

Glycogen synthase kinase–3 β inhibitors suppress leukemia cell growth

Emma Y. Song^{a,b}, Patricia Palladinetti^{b,c}, Guy Klamer^{b,c}, Kap-Hyoun Ko^{a,b}, Robert Lindeman^{b,d}, Tracey A. O'Brien^{a,b}, and Alla Dolnikov^{a,b}

^aSydney Cord and Marrow Transplant Facility, Centre for Children's Cancer and Blood Disorders, Sydney Children's Hospital, Sydney, Australia;

^bThe University of New South Wales, Sydney, Australia; ^cChildren's Cancer Institute Australia for Medical Research, Lowy Cancer Research Centre, The University of New South Wales, Sydney, Australia; ^dDepartment of Hematology, Prince of Wales Hospital, Sydney, Australia

(Received 3 March 2010; revised 1 June 2010; accepted 1 June 2010)

Objective. The objective of this study was to investigate the effect of small molecule inhibitors of glycogen synthase kinase–3 β (GSK-3 β) on leukemia cell growth and survival.

Materials and Methods. Analysis of cytotoxicity and cell proliferation was conducted using the MTS assay, cell-cycle analysis, and division tracking. Apoptosis was investigated by Annexin-V/7-aminoactinomycin D and caspase-3 expression. The effect of GSK-3 β inhibitors was also tested in vivo in an animal model of leukemia. Gene expression analysis was performed to identify the genes modulated by GSK-3 β inhibition in leukemia cells.

Results. GSK-3 β inhibitors suppress cell growth and induce apoptosis in seven leukemia cell lines of diverse origin, four acute myeloid leukemia, one myelodysplastic syndrome, and one acute lymphoblastic leukemia samples. GSK-3 β inhibitors are cytotoxic for rapidly dividing clonogenic leukemia blasts, and higher doses of the inhibitors are needed to eliminate primitive leukemia progenitor/stem cells. Slow cell-division rate, low drug uptake, and interaction with bone marrow stroma make leukemia cells more resistant to apoptosis induced by GSK-3 β inhibitors. Global gene expression analysis combined with functional approaches identified multiple genes and specific signaling pathways modulated by GSK-3 β inhibition. An important role for Bcl2 in the regulation of apoptosis induced by GSK-3 β inhibitors was defined by expression analysis and confirmed by using pharmacological inhibitors of the protein. In vivo administration of GSK-3 β inhibitors delayed tumor formation in a mouse leukemia model. GSK-3 β inhibitors did not affect hematopoietic recovery following irradiation.

Conclusions. Our data support further evaluation of GSK-3 β inhibitors as promising novel agents for therapeutic intervention in leukemia and warrant clinical investigation in leukemia patients. Crown Copyright © 2010 Published by Elsevier Inc. on behalf of the ISEH - Society for Hematology and Stem Cells. All rights reserved.

Glycogen synthase kinase-3 β (GSK-3 β) is a serine/threonine kinase that regulates a number of signaling pathways in the cell [1,2]. GSK-3 β was identified and studied initially for its role in the regulation of glycogen synthesis, however, over the past decade, interest in GSK-3 β has grown far beyond glycogen metabolism due to its role in the regulation

of many other cellular processes, particularly cell proliferation, apoptosis, and stem-cell maintenance [2–4]. GSK-3 β activity is regulated by various kinases in response to different stimuli [5]. GSK-3 β phosphorylates a broad range of substrates making them susceptible to degradation, with the specificity of GSK-3 β signalling depending on cell context [5]. The role that GSK-3 β plays in the regulation of normal and aberrant haematopoiesis, stem cell function, angiogenesis and inflammation, makes GSK-3 β a candidate therapeutic target in leukemia and inflammatory diseases [6,7].

Although inhibition of GSK-3 β was shown to be procarcinogenic in colorectal cancer because of its activation of β -catenin, in certain cancers it is the reverse that is possibly

Offprint requests to: Alla Dolnikov, Ph.D., Sydney Cord and Marrow Transplant Facility, Centre for Children's Cancer and Blood Disorders, Sydney Children's Hospital, High Street, Randwick 2031, Sydney, Australia; E-mail: Alla.Dolnikov@sesiahs.health.nsw.gov.au

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.exphem.2010.06.001.

paving the way for small molecule inhibitors of GSK-3 β as selective anti-cancer agents. GSK-3 β inhibitors were shown to suppress chronic lymphocytic leukemia, glioblastoma, breast and colorectal cancer cell lines with oncogenic phosphatidylinositol 3-kinase, catalytic, α polypeptide (PIK3CA) mutations [8–11]. In addition, GSK-3 β was shown to support mixed lineage leukemia (MLL) cell proliferation and transformation by a mechanism that involves destabilization of the cyclin-dependent kinase inhibitor p27Kip1 [5]. Inhibition of GSK-3 β in a preclinical murine model of MLL leukemia provided promising evidence of efficacy and identified GSK-3 β as a candidate cancer drug target for MLL leukemia [5].

In the present study, we demonstrate that small molecule inhibitors of GSK-3 β suppress cell growth and induce apoptosis in leukemia cell lines of diverse origin and, more importantly, primary leukemia samples. When tested in a bone marrow transplantation model, acute myeloid leukemia (AML) stem cells pretreated with GSK-3 β inhibitors exhibited reduced engraftment. In vivo administration of a GSK-3 β inhibitor delayed tumor formation in a mouse leukemia model and did not affect hematopoietic recovery following irradiation. Gene expression analysis identified multiple genes and specific molecular pathways modulated by GSK-3 β inhibition in human leukemia TF-1 cells. Our data support further evaluation of GSK-3 β inhibitors as promising novel agents for therapeutic intervention in leukemia.

Materials and methods

Cell culture

Human leukemia TF-1, U937, K562, HL-60, CEM, Jurkat, and D1.1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium. Primary bone marrow cells were obtained from patients with AML (n = 4), acute lymphoblastic leukemia (ALL) (n = 1), and myelodysplastic syndrome (MDS) (n = 1) at diagnosis after obtaining patient's consent to perform the research. This study was approved by the Institutional Review Board and met all requirements of the Declaration of Helsinki. Cells were cultured in serum-free medium StemPro-34 SFM (Invitrogen, Melbourne, Australia) with the addition of stem cell factor and interleukin (IL)-3 (R&D Systems, Minneapolis, MN, USA), both at 100 ng/mL. Bone marrow stroma, MS5 cells were cultured in α -minimal essential medium (Gibco BRL; Invitrogen, Melbourne, Australia) supplemented with 10% fetal calf serum. TF-1 cells required the addition of 10 ng/mL human IL-3. Selective adenosine-5'-triphosphate (ATP)-competitive inhibitors of GSK-3 β , i.e., 6-bromoindirubin-3'-oxime (BIO), SB-216763, and SB-415286 (Sigma-Aldrich, Sydney, Australia) were used at 1 to 5 μ M. Intracellular concentration of BIO was measured by flow cytometry based on the inherent ability of BIO to excite fluorescence (λ = 580 nm). Lithium chloride was used at a concentration of 1 to 20 mM. Bcl2 inhibitor ABT737 was used at a concentration of 0.3 to 3 μ M (Selleck Chemicals, Shanghai, China). Quercetin dihydrate was used at a concentration of 12.5 to 100 μ M (Sigma-Aldrich). Mouse anti-human integrin α 4 monoclonal antibody was purchased from Millipore (Sydney,

Australia). CD123-allophycocyanin (APC), CD34-APC, mCD45-fluorescein isothiocyanate, and hCD45-APC were purchased from Becton Dickinson (Sydney, Australia).

Cytotoxicity assay

Cytotoxicity of GSK-3 β inhibitors was analyzed using the MTS assay (Promega, Sydney, Australia). Briefly, exponentially growing leukemia cells were seeded into 96-well flat-bottomed plates in triplicate at a cell density of 1×10^4 cells in 50 μ L medium. Fifty microliters of each GSK-3 β inhibitor used at different concentrations were added to the cells. Cells were incubated with or without inhibitors for 72 hours at 37°C. Cells were then incubated with 20 μ L CellTiter 96 Aqueous One Solution reagent for 3 hours. The absorbance in each well was then recorded at 490 nm using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA). The absorbance reflects the number of viable cells.

Analysis of cell cycle and apoptosis

Cells were incubated in 1% Triton X-100 and 1 M sodium citrate followed by staining with 50 μ g/mL propidium iodide for 45 minutes at 37°C (Sigma-Aldrich). Propidium iodide fluorescence was analyzed by flow cytometry using FACSCanto and data was analyzed using FACSDiva software, both from Becton Dickinson. Annexin-V staining was performed using Annexin-V staining kit (Becton Dickinson) according to manufacturer's instructions. Annexin-V–positive/7-aminoactinomycin D–negative cells identified by two-parameter flow cytometry were considered as apoptotic. Caspase-3 activity was measured using caspase-3 assay kit (Becton Dickinson) according to manufacturer's instructions. High-resolution division tracking of AML cells stained with carboxyfluorescein succinimidyl ester (CFSE) was performed as described previously [12].

Colony-forming unit assay

The colony-forming unit assay was performed using MethoCult GF H4434 (Stem Cell Technologies, Vancouver, BC, Canada). One-thousand control or 72-hour post-5- μ M BIO-pretreated leukemia cells were plated in 1.1 mL MethoCult and analyzed 11 days after plating.

Western blot analysis

Western blot analysis was performed using standard techniques. Briefly, cell lysates were obtained from TF-1 cells incubated with BIO (1–5 μ M) for 24 hours. Protein lysates were run on a precast 10% gradient polyacrylamide gel (Bio-Rad, Sydney, Australia). Mouse anti- β -catenin and Bcl2 antibody were purchased from Becton Dickinson and anti-actin from Sigma-Aldrich. Goat anti-mouse antibody conjugated with horseradish peroxidase was used as a secondary antibody.

Gene expression analysis

RNeasy Mini-Kit (Qiagen, Melbourne, Australia) was used to extract total RNA. Biotinylated complementary RNA was prepared using an Illumina TotalPrep RNA Amplification Kit (Ambion, TX, USA). Complementary RNA was hybridized to Sentrix HumanRef-8 Expression version 2.0 BeadChips (Illumina) containing 24,526 human genes. All procedures for hybridization, signal detection, and analysis were performed according to BeadStation 500 \times system protocols. Raw data were normalized to the background and a detection *p* value of ≤ 0.01 , differential score ≥ 13 , and fold ratio change ≥ 1 -fold. The online Database for Annotation, Visualization and

Integrated Discovery 2008 [13,14] was used to identify molecular pathways modulated by the treatments.

