Halofuginone Has Anti-Proliferative Effects in Acute Promyelocytic Leukemia by Modulating the Transforming Growth Factor Beta Signaling Pathway

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Abstract

Promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) expression in acute promyelocytic leukemia (APL) impairs transforming growth factor beta (TGFβ) signaling, leading to cell growth advantage. Halofuginone (HF), a low-molecular-weight alkaloid that modulates TGFβ signaling, was used to treat APL cell lines and non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice subjected to transplantation with leukemic cells from human chorionic gonadotrophin-PML-RARα transgenic mice (TG). Cell cycle analysis using incorporated bromodeoxyuridine and 7-amino-actinomycin D showed that, in NB4 and NB4-R2 APL cell lines, HF inhibited cellular proliferation (P<0.001) and induced apoptosis (P=0.002) after a 24-hour incubation. Addition of TGFβ revealed that NB4 cells were resistant to its growth-suppressive effects and that HF induced these effects in the presence or absence of the cytokine. Cell growth inhibition was associated with up-regulation of TGFβ target genes involved in cell cycle regulation (TGFβ, TGFBRI, SMAD3, p15, and p21) and down-regulation of MYC. Additionally, TGFβ protein levels were decreased in leukemic TG animals and HF in vivo could restore TGFβ values to normal. To test the in vivo anti-leukemic activity of HF, we transplanted NOD/SCID mice with TG leukemic cells and treated them with HF for 21 days. HF induced partial hematological remission in the peripheral blood, bone marrow, and spleen. Together, these results suggest that HF has anti-proliferative and anti-leukemic effects by reversing the TGFβ blockade in APL. Since loss of the TGFβ response in leukemic cells may be an important second oncogenic hit, modulation of TGFβ signaling may be of therapeutic interest.

Introduction

Transforming growth factor beta (TGFβ) is a cytokine that regulates multiple cellular responses, including inhibition of cell proliferation and induction of differentiation, senescence, and apoptosis [1,2]. Its actions are mediated by binding to the serine/threonine kinase receptor TβRII that recruits and activates TβRI, which in turn phosphorylates downstream targets. These include the proteins SMAD2 and SMAD3, which translocate to the nucleus in a complex with the common mediator SMAD4 to regulate transcription of target genes [3,4]. The tumor suppressor responses of TGFβ are essential for maintaining homeostatic control of normal cell growth and cells in the early phases of tumorigenesis. Among the TGFβ-mediated effects in premalignant cells are the suppression of c-Myc expression [5] and the induction of the cell cycle inhibitors p15 and p21. Although these actions imply a tumor suppressor role for TGFβ, its effects are both cell- and context-dependent. In that regard, Siegel et al. have shown that activation of TGFβ delays the appearance of primary mammary tumors, and mice deficient in TGFβ signaling are prone to earlier tumor development, suggesting that the tumor suppressor response of TGFβ is important in the early stages of tumorigenesis. In contrast, mice expressing an activated TGFβ receptor exhibited increased metastatic lung foci, consistent with a pro-oncogenic effect of this pathway in late-stage disease [6]. In addition, advanced disease is accompanied by increased expression and activation of the ligand but decreased TGFβ responsiveness, thus facilitating tumor cell growth [7].

Deregulation of TGFβ signaling may alter hematopoiesis, causing a predisposition to leukemia. In contrast to solid tumors, mutations in SMAD genes are rare in leukemia and disruption of TGFβ responsiveness is commonly secondary to either (a) altered transcription, as described in acute myeloid leukemia with translocation t(8;21), in which the AML1/ETO chimeric protein...
represses transcription of TGFβ-responsive genes [8] or (b) disruption of TGFβ target gene expression such as the cell cycle regulators c-Myc, p15 and p21, which are commonly associated with leukemogenesis [9].

