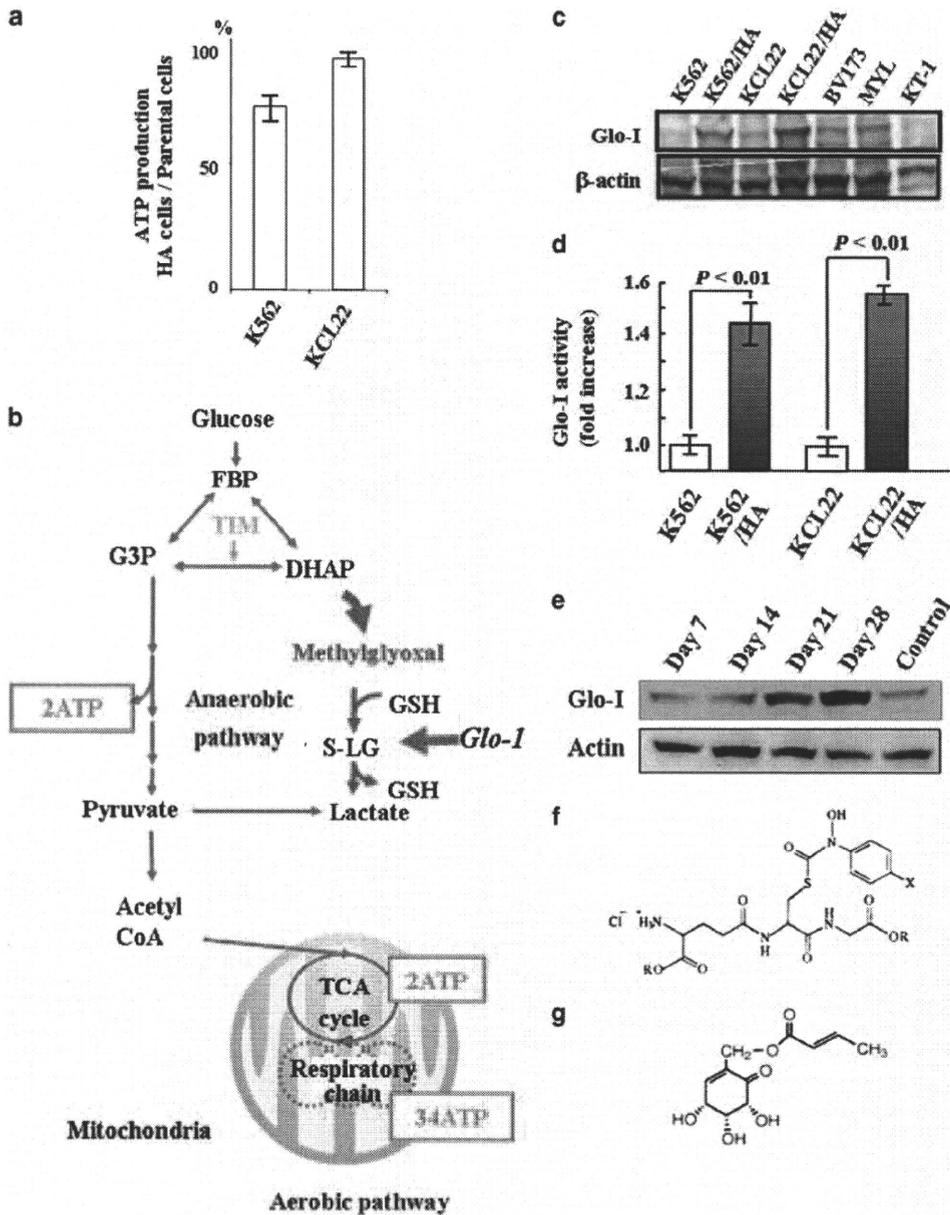


Figure 3 The influences of adaptation to hypoxia on ATP production and glyoxalase-I (Glo-I) activity. (a) ATP production is expressed as the ratio of production in hypoxia-adapted (HA) cells relative to parental chronic myeloid leukemia (CML) cells. (b) Schematic illustration of aerobic and anaerobic ATP production pathways. Methylglyoxal accumulates in cells that use the anaerobic pathway, and Glo-I functions to detoxify methylglyoxal. (c) Glo-I protein levels in parental CML cell lines and the respective HA subclones. (d) Glo-I activity in parental CML cell lines and the respective HA subclones. (e) Glo-I protein levels under hypoxic conditions. Glo-I expression was clearly evident 21 days after initiation of hypoxia (1.0% O₂). Chemical structures of *S-p*-bromobenzyl glutathione cyclopentyl diester (BBGC) (f) and 2-crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone (COTC) (g)