Real-time polymerase chain reaction

SYBR green–based quantitative real-time polymerase chain reaction (PCR) was used to validate gene expression. Complementary DNA was synthesized using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). All reactions were performed in triplicate and analyzed using iCycler iQ Real-Time PCR Detection System version 4.006 (Bio-Rad).

TCF/LEF reporter assay

HEK293-H cells were transfected with β -catenin/green fluorescent protein (GFP) or Bcl2 luciferase reporter constructs (Cignal TCF/LEF Reporter Kit, SABiosciences and Switchgear Genomics, CA, USA, respectively). HEK293-H cell transfection was performed according to manufacturer's instruction. Briefly, exponentially growing HEK293-H cells were transfected with 400 ng β -catenin/GFP DNA construct using SureFECT (SABiosciences). TCF/LEF binding sites upstream of a basal promoter element (TATA box) drive expression of GFP. BIO (1 μ M) or quercetin (25 μ M) was added to cells 24 hours after transfection. HEK293-H cells were transfected with 50 ng Bcl2/luciferase construct using Fugene (Roche-Applied Science, Sydney, Australia). GFP or luciferase expression was analyzed 16 hours after treatment with BIO and/or quercetin. GFP expression was analyzed by flow cytometry. Luciferase activity was analyzed using SteadyGlo Luciferase Assay reagent (Promega, Madison, WI, USA) according to manufacturer's recommendations.

Primary AML cell engraftment tested in a bone marrow transplantation model

Female, 6- to 8-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were obtained from Monash University. Mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by University of New South Wales Animal Care and Ethics Committee and in accordance with state regulations and standards. All animal studies were approved by Animal Care and Ethics Committee.

Ten NOD/SCID mice were irradiated with a sublethal dose of 2.5 Gy from a Cobalt-60 source 10 to 12 hours before being transplanted with AML cells as described previously [15]. AML cells were treated with BIO or dimethyl sulfoxide overnight prior to injection. Each mouse was transplanted with the equivalent of 1×10^7 unexpanded cells. Each group contained five mice. The bone marrow content of both femurs was collected 6 weeks after transplantation and analyzed by flow cytometry for human cell engraftment using species-specific anti-CD45 antibody.

Testing of GSK-3 β inhibitors in an animal model of leukemia

Female, 6- to 8-week-old BALB/c (nu/nu) athymic nude mice were obtained from the Animal Resources Centre (Western Australia). Human leukemia K562 cells were injected subcutaneously into the right flank of the mouse. BIO was injected intraperitoneally, 30 mg/kg, three times per week, starting from day 4 after cell inoculation. Control mice were injected with saline. Five mice per group were examined. Subcutaneous tumors were detectable 2 weeks after cell inoculation. Mice were culled when tumors reached 1 cm in any dimension.

Testing the effect of GSK-3 β inhibitors on hematopoietic recovery after irradiation

Both NOD/SCID and nu/nu mice were examined. Mice were sublethally irradiated as described here. BIO (30 mg/kg) or lithium chloride (200 mg/kg) ($n = 5$) was injected intraperitoneally five times per week. Peripheral blood cell counts were measured twice a week. Mice were analyzed postmortem 3 and 4 weeks after irradiation. Bone marrow was analyzed by flow cytometry using mouse-specific antibodies.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA). Results were expressed as mean \pm standard deviation. Differences between groups were examined for statistical significance using Student's *t*-test.

Results

Leukemia cell growth is suppressed by GSK-3 β inhibition

GSK-3 β inhibitors suppressed cell growth in seven leukemia cell lines, four AML, one MDS, and one ALL samples. Five leukemia cell lines and one AML (AML1), MDS, and ALL sample, as well as normal bone marrow cells are presented in Figure 1A and B. Stabilized β -catenin, GSK-3 β substrate, was used as a reliable surrogate for measuring GSK-3 β activity (Fig. 1C). Inhibitor dose escalation resulted in higher cytotoxicity and correlated with higher expression of β -catenin (Fig. 1A–C). Among several different GSK-3 β inhibitors tested, BIO exhibited the highest cytotoxicity in TF-1 cells (Fig. 1A). All cell lines treated with BIO exhibited an increased proportion of cells with subgenomic DNA content, Annexin-V– and caspase-3–positive cells representing apoptotic cells (Fig. 1E–G, data for Jurkat cells only is shown). BIO reduced the number of clonogenic leukemia blasts in TF-1 (data not shown) and HL-60 cells and primary AML (Fig. 1H). The average number of cells produced by each clonogenic blast in BIO-treated cells was lower than in control, suggesting that the proliferative capacity of leukemia blasts that survived treatment with BIO was severely impaired (Fig. 1H). BIO used at doses toxic to leukemia cells showed only a minimal reduction in cell numbers when tested on bone marrow mononuclear cells from healthy patients (Fig. 1B).

Leukemia progenitor/stem cells display differential drug sensitivity determined by division rate and intracellular concentration of the drug

It is important for anti-leukemic treatment to be effective to target progenitor/stem cells. Primitive AML progenitor cells similar to normal hematopoietic stem cells are quiescent in vivo and divide slowly in ex vivo cultures. As a surrogate marker, this can be addressed with the slow dividing fraction of AML cells. High-resolution division tracking was performed to examine the sensitivity of slowly and rapidly

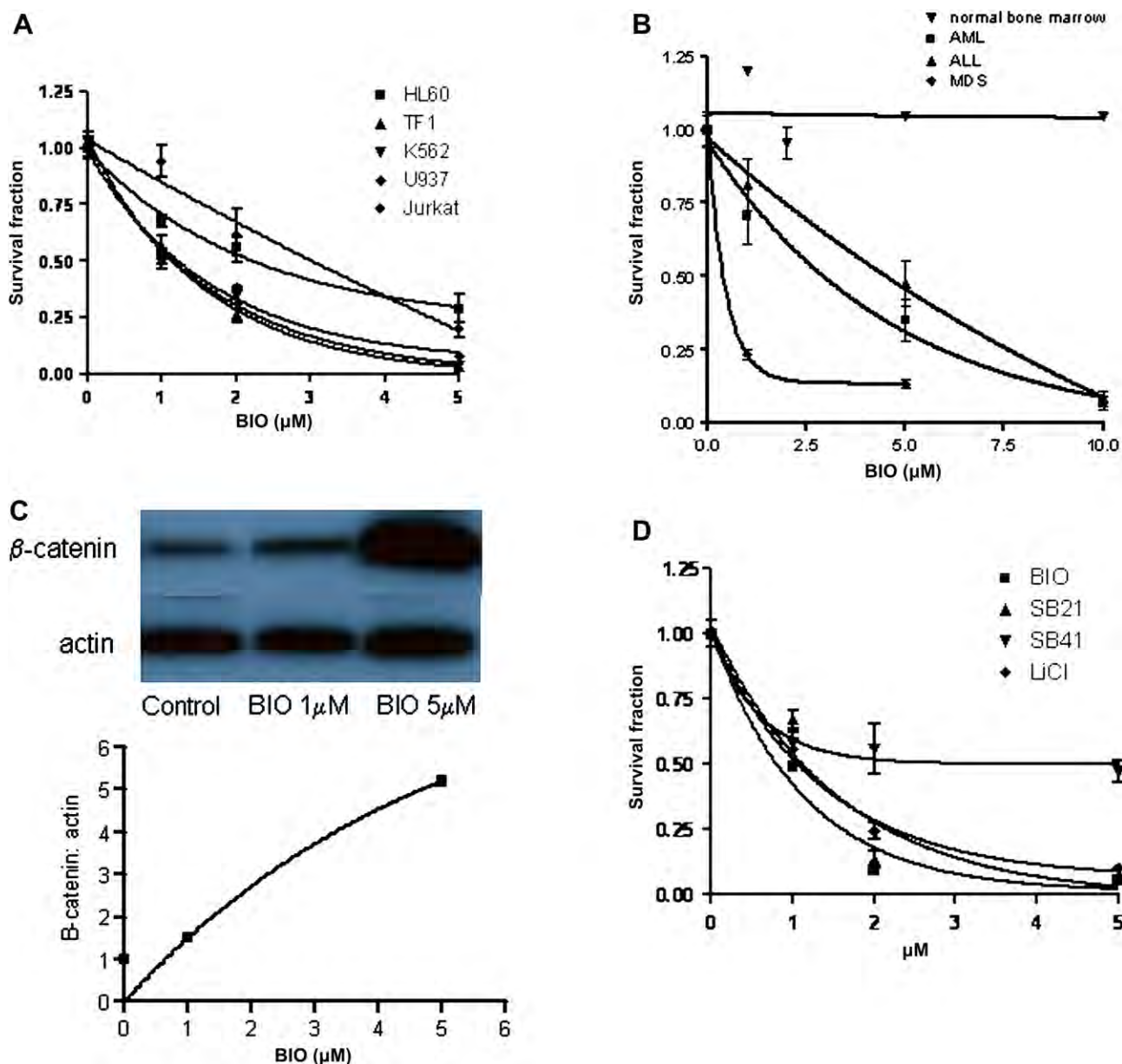


Figure 1. Leukemia cell growth is suppressed by glycogen synthase kinase-3 β (GSK-3 β) inhibitors. GSK-3 β inhibition caused cytotoxicity in leukemia cell lines (A) and primary leukemia samples (B), when incubated with increasing concentrations of 6-bromoindirubin-3'-oxime (BIO) for 72 hours. There was a significant difference in cell survival between BIO-treated and control cells ($p < 0.01$). (C) GSK-3 β inhibition was monitored by the accumulation of cellular β -catenin, the direct substrate of GSK-3 β using Western blot analysis in TF-1 cells treated with BIO for 72 hours. (D) MTS assays revealed the cytotoxicity of different GSK-3 β inhibitors in TF-1 cells after 72 hours of treatment. Of note, in contrast to BIO and other inhibitors, lithium chloride (LiCl) was used in mM doses.

dividing primary AML cells to BIO-induced cytotoxicity. CFSE-staining experiments were performed with three primary AML samples. The experiments revealed that BIO acts to reduce the proportion of viable cells and depleted AML samples from rapidly dividing CFSE^{dim} cells, suggesting that slowly dividing CFSE^{bright} cells exhibit significant resistance to BIO-induced apoptosis (Fig. 2A–C). Slowly dividing CFSE^{bright} cells exhibited lower mean intensity of fluorescence produced by BIO compared to rapidly dividing CFSE^{dim} cells (Fig. 2E). BIO dose escalation resulted in an increased intracellular concentration of the drug, suggesting

that susceptibility of leukemia cells to apoptosis depends on intracellular concentration of BIO (Fig. 2E). A similar trend was seen in all three AML samples. One representative experiment with AML1 is shown in Figure 2A–E.