The role of TGFβ in leukemogenesis has been recently studied in acute promyelocytic leukemia (APL), a distinct subtype of acute myeloid leukemia (AML) associated with t(15;17) and expression of the promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) hybrid protein. A gene expression study using microarrays has revealed that TGFβ was downregulated in APL compared with most non-APL samples [10]. In contrast, Raza et al. have described elevated TGFβ protein expression by immunohistochemistry in bone marrow biopsies of 23 APL patients [11]. Lin et al. demonstrated that the cytoplasmic isoform of PML (cPML) is essential for TGFβ signaling and Pml-null primary cells are resistant to TGFβ-dependent growth arrest, induction of cellular senescence, apoptosis, phosphorylation of Smad2/3, and induction of p15 and p21 expression. Restoration of cPML fully rescued these defects [12]. Since cPML function is impaired in APL blasts, through the formation of cPML/PML-RARα heterodimers, the authors hypothesized that this would be the molecular mechanism of resistance to TGFβ anti-proliferative responses [13].

To better characterize the deregulation of the TGFβ pathway in APL and to determine its potential as a therapeutic target, we took advantage of the human chorionic gonadotrophin (hCG)-PML/RARα transgenic model and analyzed the effects of halofuginone (HF; H-dl-trans-7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidyl)acetoxy]-4-(3H)-quinazolinone hydrobromide), which is a low-molecular-weight alkaloid that has been shown to modulate TGFβ signaling. In several cultured cell lines, this drug decreased TGFβ-induced phosphorylation of SMADs 2 and 3 and induced expression of inhibitory SMAD7 mRNA [14]. HF also reduced tumor growth in in vivo models of pheochromocytoma [15], brain tumors [16], and hepatocellular carcinoma [17]. The effects of HF in hematopoietic malignancies have not been previously described. Our results demonstrate that HF treatment induces anti-proliferative and pro-apoptotic effects, up-regulates TGFβ target gene expression, and significantly reduces the leukemic burden in vivo.

Materials and Methods

Ethics Statement
This study was approved by the Research Ethics Committee of the University Hospital of the Medical School of Ribeirão Preto, University of São Paulo, process number 3865/2005). Experiments using mice were conducted according to national guidelines for the care and use of laboratory animals (Brazilian College of Animal Experimentation) and was approved by the institutional Animal Experimentation Ethics Committee (protocol number 088/2007).

Cell culture
NB4, a permanent cell line harboring t(15;17) [18], and its derivative NB4-R2, in which all-trans retinoic acid (ATRA)-unresponsiveness is associated with a point mutation in the retinoid-binding domain of PML-RARα [19], were used for in vitro assays. Cells were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL, UK) and maintained at 37°C in a 5% CO2-humidified incubator.

Treatment of APL cell lines with HF
HF was kindly provided by Prof. Arnon Nagler (Chaim Sheba Medical Center, Tel Hashomer, Israel). Stock solutions of 1 mg/mL were kept at −80°C until use. Subsequently, working solutions of 10 ng/μL were freshly prepared by diluting the stock solution with autoclaved water (for cell culture assays) or 0.9% NaCl (for in vivo studies).

Cell suspensions containing 5×10^5 cells/mL of culture were treated with increasing doses of HF (6.25–200 ng/mL), which was directly added to the medium, and then cells were harvested after 24, 48, or 72 hours of incubation as indicated. Cell viability measurements were recorded with an initial minimum viability of at least 95% as determined by the Trypan blue assay. For cell cycle analysis and gene expression studies, NB4 cells were also subjected to concurrent treatment with TGFβ (0.5 ng/mL; Sigma-Aldrich, St. Louis, MO, EUA) as indicated.