production of CML stem cells (Figure 4f).²⁴ In addition, the K562/HA cell line contained more cells with the side population marker for cancer stem cells (Figure 4g).²⁵ These findings suggest that the adaptation to hypoxia induces putative stem/progenitor cell-like characteristics in Bcr-Abl⁺ cells.

Effect of Glo-I inhibitors on HA-CML cells. *S-p*-bromobenzyl glutathione cyclopentyl diester (BBGC) (Figure 3f) is a specific cell-permeable inhibitor of Glo-I,²⁶ and 2-crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone (COTC) (Figure 3g) is an

inhibitor of Glo-I and glutathione.²⁷ Recently, methyl-gerfelin (Supplementary Figure 7a) was also identified as a Glo-I inhibitor.²⁸ Although HA-CML cells were resistant to the cytotoxic effects of Abl TKIs and alkylating agents (Figures 2d and e, Table 1), BBGC, COTC and methyl-gerfelin were more strongly cytotoxic in K562/HA and KCL22/HA cells than in the parental cell lines (Figures 5a–d, Supplementary Figures 7b and c).

To determine the mechanism of cell death induced by BBGC, we examined Annexin V staining (Supplementary

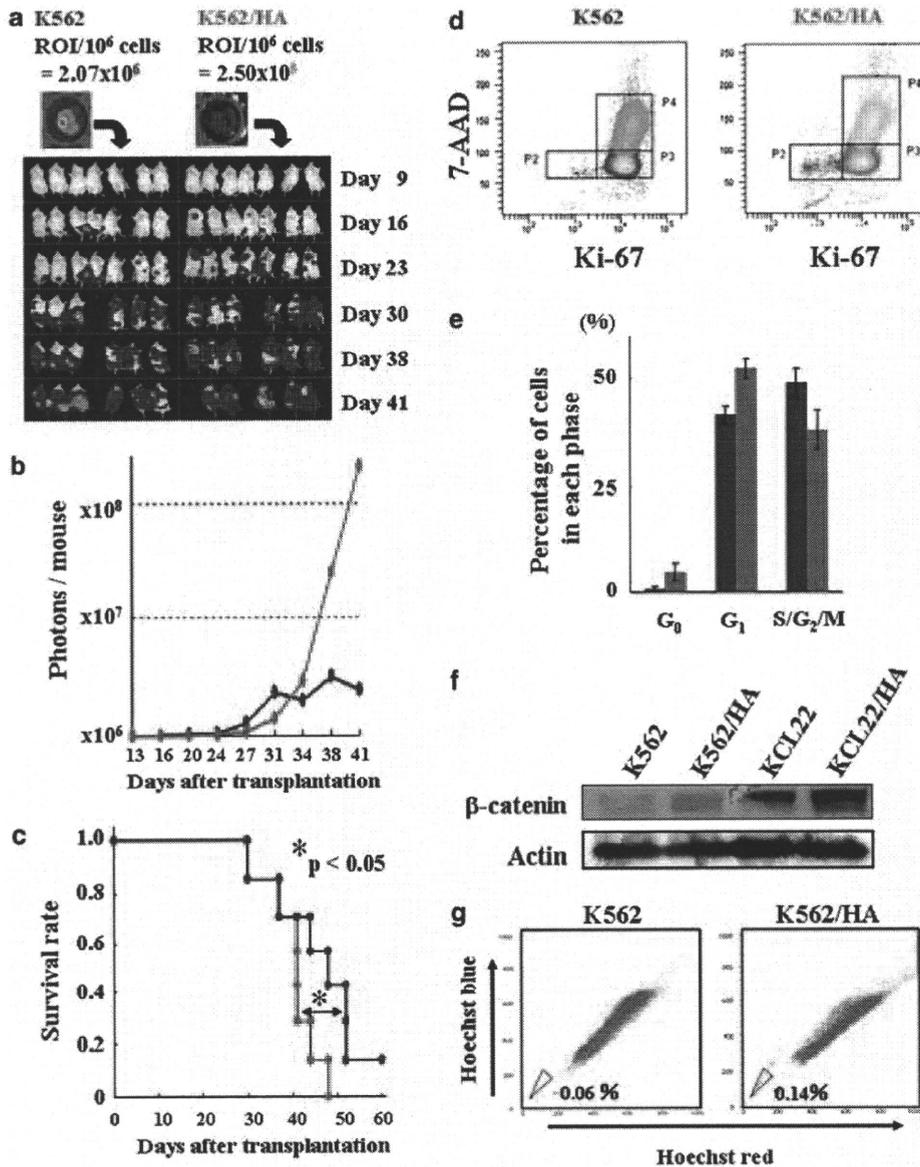


Figure 4 K562/hypoxia-adapted (HA) cells engraft more efficiently in NOD/SCID γ_c^{null} (NOG) mice. (a) Whole animal experiments using NOG mice inoculated with K562^{Luc-EGFP} or K562/HA^{Luc-EGFP} cells. Engraftment was monitored by *in vivo* imaging. (b) Total photon emission from mice inoculated with K562^{Luc-EGFP} (blue) or K562/HA^{Luc-EGFP} (red) cells. (c) Survival rates of mice inoculated with K562^{Luc-EGFP} (blue) or K562/HA^{Luc-EGFP} (red) cells. (d) Cell cycle