Finally, a slight increase in the number of slowly dividing CFSE^{high} cells was seen in the R4 gate in AML1 cells treated with 10 μM BIO (Fig. 2C). Apparently, the reduction in rapidly dividing leukemia cells promotes division of quiescent cells. In addition, the effect of BIO was examined on the CD34⁺CD38⁻ fraction of AML1 cells. The CD34⁺CD38⁻ cell phenotype is another surrogate marker

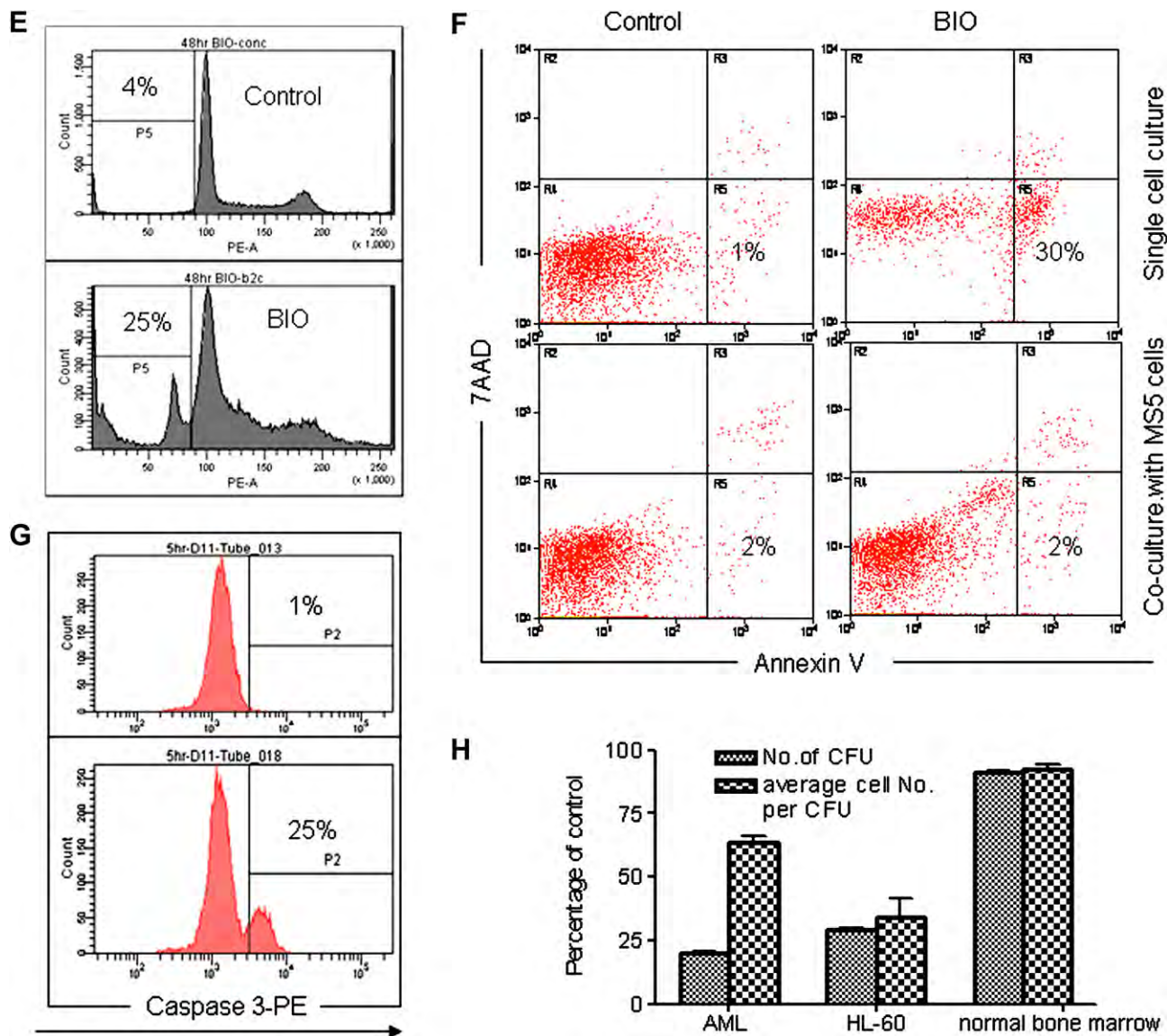


Figure 1. (Continued.) (E) Cell-cycle analysis of Jurkat cells treated with 2 μ M BIO for 48 hours revealed an increased proportion of cells with subgenomic DNA content representing apoptotic cells. (F) Expression of Annexin-V and 7-aminoactinomycin D in TF-1 cells grown in suspension culture or cocultured with MS5 stroma cells and treated with 2 μ M BIO for 72 hours was used to analyze apoptosis. (G) Apoptosis was further analyzed by caspase-3 activation in Jurkat cells treated with 2 μ M BIO for 72 hours. (H) BIO attenuated clonogenic activity of HL-60 and primary acute myeloid leukemia, but not normal bone marrow mononuclear cells.

for primitive leukemia stem cells. A reduction in CD34⁺CD38⁻ cell numbers was seen in BIO-treated AML1 cells in a BIO-dose-dependent manner, and both bulk CD34⁺ and CD34⁺CD38⁻ cells comprising 50% of total CD34⁺ cells exhibited a similar sensitivity to BIO (Fig. 2D, left panel). Thus, slowly dividing CFSE^{bright} AML1 cells appear to be an alternate surrogate marker to characterize the response of most primitive leukemia progenitor cells to BIO-induced cytotoxicity rather than a CD34⁺CD38⁻ phenotype. In the AML2 sample, however, CD34⁺CD38⁻ cells were more resistant to BIO compared to bulk CD34⁺ and AML blasts (Fig. 2D, right panel). Collectively, these results suggest that both a CD34⁺CD38⁻ phenotype and a slow

division pattern may be used independently to characterize the therapeutic response in vitro.

The engraftment of AML cells pretreated with BIO was tested in a NOD/SCID mouse transplantation model [16]. It was previously shown that only a proportion of primary AML samples engraft in an NOD/SCID mouse model. In addition, it was demonstrated that only primitive CD34⁺ AML stem cells contribute to engraftment [16]. AML samples from four patients were tested in our experiments and only two samples (AML1 and AML3) exhibited significant engraftment (Fig. 2F). Human cell engraftment was significantly reduced from 19% in control mice to <0.1% in mice transplanted with AML1 cells pretreated

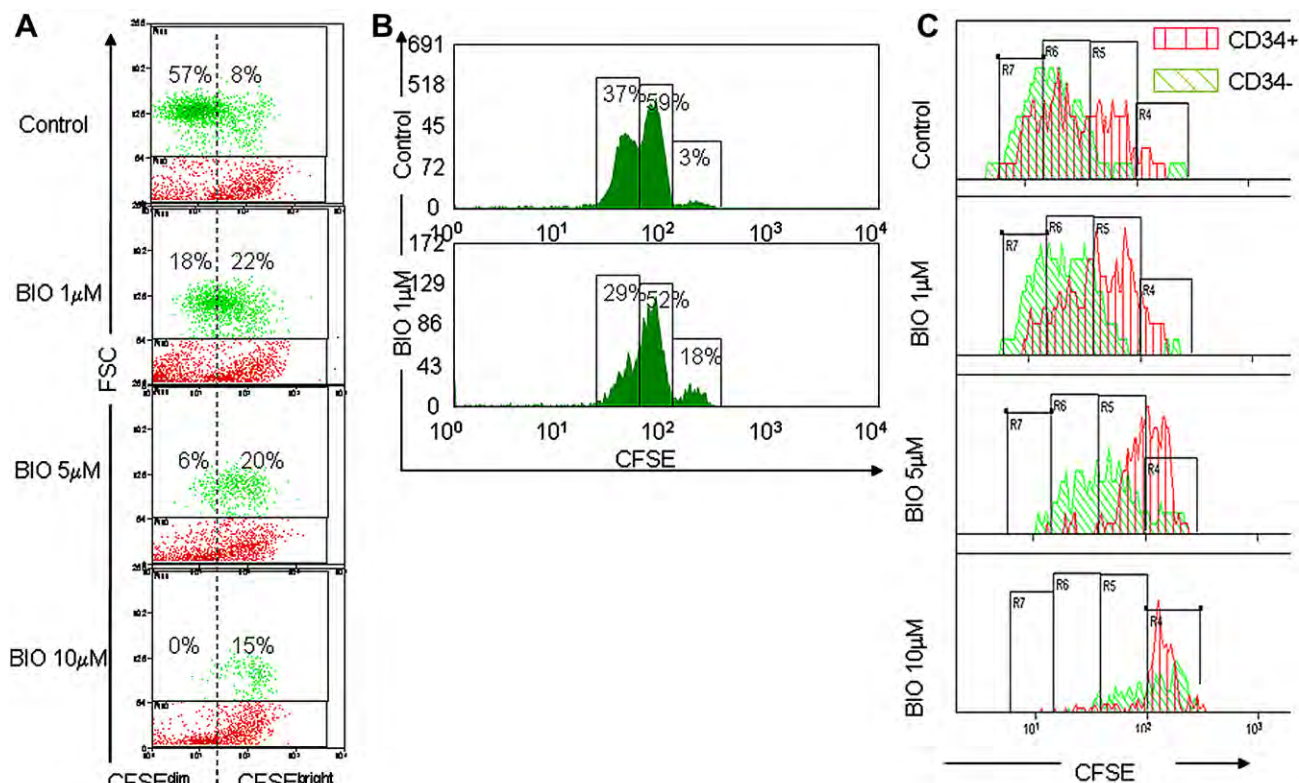


Figure 2. Growth suppression induced by 6-bromindirubin-3'-oxime (BIO) in primary acute myeloid leukemia (AML) cells. Primary AML cells stained with carboxyfluorescein succinimidyl ester (CFSE) for division tracking then treated with different concentrations of BIO for 72 hours (A–C). (A) Apoptotic cells were identified as FSC^{low} and CFSE^{dim} shown in left and right bottom quadrants. The percentage of alive cells (left and right top quadrants) is shown in each panel. Slowly dividing CFSE^{bright} cells exhibited significant resistance to BIO-induced apoptosis. (B) The histogram shows the percentage of cells in each division, slowly dividing cells are more resistant to BIO-induced cytotoxicity. (C) BIO dose escalation reduced the proportion of alive cells and depleted AML samples from rapidly dividing CFSE^{dim} cells, showing that slowly dividing CFSE^{bright} cells exhibit significant resistance to BIO-induced apoptosis. This was relevant for both CD34⁺ and CD34⁻ AML cells.

with BIO (Fig. 2F and G). Remarkably, the impaired engraftment of AML1 cells was observed at 5 μM BIO, which does not kill all CFSE^{bright} CD34⁺ cells, leaving 25.9% of cells alive (Fig. 2C). Apparently, BIO not only induces apoptosis, but also impairs engraftment of AML stem cells that survive BIO-induced apoptosis. In addition, three of five mice transplanted with AML3 cells showed engraftment compared to no engraftment in the BIO group (Fig. 2F).