Cell proliferation and apoptosis assays
For the analysis of proliferation and the cell cycle, NB4 and NB4-R2 cells were treated with HF as described above for 24 hours and then were subjected to immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and 7-amoactino-mycin (7-AAD), followed by flow cytometric analysis using the BrdU Flow Kit (BD Biosciences, San Jose, CA, USA). In this method, BrdU, an analog of the DNA precursor thymidine, is incorporated into newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cell cycle. The incorporated BrdU is stained with a specific BrdU conjugated antibody and 7-amoactino-mycin (7-AAD), a dye that binds to total DNA. With this combination, a two-color flow cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) according to their cell cycle position (i.e., G0/1, S, or G2/M phases defined by 7-AAD staining intensities). Cells were incubated with 10 μM of BrdU during the last 30 minutes of culture, and then processed according to the manufacturer’s recommendations. For the NB4 cell line only, TGFβ was added or not to the culture medium to evaluate the tumor suppressive effects of TGFβ.

For the analysis of apoptosis after 24 and 48 hours of HF treatment, NB4 cells that were treated with different concentrations of HF were evaluated using the Trypan blue exclusion assay. Concomitantly, apoptosis was determined using the Annexin V and propidium iodide (PI) binding assay (BD Biosciences), and then analyzed by flow cytometry. Each sample was washed in 1× phosphate-buffered saline (PBS), and then incubated with 5 μL of Annexin V, 5 μL of PI, or both at 4°C for 15 min. Subsequently, 400 μL of binding buffer was added to the samples. All experiments were performed in triplicate and in each sample, 10,000 events were acquired in a FACSCalibur flow cytometer (BD Biosciences) and analysis was performed using Cell Quest software.

The effective dose at 50% (ED50) of HF was calculated based on the inhibition of proliferation in the NB4 cell line as determined by the BrdU incorporation assay. This analysis was performed using CalcuSyn software (Biosoft, Great Shelford, UK).

Analysis of TGFβ target gene expression by real-time polymerase chain reaction (PCR)
NB4 and NB4-R2 cells were treated as above for 24 or 72 hours, and wherever indicated, TGFβ (0.5 ng/mL) was added 1 hour before HF to the culture. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Reverse transcription of 500 ng of RNA was performed using the cDNA High Capacity Archive kit (Applied Biosystems, Foster City, CA, USA). Subsequently, the mRNA expression of TGFβ target genes that are involved in cell cycle regulation (TGFβ, TGFβRII, p15, p21, SMAD3, and MYC) was
evaluated by real-time PCR using the Taqman method. All the cDNA samples were diluted five times and were processed in duplicate. The PCR amplification was performed in 40 cycles, using the Taqman PCR master mix, in an SDS (Sequence Detection System) 5700 platform connected to a 7300 Real-Time PCR System (Applied Biosystems). The probes used for amplification were synthesized using the Assay-on-Demand System (Applied Biosystems) with the following GeneBank sequences: TGFβ (NM_003236, FAM-TGAAAGGAGGAGGAGATACACGG-NFQ), TGFβRI (NM_006612.2, FAM-TGGCGAGCGTT-GAAGCCTTGGAG-NFQ), Smad3 (NM_005902.3, FAM-GAGGCGGGAAGCGAGCAGACCT-NFQ), p15 (NM_006428.3, FAM-CCAAAGGCAAGCTCCTCCAAGAG-NFQ), p21 (NM_000859.2, FAM-GGCAGCACGATGACGATTTC-TA-NFQ), and MDC (NM_002467.3, FAM-AACAGGACCGCT-CGGCGACGATGC-NFQ). The expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was determined using the PDL reagent (Pre-Developed Assay Reagent; Applied Biosystems) and was used to normalize the data. The 2^(-ΔΔCt) method was used in the analysis of the PCR data and the relative gene expression in a particular sample was determined as follows: relative amount of target = 2^(-ΔΔCt) value.