distributions of K562 and K562/HA determined by double staining with Ki-67 and 7-AAD. (e) The percentages of cells in each phase of the cell cycle in K562 (blue) and K562/HA (red), (f) β -catenin protein expression levels in K562, K562/HA, KCL22 and KCL22/HA cells. (g) Number of side population cells in K562 and K562/HA cells

Figures 8a–d) and mitochondrial outer membrane permeabilization (Supplementary Figures 8e–h). The proportion of cells undergoing apoptosis was greater in the BBGC- and COTC-treated K562/HA and KCL22/HA cells than in the parental cells (Supplementary Figures 9a–d). These results indicate that HA-CML cells were dependent on Glo-I activity for survival under hypoxic conditions, whereas the parental cells, cultured in normoxic conditions, were not dependent on Glo-I activity.

To evaluate the *in vivo* antileukemic activity of BBGC, we treated NOG mice inoculated with K562^{Luc-EGFP} or K562/HA^{Luc-EGFP} cells with BBGC. BBGC had no apparent effect on the survival of K562^{Luc-EGFP}-engrafted mice (Figure 5e).

However, it significantly prolonged the survival of K562/HA^{Luc-EGFP}-transplanted mice (Figure 5f). The body weight of BBGC-treated mice did not decrease during the course of treatment (data not shown). These findings indicate that BBGC has potent antileukemic effects *in vivo*, and preferentially targets CML cells with higher Glo-I activity, with minimal associated toxicity.

Discussion

Abl TKIs can induce apoptosis only in actively proliferating Bcr-Abl⁺ cells, making these drugs much less active against CML stem cells, which are predominantly in a quiescent