Testing GSK-3β inhibitors in an animal model of leukemia

Although most in vitro experiments are conducted with TF-1 cells, the in vivo experiments were done with K562 cells because TF-1 cells do not engraft in a xenograft murine model. Continuous systemic administration of BIO (30 mg/kg, three times a week) was tested in mice injected with human leukemia K562 cells forming subcutaneous tumors [17]. The median survival for control mice injected with saline was 40 days, while all mice injected with BIO survived >70 days with no signs of tumor formation (Fig. 2H). In addition, GSK-3β inhibition did not affect hematopoietic recovery following irradiation in Balb/c

nu/nu mice injected with BIO or LiCl (Supplementary Figure E1, online only, available at www.exphem.org) or in NOD/SCID mice injected with BIO (data not shown).

Coculture with bone marrow stroma MS5 cells delay BIO-induced apoptosis

The proportion of TF-1 cells that survived treatment with BIO was significantly higher when leukemia cells were cocultured with bone marrow stroma MS5 cells as compared to suspension culture (Fig. 3A). Both adherent and suspension fractions of TF-1 cells cocultured with the stroma were resistant to BIO, suggesting that the protective effect of stroma is mediated through secreted factors and adhesion of leukemia cells to the stroma (data not shown). Integrin-mediated adhesion of leukemia cells to the extracellular matrix in the bone marrow was shown to promote drug resistance [18,19]. TF-1 cells express high levels of the integrin very late activation antigen 4 (VLA4) and rapidly adhere to the surface of plates covered with retronectin, the fragment of fibronectin that specifically binds to VLA4 (Fig. 3A and B). The VLA4/fibronectin axis was shown to activate integrin-linked kinase signaling and promote leukemia cell resistance to chemotherapy

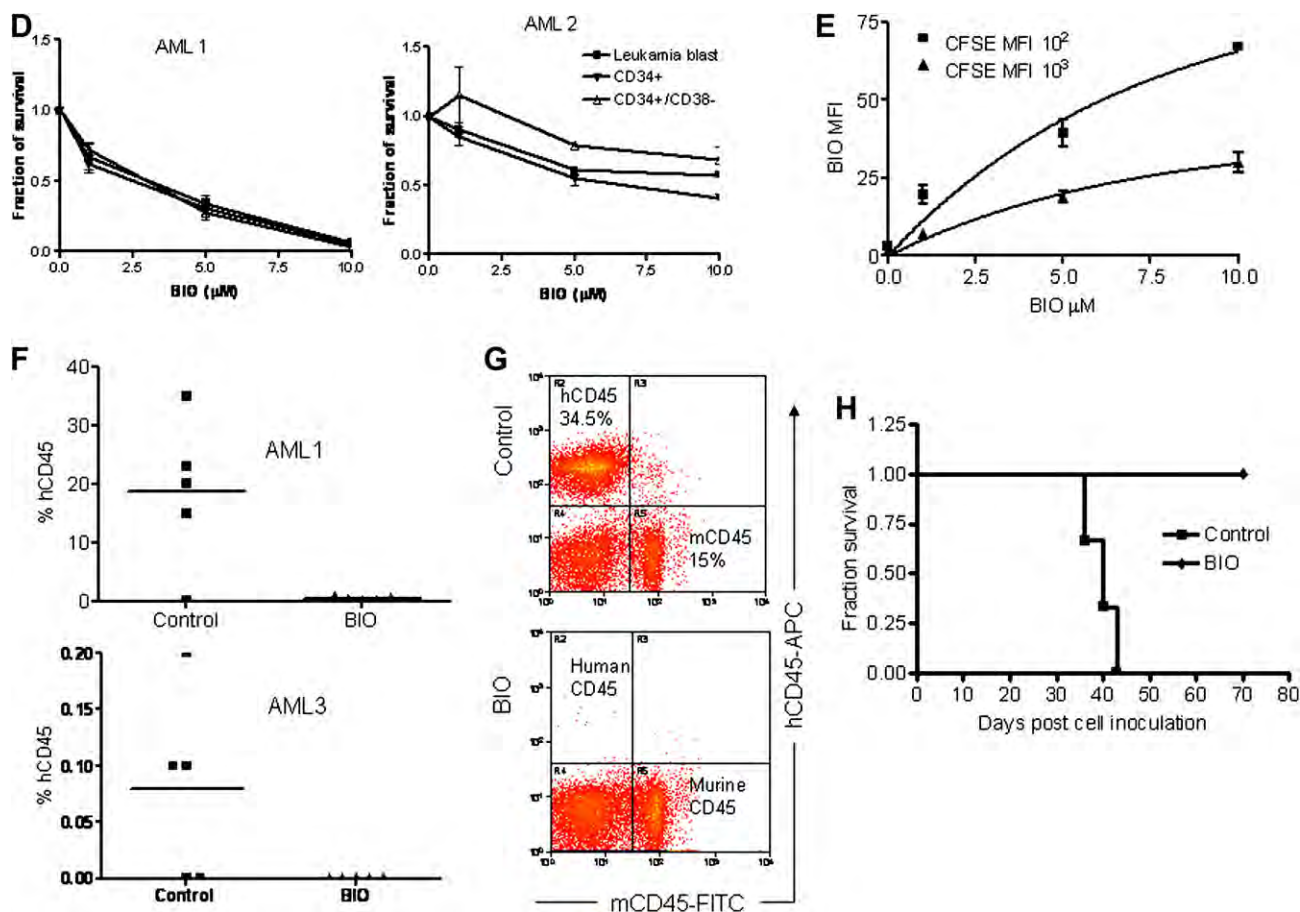


Figure 2. (Continued.) (D) AML1 showed similar sensitivity to BIO in bulk blast, CD34⁺ and CD34⁺CD38⁻ cells, while AML2 showed CD34⁺CD38⁻ cells are more resistant to BIO. (E) The intracellular concentration of BIO increased with BIO dose escalation (x-axis). Intracellular concentration of BIO was measured by flow cytometry using the FL2 channel (y-axis) after 6 hours of BIO incubation. Slowly dividing cells (CFSE mean fluorescence intensity [MFI] 10³) exhibited lower BIO plasma concentration compared to rapidly dividing cells (CFSE MFI 10²). CFSE staining experiments were performed with three primary AML samples. A similar trend was seen in all three AML samples. One representative experiment is shown. (F) Cells from two AML patients pretreated with BIO (5 μM, overnight) exhibited reduced engraftment in a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse transplantation model. (G) Human CD45⁺ cell engraftment in the bone marrow of NOD/SCID mice, one representative mouse from control and BIO groups is shown. (H) Continuous systemic administration of BIO (30 mg/kg, every second day) prolonged the survival of mice inoculated with human leukemia K562 cells.

[20]. VLA4 ablation with VLA4-neutralizing antibody (anti- α 4) abrogated TF-1 cell attachment to the retronectin, but did not prevent BIO-induced apoptosis in TF-1 cells cocultured with the stroma, suggesting that stroma-mediated resistance to BIO-induced apoptosis is mediated through alternative mechanisms. VLA4 signaling, however, appears to play a role in protection from BIO-induced apoptosis in TF-1 cells cultured in suspension because the ablation of VLA4 made TF-1 cells more sensitive to BIO-induced apoptosis (Fig. 3C).

Gene expression profiling of TF-1 cells treated with BIO

Because GSK-3 β regulates the turnover and activity of several transcriptional factors, such as β -catenin, nuclear factor- κ B (NF- κ B), nuclear factor for activated T cell, cAMP response element-binding (CREB), etc., we hypothesized that growth suppression in BIO-treated cells may be

mediated through the transcriptional reprogramming initiated by GSK-3 β inhibition. Gene expression analysis of TF-1 cells treated with 1 and 2 μM BIO performed 6 and 24 hours after treatment identified numerous genes and several signaling pathways modulated by BIO. The expression of ~10,000 genes, comprising 40% of array probes, was detected in TF-1 cells (Supplementary Tables E1 and E2, online only, available at www.exphem.org). The 1555 transcripts (5.2% of expressed genes) were modulated by BIO in TF-1 cells; 17.7% of modulated genes (276 genes) were upregulated and 82.3% (1249 genes) were downregulated in at least one of the conditions (Supplementary Tables E1 and E2). Thus, global transcriptional suppression observed in BIO-treated TF-1 cells is consistent with growth suppression mediated by BIO. Transcriptional suppression was already seen at 6 hours after the addition of BIO, and the number of suppressed genes and the magnitude of gene