Detection of TGFβ protein expression

After treatment with HF as described above, total protein extracts were obtained according to Schreiber et al. [20]. Briefly, NB4 cells were washed twice in cold PBS, lysed with lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM Na3VO4, 1 mg/mL leupeptin) containing a protease inhibitor mixture (Sigma, St. Louis, MO, USA) and homogenized in a Dounce system (model D-130, Biosystems) for one minute on ice. Lysates were centrifuged at 20,000 g for 30 minutes at 4°C and the supernatants were removed. Protein concentration was determined by the Bradford’s method [21]. Proteins were submitted to SDS–PAGE and a total of 30 μg of protein from each sample was transferred to polyvinylidene difluoride (PVDF) membranes (GE Lifesciences, Pittsburgh, PA, USA) [22]. Membranes were blocked with 5% non-fat dry milk in 0.1% Tween-TBS and incubated with the specific antibodies. Mouse anti-β-actin was purchased from Santa Cruz Biotechnology (California, USA). Rabbit anti-TGFβ, anti-TGFβ receptor II, anti-Smad3 and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody were purchased from Cell Signaling (Beverly, MA, USA), and goat anti-mouse IgG secondary antibody from GE Lifesciences (Pittsburgh, PA, USA). The antibody-protein complex was detected using the ECL Western Blotting Detection Reagents (GE Lifesciences).

HF treatment in an APL transplant model

To analyze in vivo effects of HF, irradiated immunodeficient non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were injected with leukemia cells from hCG-PML-RARα transgenic mice (TM). The TM were kindly provided by Prof. Pier Paolo Pandolfi (Beth Israel Deaconess Medical Center, Harvard Stem Cell Institute, Boston, MA, USA) and their generation has been described elsewhere [23]. Notably, in this transgenic model, a lethal form of leukemia that closely resembles human APL occurs after a long pre-leukemic phase (12–15 months) and affects only 10–15% of the TM [24]. We have established a transplant model, in which all animals develop leukemia after 14 days from the transplant. Briefly, leukemic cells, previously maintained at ~80°C, were thawed and suspended in RPMI 1640 with 10% FCS. After Trypan blue exclusion testing and 12 hours after sublethal cobalt irradiation with 250 cGy, 2 × 10^8 viable cells were intravenously injected into the ocular plexus of CB17-Prkdcscid/J 10–12-week-old NOD/SCID mice (The Jackson Laboratory, Bar Harbor, Maine, USA). Animals were maintained under pathogen-free conditions and received autoclaved food and water ad libitum. Experiments were conducted according to institutional and national guidelines for the care and use of laboratory animals.

The definition of the dose and mode of administration of HF was based on previous reports of the in vivo use of the drug in solid tumors and fibrosis models [14,15,17,25,26,27,28], and pilot experiments were performed to confirm leukemic infiltration in transplanted animals and to test the efficacy and toxicity of HF. Twenty-four hours after the transplant procedure, NOD/SCID mice received treatment with vehicle only (0.9% NaCl; n = 5) or 150 μg/kg/day HF (n = 5) as an intraperitoneal injection for 21 consecutive days. At the end of the experiment (day 21), mice were sacrificed under ketamine anesthesia after being subjected to a cardiac puncture to obtain peripheral blood (PB) samples. Non-leukemic, age-matched NOD/SCID were used as controls (n = 5). Animals were maintained under pathogen-free conditions and received autoclaved food and water ad libitum.

Analysis of the hematological and molecular responses to HF in vivo

For monitoring PB counts, mice were bled from the tail before transplantation and 10 days after the beginning of HF injections. Automated counts (hemoglobin, white blood cells, and platelets) were performed using a T-890 Coulter cell counter (Coulter Corporation, Hialeah, FL, USA), and differential counts were obtained from Leishman-Wright-Giemsa-stained smears. In addition, after euthanasia, bone marrow (BM) cells were obtained by flushing the bone cavities of femurs and tibiae with RPMI 1640 containing 10% FCS. Cells were washed once, and then the pellet was resuspended in PBS at a concentration of 10^6/mL. Approximately 10^7 cells were used for cytospin slide preparation and staining with Leishman-Wright-Giemsa, and then 10^5 cells were used for DNA extraction and PCR analysis to detect PMI-L-RARα, as previously described by van Dongen [29].