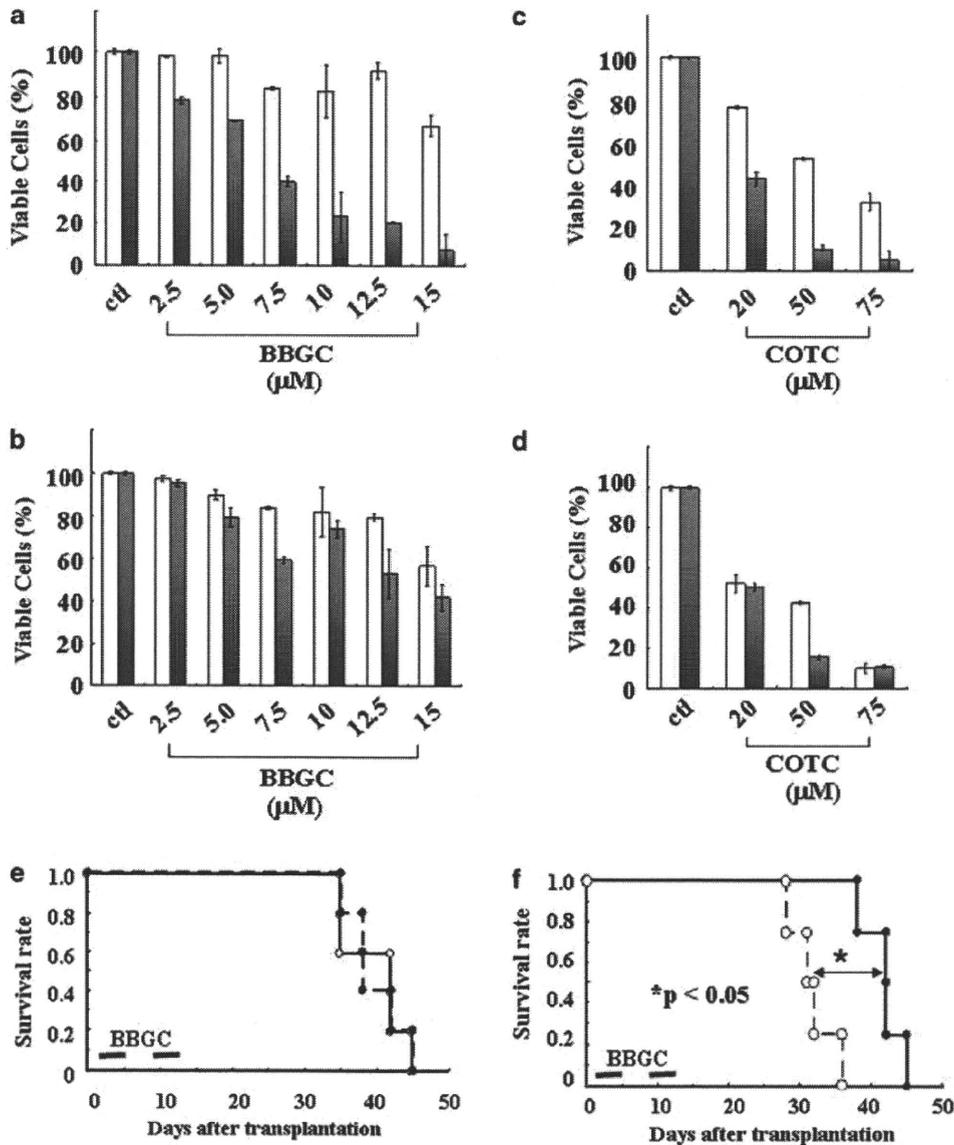


Figure 5 Effect of glyoxalase-I (Glo-I) inhibitors on hypoxia-adapted chronic myeloid leukemia (HA-CML) cells. *In vitro* cytotoxic effects of (a) *S*-*p*-bromobenzyl glutathione cyclopentyl diester (BBGC) and (c) 2-crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone (COTC) on parental K562 (white column) and K562/HA (black column) cells. *In vitro* cytotoxic effects of (b) BBGC and (d) COTC on parental KCL22 (white column) and KCL22/HA (black column) cells. *In vivo* effects of BBGC on transplanted (e) parental K562 and (f) K562/HA cells in NOD/SCID γ_c^{null} (NOG) mice. Vehicle-treated mice, ○; BBGC-treated mice, ●

state.^{10,11,29} Hypoxia favors the self-renewal of normal hematopoietic stem cells,³⁰ and resistance to hypoxia is one of the defining features of leukemic stem cells.³¹ Therefore, it may be more important to search for new antileukemic agents that target quiescent CML stem cells residing in the hypoxic BM milieu. To this end, we have established two HA-CML sublines (Figures 2a–c). Previously, investigators have studied the role of hypoxia in leukemia using relatively short-term assays.^{30–32} Our results suggest that transient hypoxia may not adequately mimic the physiological environment of CML cells (Figure 3e). Furthermore, in the mouse CML xenograft model, the oxygen concentration of engrafted leukemic cells in the BM was <10 mm Hg (~1.3% O₂) (Figure 1f). Interestingly, the HA-CML cells exhibited characteristics similar to CML stem cells, including greater

numbers of cells in a dormant, side population fraction, higher β -catenin expression, resistance to Abl TKIs and higher transplantation efficiency (Figures 2d, e and 4, Supplementary Figures 8 and 9).