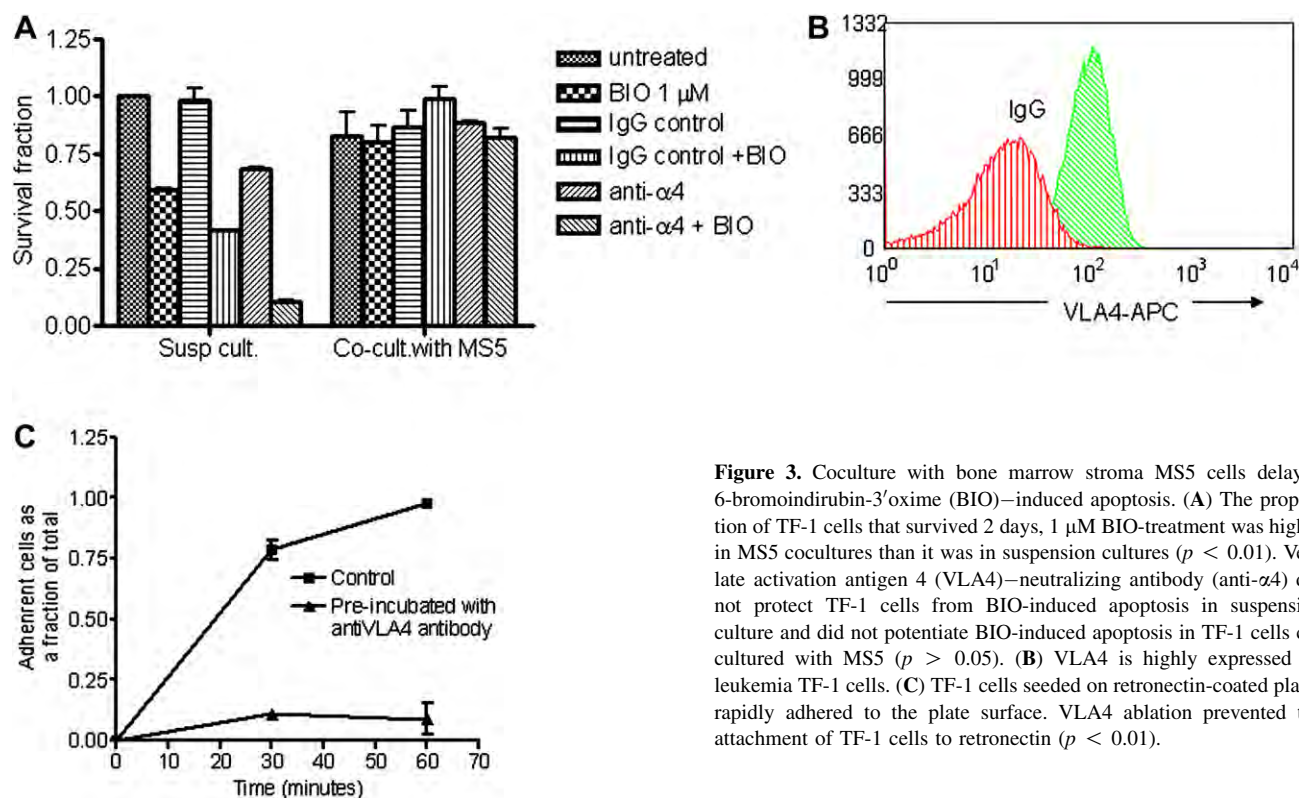


Figure 3. Coculture with bone marrow stroma MS5 cells delayed 6-bromoindirubin-3'-oxime (BIO)-induced apoptosis. (A) The proportion of TF-1 cells that survived 2 days, 1 μ M BIO-treatment was higher in MS5 cocultures than it was in suspension cultures ($p < 0.01$). Very late activation antigen 4 (VLA4)-neutralizing antibody (anti- α 4) did not protect TF-1 cells from BIO-induced apoptosis in suspension culture and did not potentiate BIO-induced apoptosis in TF-1 cells cocultured with MS5 ($p > 0.05$). (B) VLA4 is highly expressed by leukemia TF-1 cells. (C) TF-1 cells seeded on retromectin-coated plates rapidly adhered to the plate surface. VLA4 ablation prevented the attachment of TF-1 cells to retromectin ($p < 0.01$).

suppression increased with an increase in BIO dose (Supplementary Tables E1 and E2). Gene annotation enrichment analysis revealed that a significant proportion of modulated genes belonged to categories related to regulation of cellular and metabolic processes, transcription, apoptosis, and cell cycle, consistent with growth suppression and apoptosis induced by BIO. At the top of the list of downregulated genes were the genes encoding pro-mitotic and/or anti-apoptotic protein kinases Janus activating kinase 2, mitogen-activated protein kinase kinase 3 (MAP3K3), two domains of PIK3 kinase, i.e., regulatory PIK3 subunit 1 (PIK3R1) and catalytic β polypeptide PIK3; oncogenic kinases, PIM1; and Rho-associated protein kinase, Rho-associated, coiled-coil containing protein kinase 1 (ROCK1), previously shown to promote leukemia cell growth [10,21]. All of these genes were downregulated in TF-1 cells treated with 1 and 2 μ M BIO as early as 6 hours after treatment (Table 1). In addition, downregulation of the gene encoding the IL-3 receptor (IL-3RA) was seen in cells treated with 1 μ M BIO. BIO treatment also downregulated expression of histone deacetylase 7A (HDAC7A), adenylate cyclase activating polypeptide 1, angiopoietin 2, BCL2, MLL5, MLL, transcription factor 4 (TCF-4), and interferon regulatory factor (IRF) 2, at both doses used and as early as 6 hours after treatment (Table 1). Despite global transcriptional suppression, upregulation of a number of negative regulators of cell growth, including IL-8; transforming growth factor- β ; IRF1; IL-1 α ; thrombospondin; bone

morphogenetic protein 6; BCRA1; DOC1; tumor necrosis factor (ligand) superfamily, member 12; and tumor necrosis factor α -induced protein was seen in BIO-treated cells (Table 1) [22–31]. In addition, genes encoding chemokines and chemokine receptors with a C-C motif (eg, CCL2 and CCR7), IL-18R1, IL-18RAP, IL-4R, and IL-9R were upregulated in BIO-treated cells, suggesting upregulation of proinflammatory responses (Table 1).

Despite significant β -catenin accumulation in BIO-treated TF-1 cells, β -catenin transcriptional target genes, such as cyclin D1, c-myc, c-jun, fra-1, etc., were not upregulated (Supplementary Tables E1 and E2). To examine the effect of BIO on β -catenin transcription activity, the reporter assay analyzing β -catenin binding to its cofactor TCF-4 was used. Because TF-1 cells are known to be difficult to transfect, the TCF-4 reporter assay was performed using HEK293-H cells. Reporter GFP expression was very low in control cells transfected with the reporter construct and significantly upregulated by BIO (Fig. 4A). Thus, BIO acts to upregulate β -catenin transcription activity in HEK293-H cells. It is relevant that BIO used at 1 μ M did not affect HEK293-H cell viability or growth (data not shown). The reduced expression of TCF-4, the cofactor for β -catenin, was seen in BIO-treated TF-1 cells and validated by real-time polymerase chain reaction (Table 1 and Fig. 4B). We speculate that downregulation of TCF-4 may account for the abandoned β -catenin target gene activation in TF-1 cells. In addition, suppression of β -catenin transcriptional activity

Table 1. List of the selected genes modulated by 6-bromindirubin-3'oxime in TF-1 cells

Gene symbol	Negative stroma		Positive stroma		Genbank name	Definition
	6-h B1	6-h B2	6-h B1	6-h B2		
HDAC7A	-5.13	-2.36	NM	-1.67	NM_015401.1	Histone deacetylase 7A
ADCYAP1	-4.46	-4.7	0.4	0.9	NM_001117.2	Adenylate cyclase activating polypeptide 1
PIK3R1	-3.69	-5	NM	NM	NM_181523.1	phosphoinositide-3-kinase, regulatory subunit 1
ANGPT2	-2.5	-3.13	-1.7	-5	NM_001147.1	Angiopoietin 2
MAP3K3	-2.14	-2.42	NM	NM	NM_203351.1	Mitogen-activated protein kinase kinase kinase 3
IRF2	-2.08	-2.1	NM	NM	NM_002199.2	Interferon regulatory factor 2
TCF7L2	-1.57	-2.55	NM	NM	NM_030756.1	Transcription factor 7-like 2 (T-cell specific, HMG-box)
TCF4	-1.5	-2.11	-1.25	NM	NM_003199.1	Transcription factor 4
PIK3CB	-1.34	-1.82	NM	NM	NM_006219.1	Phosphoinositide-3-kinase, catalytic, β polypeptide
MLL5	-1.3	-1.28	NM	NM	NM_018682.2	Myeloid/lymphoid or mixed-lineage leukemia 5
BCL2	-1.21	-1.37	NM	NM	NM_000633.2	B-cell CLL/lymphoma 2
PIM1	-1.155	-2.316	-0.45	-2.1	NM_002648.2	PIM-1 oncogene
ROCK1	-1.15	-1.35	NM	NM	NM_005406.1	Rho-associated, coiled-coil containing protein kinase 1
JAK2	-1.14	-1.21	-1.14	NM	NM_004972.2	Janus kinase 2
MLL	-1	-1.6	NM	NM	NM_005933.2	Myeloid/lymphoid or mixed-lineage leukemia
MAPK8IP2	1.15	1.02	NM	NM	NM_012324.2	Mitogen-activated protein kinase 8 interacting protein 2
IL4R	1.59	1.34	1.35	NM	NM_000418.2	Interleukin 4 receptor
IRF1	2.03	2.12	1	-0.33	NM_002198.1	Interferon regulator factor 1
TNFSF12	2.37	1.72	2.75	1.01	NM_003809.2	Tumour necrosis factor (ligand) superfamily, member 12
BCAR1	2.61	2.52	1.71	1.44	NM_014567.2	Breast cancer anti-estrogen resistance 1
CCL2	2.73	1.52	2.73	1.52	NM_002982.3	Chemokine (C-C motif) ligand 2
IL8	2.78	4.22	2.78	4.22	NM_000584.2	Interleukin 8
IL9R	2.79	4.18	7.74	8.65	NM_002186.2	Interleukin 9 receptor
CCR7	4.58	4.46	2.95	2.89	NM_001838.2	Chemokine (C-C motif) receptor 7
CYP1A1	7.5	7.4	7.6	7.7	NM_000499.2	Cytochrome P450

B1 = 6-bromindirubin-3'oxime, 1 μ M; B2 = 6-bromindirubin-3'oxime, 2 μ M; NM = not modified.

by the small molecule inhibitor quercetin induced cytotoxicity in TF-1 cells (Fig. 4C), suggesting that the abandoned β -catenin signaling per se may contribute to BIO-induced cytotoxicity.

Gene expression analysis identified a subset of genes modulated by BIO in TF-1 cells cocultured with stroma: 90 genes (58% of the modulated genes) were upregulated and 66 (42%) were downregulated by BIO in TF-1 cells cocultured with stroma, while 181 (35%) were upregulated and 327 (65%) downregulated in suspension culture treated with the same dose of BIO without stroma (Table 2). Importantly, coculture with stroma abrogated transcriptional suppression of genes encoding anti-apoptotic kinases MAP3K3, PIK3R1, ROCK1, and BCL2 seen in suspension culture (Table 2). In addition, coculture with stroma attenuated HDAC7A inhibition observed in suspension culture treated with BIO (Table 1). The transcriptional suppression of TCF7L2, another cofactor for β -catenin downregulated by BIO in suspension culture, was abrogated by stroma (Table 1). Collectively, these results demonstrate that attenuation of BIO-induced apoptosis in TF-1 cells cocultured with stroma correlates with the modulation of BIO-induced transcriptome.

Role for Bcl2 in BIO-induced apoptosis

Bcl2 gene and protein expression was reduced following 6 hours treatment with BIO (Table 2, Figs. 5A and B). BIO

downregulates Bcl2 promoter activity HEK293-H cells transfected with a Bcl2-promoter/reporter construct (Fig. 5C). Downregulation of Bcl2 promoter activity in TF-1 cells does not appear to result from BIO-induced apoptosis because BIO does not induce apoptosis in HEK293-H cells, but downregulates Bcl2 promoter. Bcl2 ablation using small molecule inhibitor ABT737 induced apoptosis in TF-1 cells in suspension and cocultures with MS5 stroma cells (Fig. 5D). We also show that the combination of two reagents, ABT737 and BIO, both downregulating Bcl2 in TF-1 cells, is more efficient than the effect of each single reagent used at the same dose (Fig. 5D). Although these experiments did not demonstrate the real synergism in ABT737 and BIO, we speculate that ABT737-mediated inactivation of Bcl2 in TF-1 cells cocultured with stroma makes leukemia cells more responsive to BIO-induced apoptosis. Of note, Bcl2 expression in TF-1 cells cocultured with stroma was somewhat lower than in single-cell suspension (Fig. 5B). It is relevant that coculture with stroma inhibits TF-1 cell growth compared to suspension culture. The reduced cell growth apparently diminishes secretion of prosurvival factors activating Bcl2 in cocultures with stroma. This hypothesis will be tested in the future.