For morphological analysis of PB and bone marrow slides, a minimum of 100 PB and 200 BM cells were counted, and then myeloid cells were classified as immature, intermediate, or mature, according to the Bethesda proposals for classification of non-lymphoid hematopoietic neoplasms in mice [30]. Because extensive spleen infiltration was observed in leukemic animals, the relation between the spleen and body weight was also assessed to further quantify the hematological response to HF.

Quantification of TGFβ in the serum of leukemic mice treated with HF

Serum and BM samples of transplanted and control NOD/SCID mice were obtained after euthanasia, as previously described, and used to quantify TGFβ using the enzyme-linked immunosorbent assay (ELISA). Serum samples were stocked at ~80°C until use. BM cells were washed twice with PBS and total protein extracts were prepared by suspending cell pellets in a lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM PMSF) supplemented with a cocktail of protease and phosphatase inhibitors (Sigma Aldrich), and then subjecting the samples to three cycles of sonication. The resulting lysates were centrifuged at 13000 rpm for 10 minutes at 4°C, and then supernatants (a minimum total protein of 1 μg) were stocked at ~20°C until use. Subsequently, serum and
Figure 1. Haloferonone (HF) exerts antiproliferative effects in acute promyelocytic leukemia (APL) cell lines NB4 and NB4-R2. (A) Representative example of one out of three experiments of flow cytometric analysis of bromodeoxyuridine (BrdU; y-axis) and 7-amino-actinomycin (7-AAD; x-axis) staining of NB4 cells incubated with increasing doses of HF or all-trans retinoic acid (ATRA, as a control), showing HF-induced growth arrest at the G1/S transition. Cell subpopulations were identified as R1 (sub G0/G1, apoptotic cells), R2 (G0/G1), R3 (G2+/M), and R4 (S phase). (B–C) Cell cycle status of NB4 (B) and NB4-R2 (C) cells after treatment with increasing doses of HF and BrdU incorporation. Percentage of cells in S phase or
BM protein samples were subjected to activation of latent TGFβ1 to immunoreactive TGFβ by acidification, and then used in a sandwich ELISA assay using the Quantikine TGFβ1 Immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s recommendations. The optical density was determined using a microplate reader set at an absorbance of 450 nm and a standard curve was generated to report the results of TGFβ quantification in pg/mL.

Statistical analysis

The effects of HF on APL cell lines regarding cell proliferation, apoptosis, BCL-2 expression, and transcription of TGFβ target genes were analyzed by multivariate analysis using the mixed linear model. Comparisons simultaneously included the following variables: treatment with variable doses of HF, cell type (either NB4 or NB4-R2), and addition of TGFβ. For in vivo experiments, differences in blood cell counts, percentage of immature cells, comparisons of the spleen/body weight ratio, and TGFβ quantification in leukemic and control mice were evaluated by analysis of the variance (ANOVA) between groups followed by the Bonferroni’s correction post-test. In all comparisons, a significance level of P < 0.05 was considered to be significant. Statistical analyses were performed using SPSS 13.0 software.

Results

HF exerts anti-proliferative actions on APL

Cell cycle analysis, evaluated by the immunofluorescent staining of incorporated BrdU and 7-AAD, showed that, in both NB4 and NB4-R2 cell lines, HF inhibited cellular proliferation (P < 0.001) and induced apoptosis (P = 0.002), although the pro-apoptotic effect in NB4 cells was visually less evident than the one observed in NB4-R2. (D–E) Analysis of the number of viable NB4 cells by Trypan blue exclusion according to the incubation time (P < 0.001) with various doses of HF (P < 0.001), indicated by the right-sided legend. Forty-eight hours after incubation with doses greater than 50 ng/mL of halofuginone, the number of viable NB4 cells significantly reduced.

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Halofuginone Effects on Acute Promyelocytic Leukemia

Figure 2. Halofuginone (HF) induces apoptosis in a dose-dependent manner in acute promyelocytic leukemia (APL) cell line NB4. (A, B) Analysis of the number of apoptotic NB4 cells by Annexin V and propidium iodide binding assay according to the incubation time (P < 0.001) with various doses of halofuginone (P < 0.001), indicated by the right-sided legend. Apoptosis was more pronounced after the 48-hour treatment (A) and occurred particularly when doses greater than 12.5 ng/mL of HF. Although the multivariate analysis by the mixed linear model revealed that

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addition of TGFβ is statistically relevant for apoptosis \( (P = 0.013) \), this was due to the results detected with 12.5 ng/mL of HF.