The level of Bcr-Abl phosphorylation was lower in HA-CML cells, which may explain why those cells are less sensitive to Abl TKIs (Figure 2f). Giuntoli *et al.*³² have also reported that the hypoxic selection of CML cells resulted in decreased cell sensitivity to imatinib and activation of Bcr-Abl-independent survival signaling pathways. Erk, a downstream effector of Bcr-Abl, was also less phosphorylated in HA-CML cells compared with parental cells, whereas the levels of p-Akt and p-Stat5 were similar (Figure 2f). These observations suggest that alternative mechanisms of activation exist for these signaling molecules in HA-CML cells.³³ In addition to

the phosphorylation status of Bcr-Abl, the sensitivity to antileukemic agents depends on the balance of pro- and antiapoptotic molecules.⁸ Akt pro-survival effects have been reported to be dependent on the first step in glycolysis.³⁴ However, Glo-1 induction in HA-CML cells was not directly controlled by Akt because Akt phosphorylation was unchanged in both HA cell lines (Figure 2f). The adaptation to hypoxia may also alter the status of Bcl-2 family members, because the expression levels of several proteins were altered in K562/HA cells. However, there were no changes in KCL22/HA cells. As the parental KCL22 cells are intrinsically resistant to imatinib and exhibit very high Bcl-2 expression, alterations in Bcl-2 family proteins may not be obvious. Further experiments will be required to clarify the hypoxia-induced changes.

We tried to identify a specific target in HA-CML cells that could be inhibited by small molecules with therapeutic potential. The dependence on glycolysis-mediated ATP production for uncontrolled cellular growth under limited O₂ conditions is a hallmark of malignant cells (Figure 3b).^{35,36} Reduced ATP production (Figure 3a), as well as increased glucose consumption and lactate production (Supplementary Figure 4) in HA-CML cells, suggested that there was preferential utilization of glycolysis for ATP production in these cells. We focused on the components of glycolysis to identify leukemia cell targets that would circumvent drug resistance acquired through adaptation to hypoxia.^{37,38} Glo-1 is an enzyme that detoxifies methylglyoxal, a cytotoxic α -oxoaldehyde side product of glycolysis. Accumulation of methylglyoxal damages cells through multi-base DNA deletions and base-pair substitutions. The overexpression of Glo-1 induces drug resistance to alkylating agents in leukemia as well as other solid tumors.^{26,39} Glo-1 activity was elevated in both K562/HA and KCL22/HA cells (Figures 3c and d), possibly because of their increased dependence on glycolysis during adaptation to hypoxia. Both K562/HA and KCL22/HA cells were more sensitive to cell killing by Glo-1 inhibitors, indicating that these cells are indeed dependent on Glo-1 activity for survival under hypoxic conditions (Figures 5a–d, Supplementary Figures 7b, c, 8 and 9).

All the Glo-1 inhibitors examined were effective for killing HA-CML cells *in vitro*. We selected BBGC for *in vivo* analysis because BBGC was the most potent compound *in vitro*, and because BBGC has previously been used against lung cancer *in vivo*.⁴⁰ As engrafted K562 cells survive in the hypoxic environment of the BM (Figure 1f), we expected that BBGC would be effective against the parental K562^{Luc-EGFP} cells, even if Glo-1 activity in these cells was low. However, BBGC had no effect on the survival of K562^{Luc-EGFP}-engrafted mice (Figure 5e). This result may be due to the time differences between Glo-1 induction by hypoxia and administration of BBGC. BBGC was administered from day 1 to day 11 after transplantation, whereas, in *in vitro* cultures, Glo-1 was not induced until 21 days after the initiation of hypoxia (Figure 3e). Intriguingly, BBGC significantly prolonged the survival of K562/HA^{Luc-EGFP}-engrafted mice compared with untreated mice (Figure 5b). Thus, BBGC may be a promising therapeutic agent for use against CML cells with high Glo-1 activity, which also more frequently accompanies the quiescent status. As the Abl inhibitor imatinib is currently the drug of

choice for CML treatment, we examined the combined effects of BBGC with imatinib *in vitro*. BBGC augmented the effects of imatinib in killing CML cells *in vitro* (Supplementary Figure 10).