BIO downregulates expression of the IL-3 receptor in leukemia TF-1 cells

Bcl2 expression is regulated by IL-3 in TF-1 cells [32]. TF-1 cells express high levels of IL-3 receptor α chain CD123

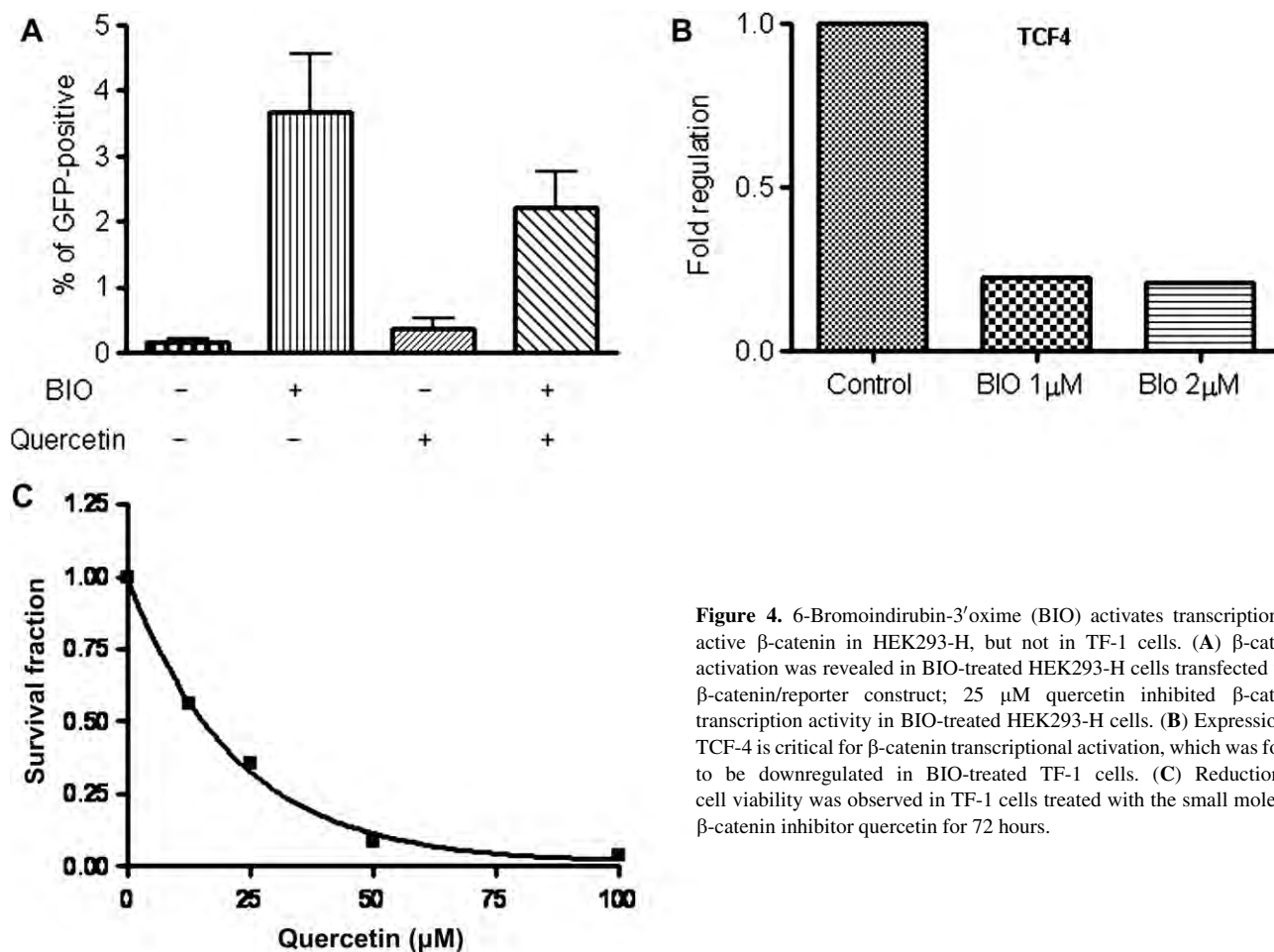


Figure 4. 6-Bromoindirubin-3'-oxime (BIO) activates transcriptionally active β -catenin in HEK293-H, but not in TF-1 cells. (A) β -catenin activation was revealed in BIO-treated HEK293-H cells transfected with β -catenin/reporter construct; 25 μ M quercetin inhibited β -catenin transcription activity in BIO-treated HEK293-H cells. (B) Expression of TCF-4 is critical for β -catenin transcriptional activation, which was found to be downregulated in BIO-treated TF-1 cells. (C) Reduction in cell viability was observed in TF-1 cells treated with the small molecule β -catenin inhibitor quercetin for 72 hours.

(Fig. 6A) and are reliant on IL3–IL3 withdrawal results in a significant reduction in cell numbers (Fig. 6B). Treatment with BIO that induced apoptosis was associated with reduced CD123 expression in TF-1 cells (Fig. 6A), suggesting that suppression of IL-3 signaling contributes to BIO-induced growth suppression through downregulation of Bcl2.

Discussion

Here we demonstrate that small molecule GSK-3 β inhibitors suppress cell growth and induce apoptosis in seven leukemia cell lines, including myeloid and T-cell leukemia, and more importantly, in six primary leukemia samples, including four AMLs, one ALL, and one MDS. It is considered to be important for anti-leukemic treatment to be effective against progenitor/stem cells. Primitive AML progenitor cells similar to normal hematopoietic stem cells are quiescent *in vivo* and divide slowly in *ex vivo* cultures. Thus, as a surrogate marker, this can be addressed with the slow dividing fraction of AML cells. High-resolution division tracking was performed to examine the sensitivity of slowly and rapidly dividing primary AML cells to BIO-induced cytotoxicity. CFSE staining experiments were performed with three primary AML

samples. The experiments revealed that BIO acts to reduce the proportion of viable cells and depleted AML samples from rapidly dividing CFSE^{dim} cells, suggesting that slowly dividing CFSE^{bright} cells exhibit significant resistance to BIO-induced apoptosis. Slowly dividing CD34⁺ AML progenitor cells exhibit a lower intracellular concentration of BIO compared to rapidly dividing CD34⁻ leukemia blasts. A similar finding was recently demonstrated comparing the resistance of slowly and rapidly dividing chronic myelogenous leukemia cells to imatinib-induced apoptosis [33]. BIO dose escalation was needed to kill slowly dividing CD34⁺ AML cells.

Slightly increased numbers of slowly dividing CD34⁺ cells were observed at high dose of BIO (10 μ M). This may be a result of activation of a feedback mechanism triggered by the reduction in rapidly dividing leukemia cells promoting the division of quiescent cells. The latter may induce disease relapse in AML patients treated with GSK-3 β inhibitors similarly to conventional chemotherapy.

The effect of BIO was also examined on the CD34⁺CD38⁻ fraction of AML cells. CD34⁺CD38⁻ cell phenotype is another surrogate marker for primitive leukemia stem cells. A reduction in CD34⁺CD38⁻ cell numbers was seen in

Table 2. 6-Bromindirubin-3'oxime modulates gene expression in TF-1 cells

BIO dose ^a	Suspension				Coculture with MS5 stroma cells			
	No. of upregulated genes		No. of downregulated genes		No. of upregulated genes		No. of downregulated genes	
	n	%	n	%	n	%	n	%
1 μ M	181	35	337	65	90	58	66	42
2 μ M	261	34	504	66	273	45	329	55

^aAnalysis was performed 6 hours after addition of 6-bromindirubin-3'oxime (BIO). Fold-change (Log) ≥ 1.0 , p value ≤ 0.05 .

BIO-treated AML1 cells in a BIO–dose-dependent manner (Fig. 2C, bottom left panel). Surprisingly, CD34⁺CD38[−] cells comprising 50% of total CD34⁺ cells were as sensitive to BIO as bulk CD34⁺ cells (Fig. 2C, bottom panel). Thus, slowly dividing CFSE^{bright} cells appear to be a better surrogate marker than the CD34⁺CD38[−] phenotype to characterize the response of most primitive leukemia progenitor cells to BIO-induced cytotoxicity. In another AML sample (AML2), however, CD34⁺CD38[−] cells were more resistant to BIO compared to bulk CD34⁺ and bulk AML blasts (Fig. 2C, bottom right panel). It is relevant that not all AML samples

contain a significant fraction of CD34⁺CD38[−] cells available for analysis. Collectively, these results suggest that both a CD34⁺CD38[−] phenotype and a slow division pattern can be used independently to characterize the therapeutic response in vitro.

BIO also impaired the engraftment of AML stem cells that survived BIO-induced apoptosis, as demonstrated in an NOD/SCID mouse transplantation model. In addition, in vivo administration of BIO produced a curative effect in the mouse leukemia model and did not affect normal bone marrow cell viability and hematopoietic recovery following

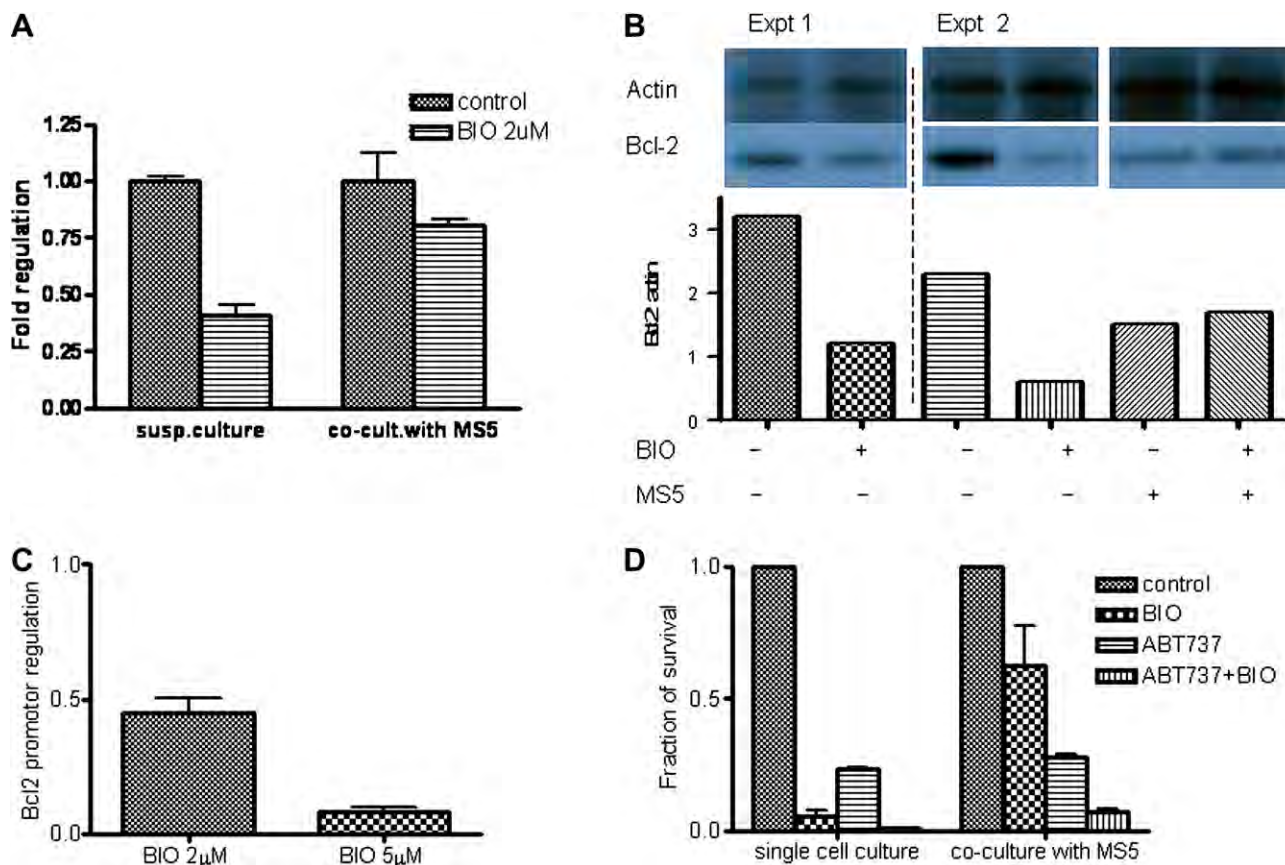


Figure 5. The role of Bcl2 in 6-bromindirubin-3'oxime (BIO)-induced apoptosis. Reduced expression of the antiapoptotic Bcl2 gene in BIO-treated TF-1 cells was validated by real-time polymerase chain reaction (A) and Western blot analysis (B), respectively. There were no significant differences of Bcl2 expression in BIO-treated TF-1 cells cocultured with MS5 ($p > 0.05$). (C) Downregulation of Bcl2 reporter expression was seen in BIO-treated HEK293-H cells transfected with the reporter construct ($p > 0.01$). (D) 3 μ M ABT737 inhibited growth and viability of TF-1 cells and potentiated proapoptotic effects of 1 μ M BIO after 72 hours treatment. TF-1 cells treated with both drugs exhibited more apoptotic cells compared to those treated with each single drug. ABT737 abrogated the protection from BIO-induced apoptosis provided by MS5 coculture.

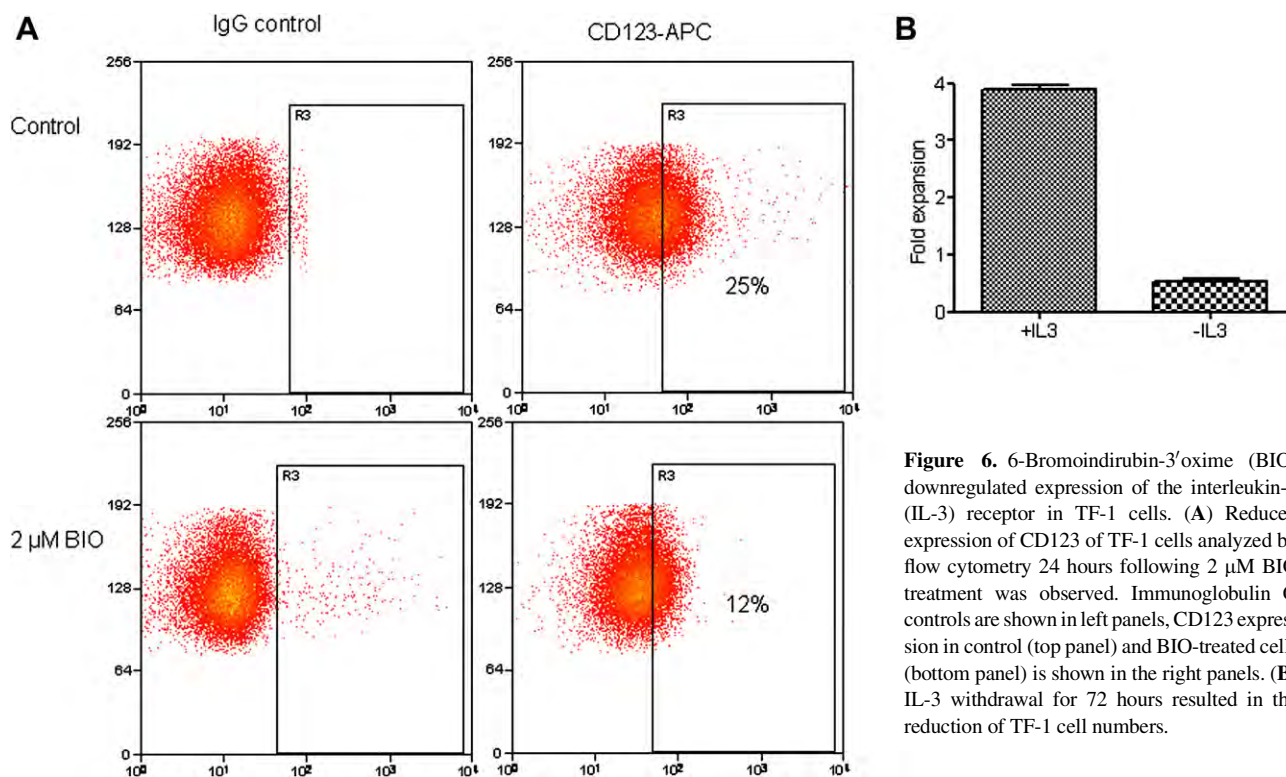


Figure 6. 6-Bromindirubin-3'-oxime (BIO) downregulated expression of the interleukin-3 (IL-3) receptor in TF-1 cells. (A) Reduced expression of CD123 of TF-1 cells analyzed by flow cytometry 24 hours following 2 μ M BIO treatment was observed. Immunoglobulin G controls are shown in left panels, CD123 expression in control (top panel) and BIO-treated cells (bottom panel) is shown in the right panels. (B) IL-3 withdrawal for 72 hours resulted in the reduction of TF-1 cell numbers.

irradiation, suggesting that the cytotoxic effect was specific for leukemia cells only. The nature of the apparent resistance of normal bone marrow cells to apoptosis induced by GSK-3 β inhibitors needs to be addressed specifically. It has previously been shown that the growth and viability of leukemia cells depend on NF- κ B activity, and inhibition of GSK-3 β acts to downregulate NF- κ B activity in primary AML cells but not in normal hematopoietic cells, which are less reliant on NF- κ B for their survival [34].

Gene expression analysis revealed global transcriptional suppression consistent with growth suppression and induction of apoptosis in TF-1 cells treated with BIO. Downregulation of genes linked to cellular and biological processes, cell cycle and apoptosis, and, more specifically, reduced expression of genes encoding HDAC7A; adenylate cyclase activating polypeptide 1; angiopoietin 2; Bcl2; MLL5; MLL; TCF-4; IRF2; PIM1; Janus activating kinase 2; MAP3K3; PIK3R1; and phosphoinositide 3-kinase β , Rho-associated protein kinase ROCK1—all shown to maintain cell growth and survival—was observed in BIO-treated TF-1 cells (Table 1) [1,21–31,35,36]. Remarkably, despite the induction of global transcriptional suppression, BIO upregulates several negative regulators of cell growth, such as thrombospondin, BCAR1, IRF1, IL-8, and others (Table 1) [21–31]. Thus, in a sense, BIO acts as a combined chemotherapy targeting HDACs, BCL2, MAP3K8, ROCK1, PIK3, IL-3 signaling, and angiogenesis. BIO also increases expression of the CC chemokine CCL2 and the receptor CCR7, as well as expression of IL-9R

and IL-18R, which can potentially make leukemia cells more susceptible to the cytotoxic effects of immune cells.

GSK-3 β inhibition was shown to activate Wnt/ β -catenin signaling in different cell types, including embryonic stem cells [37]. Despite significant β -catenin accumulation in BIO-treated TF-1 cells, β -catenin transcriptional target genes, such as cyclin D1, c-myc, c-jun, fra-1, etc., were not upregulated. Apparently, GSK-3 β inhibition is not sufficient to induce β -catenin signaling in leukemia TF-1 cells. Using a β -catenin/reporter assay, we demonstrated that BIO upregulates β -catenin transcription activity in HEK293-H cells. Reduced expression of TCF-4, the cofactor for β -catenin, was seen in BIO-treated TF-1 cells and validated by real-time polymerase chain reaction. We speculate that downregulation of TCF-4 may account for the abandoned β -catenin target gene activation in TF-1 cells. It is relevant that another potent inhibitor of GSK-3 β , lithium chloride, also did not promote β -catenin target gene transcription in human T cells, but induced it in human fibroblasts [38]. Insufficiency of GSK-3 β inhibition to activate β -catenin target gene transcription, however, does not completely exclude a role for β -catenin in the effects mediated by BIO. β -catenin loss-of-function experiments are needed to define the role of this protein in BIO-induced apoptosis in leukemia cells.

Gene expression analysis revealed a role for Bcl2 in BIO-induced apoptosis. BIO downregulated Bcl2 expression in TF-1 cells and attenuated activity of the Bcl2-promoter region. Levels of other anti-apoptotic genes, such as Bcl-x

and XIAP, as well as proapoptotic Bax and Bac, were unchanged in BIO-treated TF-1 cells. It is unlikely that Bcl2 protein downregulation is a consequence of caspase-mediated cleavage of Bcl2 because Bcl2 downregulation was seen at the transcription level and correlated with BIO-mediated global transcriptional suppression. These data suggest that BIO induces apoptosis in leukemia cells through a mechanism involving Bcl2 reduction. Coculture with stroma cells delayed BIO-induced apoptosis and attenuated BIO-induced suppression of Bcl2. Bcl2 ablation by ABT737, which is itself cytotoxic for TF-1 cells, increased apoptosis in BIO-treated TF-1 cells cocultured with stroma. BIO-induced apoptosis was also associated with downregulation of CD123 in TF-1 cells reliant on IL-3/CD123 signaling for their growth and survival. IL-3 regulates Bcl2 expression in TF-1 cells. Downregulation of both CD123 and Bcl2 in BIO-treated TF-1 cells suggests a role for these two molecules in the regulation of apoptosis induced by a GSK-3 β inhibitor. Targeting of CD123 using CD123-blocking antibody was shown to preferentially ablate AML cells expressing high CD123 [39]. Therefore, GSK-3 β -mediated downregulation of CD123 is a novel candidate alternative therapy to neutralizing antibody treatment.