In conclusion, the survival of engrafted leukemic cells in the BM under severe hypoxia depends on the induction of Glo-1 activity, and adaptation to hypoxia seems to result in the acquisition of Abl TKI resistance in CML cells. Glo-1 inhibitors were much more effective against HA-CML cells than parental cells both *in vitro* and *in vivo*. These findings indicate the importance of the hypoxic environment for maintaining quiescent CML cells, and suggest that Glo-1 is a novel target for CML treatment.

Materials and Methods

Reagents and cell lines. The Glo-1 inhibitors, BBGC, COTC and methylglyoxal, were synthesized and purified as previously described.^{26–28} The K562 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The other CML-derived cell lines (KCL22, BV173 and MYL) were kindly provided by Dr Tadashi Nagai (Jichi Medical School, Tochigi, Japan), Dr Oliver G Ottmann (Frankfurt University, Frankfurt, Germany) and Dr Hideo Tanaka (Hiroshima University, Hiroshima, Japan), respectively. The CML cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (Vitromex, Vilshofen, Germany) at 37°C in a humidified atmosphere of 20% O₂, 5% CO₂ and 75% N₂. CML cells were subjected to continuous culture in 1.0% O₂ (7.2 mm Hg), 5% CO₂ and 94% N₂, and HA subclones of K562 (K562/HA) and KCL22 (KCL22/HA) were selected and maintained in suspension in low O₂ conditions for more than 6 months. Parental K562 and K562/HA cells were cotransfected with pGL3, a control luciferase (Luc) reporter vector (Promega, Madison, WI, USA) and pCAG.*egfp.neo* using the Nucleofector Kit V, protocol T-03 (Amaxa AG, Cologne, Germany). Stable transfectants (K562^{Luc-EGFP} and K562/HA^{Luc-EGFP}) were selected by culturing in medium containing G418 (1 mg/ml, Sigma Aldrich, Tokyo, Japan) and isolated by agarose gel cloning assays. Human primary Bcr-Abl⁺ leukemic cells were obtained from patients with informed consent, according to the Declaration of Helsinki.

***In vivo* engraftment of CML cells and histological analysis.** Animal studies were performed in accordance with the guidelines of the Institutional Review Board for animal studies at Kyoto University. To evaluate the oxygen status of engrafted leukemic cells, 1.0 × 10⁶ cells were injected into sublethally irradiated (2 Gy) male NOG or NOD/SCID mice at 6–8 weeks of age. At 35 days (NOG mice) or 50 days (NOD/SCID mice) post-inoculation, the mice were injected intraperitoneally with pimonidazole hydrochloride (Pimo, Chemicon, Temecula, CA, USA) (60 mg/kg), and 60 min later, the animals were killed and the femur and liver removed. Tissues were subjected to hematoxylin–eosin staining and immunohistochemical analysis using anti-Pimo (Chemicon) and antihuman Ki67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, and examined by microscopy. *In vivo*, leukemia cell proliferation was detected by monitoring luciferase expression using an *in vivo* imaging system (Xenogen, Berkeley, CA, USA), as previously described.⁴¹ For survival analysis, time of death was determined either by spontaneous death, or by date of euthanasia due to pain or suffering, according to established criteria. We also confirmed the oxygen concentration of primary Bcr-Abl⁺ leukemic cells that were obtained from a Ph⁺ ALL patient and engrafted in the BM of NOD/SCID mice. Primary Bcr-Abl⁺ leukemic cells (1 × 10⁶) were transplanted into four NOD/SCID mice, which were killed 50 days after transplantation, and the BM sections were stained as above.

Cell death. Cell viability was measured by incorporation of propidium iodide (PI). Mitochondrial transmembrane potential ($\Delta\psi/m$) was determined by staining with 3,3'-dihexyloxycarbocyanine iodide (Molecular Probes, Eugene, OR, USA), as previously described.⁹ For analysis of DNA content, cells were fixed with ice-cold 70% ethanol and then incubated with PI as previously described.⁴² The percentage of cells that incorporated PI, the $\Delta\psi/m$, and the percentage of cells in sub-G₁ of the cell cycle were determined by FACS using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Western blot analysis. Proteins were separated by SDS-PAGE and then electroblotted onto a Hybond-PDVF membrane (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated with 5% (wt/vol) nonfat dry milk in

phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 (Sigma, Saint Louis, MO, USA). Antibodies specific for Akt (#9272, 60 kDa), Erk1/2 (#9102, 42, 44 kDa), phospho (p)-Akt (#9271, 60 kDa), p-Erk1/2 (#9101, 42, 44 kDa) and p-Stat5 (#9351, 90 kDa, Cell Signaling Technologies, Beverly, MA, USA); for Bcl-2 (clone 100, #05-729, 26 kDa) and p-tyrosine (#05-321, Upstate, Lake Placid, NY, USA); as well as for Stat5 (#sc-835, 92 kDa) and c-Abl (#sc-23, 120 kDa, Santa Cruz Biotechnology); Bcl-X_L (#AAM-080, 26 kDa, Stressgen, Victoria, British Columbia, Canada); Glo-I (#H00002739-A01, 28 kDa, Novus Bio, Littleton, CO, USA); β-actin (#A2066, 42 kDa, Sigma); Mcl-1L (38 kDa) and Mcl-1S (30 kDa) (#LS-C43163, Life Span Biosciences, Seattle, WA, USA); and for β-catenin (#610153, 92 kDa, BD Biosciences, San Diego, CA, USA) were used as indicated. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies using enhanced chemiluminescence (ECL Advance, Amersham Biosciences).

Measurement of Glo-I activity. Cells were lysed in PBS containing 1 mM phenylmethylsulfonyl fluoride by freezing and thawing, followed by sonication. The lysates were centrifuged at 12 000 × g for 20 min and the supernatant was used as the cytosolic fraction. The Glo-I assay was performed in 0.1 M sodium phosphate (pH 7.0), 7.9 mM methylglyoxal (Sigma), 1 mM GSH and 14.6 mM MgSO₄ at 25 °C. An increase in absorbance at 240 nm because of the formation of S-D-lactoylglutathione was measured using a temperature-controlled spectrophotometer (Beckman Coulter, Brea, CA, USA, DU640).

ATP assay. ATP levels were measured using an ATP assay kit (TOYO INK, Tokyo, Japan), according to the manufacturer's instructions.

Measurement of glucose consumption and lactate production. Cells were suspended in fresh culture medium. After 6 h, the cells were collected by centrifugation and resuspended in 5 ml of RPMI at a density of 2 × 10⁶ cells/ml. Cells were incubated for 24 h, and the culture medium was collected for measurement. Glucose levels were determined using a glucose assay kit (GO, Sigma). Glucose consumption was determined from the difference in glucose concentration compared with the starting medium. Lactate levels were determined using a lactate assay (F-kit L-lactate, JK International, Tokyo, Japan).

Determination of quiescent cells. Numbers of quiescent leukemic cells (in G₀ phase) were determined by double staining with Ki-67 and 7-AAD as previously described.⁴³ Briefly, 1 × 10⁶ K562 or K562/HA cells were fixed in ice-cold 70% EtOH for at least 12 h, and then resuspended in 100 μl PBS. Cells were stained with 20 μl Ki-67 antibody (BD Biosciences) and incubated for 30 min at RT. Subsequently, 20 μl 7-AAD (BD Biosciences) was added, and the cells were resuspended in 500 μl PBS with 1% FBS and analyzed by FACS. Three independent analyses were performed.

BBGC treatment of CML mice. NOG mice 6–8 weeks of age were sublethally irradiated (2 Gy) and inoculated with 1.0 × 10⁶ K562/HA^{Luc-EGFP} or K562/HA^{Luc-EGFP} cells by intravenous tail vein injection. Therapeutic treatments (seven mice per group) were started 1 day (day 1) after transplantation. To prepare the BBGC solution, BBGC dissolved in cremophor EL/DMSO (1 : 1) was diluted to 10 mg/ml using DMSO and PBS. Within each group, half of the mice were administered EL/DMSO only (vehicle controls), and the remainder were administered 10 mg/kg BBGC on days 1 through 4, and days 8 through 11 after transplantation. For survival analysis, the time of death was determined either by spontaneous death or by date of euthanasia due to pain or suffering, according to established criteria.

Statistical analysis. Survival curves were drawn using the Kaplan–Meier method and compared using the log-rank test. *P*-values were derived from two-sided tests and a *P*-value < 0.05 was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)