In summary, our findings provide some mechanistic insight into the anti-leukemic effect of GSK-3 β inhibitors and supports further evaluation of these molecules as promising agents for novel therapeutic interventions either alone or in combination with other chemotherapeutic agents for the treatment of leukemia. It is relevant that BIO is a derivative of a natural compound widely used in Chinese medicine to treat chronic myelogenous leukemia [40].

Acknowledgments

The authors would like to thank Soo Min Heng, Thu Tran, and Dean Inwood (Department of Radiation Oncology, Prince of Wales Hospital, Sydney, Australia) for mouse irradiation.

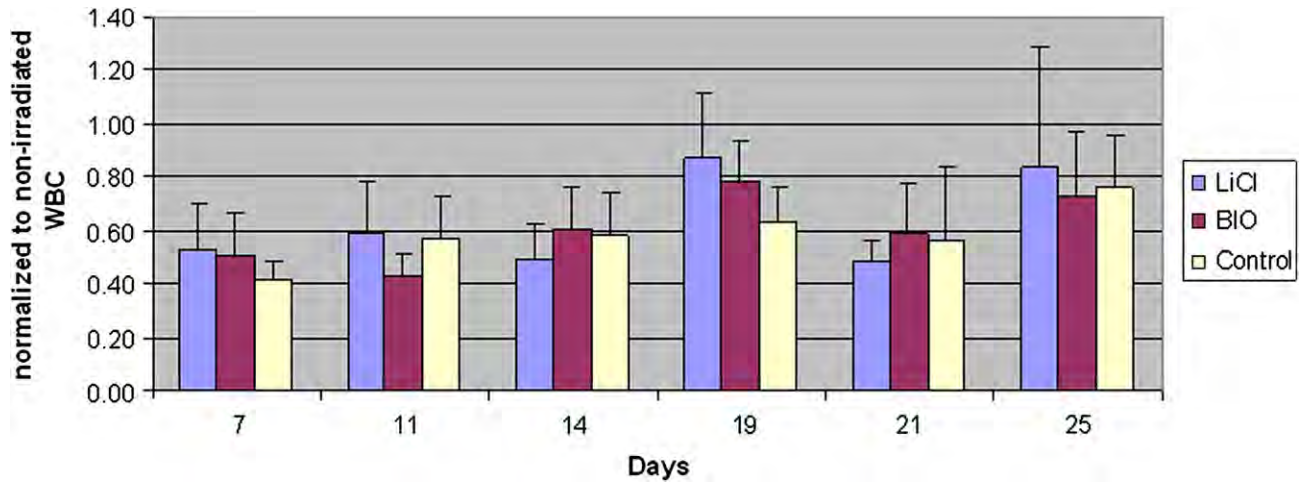
Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

- Ougolkov AV, Billadeau DD. Inhibition of Glycogen Synthase Kinase-3. Wnt Signaling. Clifton, NJ: Humana Press; 2009. p. 67–75.
- Holmes T, O'Brien TA, Knight R, Lindeman R, Symonds G, Dolnikov A. The role of glycogen synthase kinase-3b in normal haematopoiesis, angiogenesis and leukaemia. *Curr Med Chem*. 2008;15:1493–1499.
- Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J*. 2001;359:1–16.
- Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A. Glycogen synthase kinase 3: more than a namesake. *Br J Pharmacol*. 2009;156:885–898.
- Wang Z, Smith KS, Murphy M, Piloto O, Somerville TCP, Cleary ML. Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. *Nature*. 2008;455:1205–1209.
- Cline GW, Johnson K, Regittinig W, et al. Effects of a novel glycogen synthase kinase-3 inhibitor on insulin-stimulated glucose metabolism in Zucker diabetic fatty (fa/fa) rats. *Diabetes*. 2002;51:2903–2910.
- Ring DB, Johnson KW, Henriksen EJ, et al. Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. *Diabetes*. 2003;52:588–595.
- Ougolkov AV, Bone ND, Fernandez-Zapico ME, Kay NE, Billadeau DD. Inhibition of glycogen synthase kinase-3 activity leads to epigenetic silencing of nuclear factor kB target genes and induction of apoptosis in chronic lymphocytic leukemia B cells. *Blood*. 2007;110:735–742.
- Abrahamsson AE, Geron I, Gotlib J, et al. Glycogen synthase kinase 3b missplicing contributes to leukemia stem cell generation. *Proc Nat Acad Sci U S A*. 2009;106:3925–3929.
- Gustin JP, Karakas B, Weiss MB, et al. Knockin of mutant PIK3CA activates multiple oncogenic pathways. *Proc Nat Acad Sci U S A*. 2009;106:2835–2840.
- Miyashita K, Kawakami K, Nakada M, et al. Potential therapeutic effect of glycogen synthase kinase 3b inhibition against human glioblastoma. *Clin Cancer Res*. 2009;15:887–897.
- Ko K-H, Odell R, Nordon RE. Analysis of cell differentiation by division tracking cytometry. *Cytometry Part A*. 2007;71A:773–782.
- Dennis G, Sherman B, Hosack D, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biology*. 2003;4:R60.
- Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2008;4:44–57.
- Holmes T, O'Brien TA, Knight R, et al. Glycogen synthase kinase-3b inhibition preserves hematopoietic stem cell activity and inhibits leukemic cell growth. *Stem Cells*. 2008;26:1288–1297.
- Lapidot T, Fajerman Y, Kollet O. Immune-deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis. *J Mol Med*. 1997;75:664–673.
- Terui Y, Ikeda M, Tomizuka H, et al. Activated endothelial cells induce apoptosis in leukemic cells by endothelial interleukin-8. *Blood*. 1998;92:2672–2680.
- Hazlehurst LA, Dalton WS. Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. *Cancer Metast Rev*. 2001;20:43–50.
- De Toni F, Racaud-Sultan C, Chicanne G, et al. A crosstalk between the Wnt and the adhesion-dependent signaling pathways governs the chemosensitivity of acute myeloid leukemia. *Oncogene*. 2006;25:3113–3122.
- Matsunaga T, Takemoto N, Sato T, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nat Med*. 2003;9:1158–1165.
- Deng X, Ruvolo P, Carr B, May WS. Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proc Nat Acad Sci U S A*. 2000;97:1578–1583.
- Wiseman A. Therapeutic-antagonists of oestrogens can be produced for cancer and other therapies using cytochromes P450 (CYP). *Med Hypotheses*. 2005;65:1088–1090.
- Bauer S, Adrian N, Siebenborn U, et al. Sequential cancer immunotherapy: targeted activity of dimeric TNF and IL-8. *Cancer Immun*. 2009;9:2.
- Kaminska B, Wesolowska A, Danilkiewicz M. TGFbeta signalling and its role in tumour pathogenesis. *Acta Biochim Pol*. 2005;52:329–337.
- Park J, Kim K, Lee E-J, et al. Elevated level of SUMOylated IRF-1 in tumor cells interferes with IRF-1-mediated apoptosis. *Proc Nat Acad Sci U S A*. 2007;104:17028–17033.
- Dinarelli CA. The paradox of pro-inflammatory cytokines in cancer. *Cancer Metast Rev*. 2006;25:307–313.
- Lawler J, Detmar M. Tumor progression: the effects of thrombospondin-1 and -2. *Int J Biochem Cell Biol*. 2004;36:1038–1045.
- Li B. Bone morphogenetic protein-Smad pathway as drug targets for osteoporosis and cancer therapy. *Endocr Metab Immune Disord Drug Targets*. 2008;8:208–219.

29. Hussain SP, Harris CC. Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res.* 1998;58:4023–4037.
30. Rimon E, Sasson R, Dantes A, Land-Bracha A, Amsterdam A. Gonadotropin-induced gene regulation in human granulosa cells obtained from IVF patients: modulation of genes coding for growth factors and their receptors and genes involved in cancer and other diseases. *Int J Oncol.* 2004;24:1325–1338.
31. Wang S. The promise of cancer therapeutics targeting the TNF-related apoptosis-inducing ligand and TRAIL receptor pathway. *Oncogene.* 2008;27:6207–6215.
32. Rinaudo MS, Su K, Falk LA, Halder S, Mufson RA. Human interleukin-3 receptor modulates bcl-2 mRNA and protein levels through protein kinase C in TF-1 cells. *Blood.* 1995;86:80–88.
33. Jorgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. *Blood.* 2007;109:4016–4019.
34. Guzman ML, Swiderski CF, Howard DS, et al. Preferential induction of apoptosis for primary human leukemic stem cells. *Proc Natl Acad Sci U S A.* 2002;99:16220–16225.
35. Osakada F, Takahashi M. Drug development targeting the glycogen synthase kinase-3b (GSK-3b)-mediated signal transduction pathway: targeting the Wnt pathway and transplantation therapy as strategies for retinal repair. *J Pharmacol Sci.* 2009;109:168–173.
36. Phelps RA, Broadbent TJ, Stafforini DM, Jones DA. New perspectives on APC control of cell fate and proliferation in colorectal cancer. *Cell Cycle.* 2009;15:2549–2556.
37. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med.* 2004;10:55–63.
38. Staal FJT, Burgering BMT, van de Wetering M, Clevers HC. TCF-1-mediated transcription in T lymphocytes: differential role for glycogen synthase kinase-3 in fibroblasts and T cells. *Int Immunol.* 1999;11:317–323.
39. Jin L, Lee EM, Ramshaw HS, et al. Monoclonal antibody-mediated targeting of CD123, IL-3 receptor α chain, eliminates human acute myeloid leukemic stem cells. *Cell Stem Cell.* 2009;5:31–42.
40. Meijer L, Guyard N, Skaltsounis L, Eisenbrand G. Indirubin, the Red Shade of Indigo. Roscoff, France: Life in Progress Editions; 2006.



Supplementary Figure E1. The effect of systemic administration of 6-bromoindirubin-3'-oxime (BIO) or lithium chloride (LiCl) on hematopoietic recovery in irradiated mice. White blood cell (WBC) counts were measured in mice twice a week from day 7 to day 25 after irradiation. There was no significant difference in WBC counts between controls and mice injected with BIO or LiCl ($p > 0.05$).