The ED<sub>50</sub> of HF for the inhibition of proliferation was 18 ng/mL, whereas, in the presence of TGFβ, the ED<sub>50</sub> was reduced to 10 ng/mL, thus suggesting a potential additive anti-proliferative action of TGFβ and HF. Data represent the results of three independent experiments.

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Halofuginone up-regulates the expression of TGFβ target genes and TGFβ protein

HF dose-dependent up-regulation of TGFβ \( (P < 0.001) \), TGFβRI \( (P < 0.001) \), SMAD3 \( (P < 0.001) \), p15 \( (P < 0.001) \), and p21 \( (P < 0.001) \)

gene expression was detected after 72 hours of incubation. In addition, treatment resulted in the down-regulation of MYC \( (P < 0.001) \) with concentrations higher than 25 ng/mL (Fig. 4A-F).

The expression of TGFβ receptor II and Smad3 expression in NB4 cells (Fig. 5).

To verify that these effects were caused by HF treatment and not a late cellular response to cell growth inhibition, some genes were selected for a 24-hour HF incubation and real-time PCR analysis, including the addition of exogenous TGFβ to NB4 cultures. Consistent with the 72-hour treatment assay, after 24-hour treatment, HF significantly induced the transcription of TGFβ target genes (TGFβRI and p21) and repressed MYC expression in NB4, regardless of the addition of exogenous TGFβ (Fig. 6). Indeed, as analyzed using the mixed linear model, the results were similar in the presence or absence of TGFβ for TGFβRI \( (P = 0.699) \), SMAD3 \( (P = 0.963) \), p15 \( (P = 0.295) \), and MYC \( (P = 0.768) \).

HF demonstrates anti-leukemic effects in the in vivo model of APL

Table 1 shows that HF treatment for 21 days resulted in an increase to normal hemoglobin levels and platelet counts in leukemic mice. The WBC was significantly lower in leukemic mice treated with HF compared with untreated controls. However, the cytomorphological analysis of Leishman-stained PB smears demonstrated that HF treatment reduced the percentage of immature cells in the bone marrow (66.3 ± 17.9% versus 27 ± 9.3%; \( P < 0.01 \)) and peripheral blood samples (36.5 ± 21.04% versus 15.5 ± 9.09%; \( P = 0.096 \), although blasts were still detectable (Table 1). PML-RARα detection by PCR confirmed the engraftment of leukemic cells in all transplanted animals. As expected by the morphological analysis of the bone marrow, samples obtained after treatment tested positive for PML-RARα.

Since extensive spleen infiltration has been observed in the hCG-PML-RARα mouse model [23], the relation between the spleen and body weight was assessed to further quantify the hematological response to HF. Compared with NOD/SCID wild-type (WT) control mice, as expected, leukemic animals showed a higher spleen/body weight ratio \( (P < 0.001) \). Treatment with HF resulted in a significant reduction of the spleen/body weight ratio \( (P < 0.05); \) Table 1. In addition, morphological analysis of Leishman-stained slides of spleen imprints confirmed the infiltration of immature cells resembling promyelocytes in leukemic animals and demonstrated normal cellular distribution in the HF-treated mice.

HF reverses TGFβ inhibition in PML-RARα leukemic mice

TGFβ quantification in serum samples showed lower levels of the cytokine in leukemic mice compared with WT controls \( (P < 0.0001) \). In animals that received HF treatment, TGFβ levels were similar to those in controls, suggesting that the drug could reverse TGFβ inhibition in PML-RARα leukemic mice. When TGFβ protein expression was verified in the bone marrow lysate, a similar tendency of TGFβ down-regulation in leukemic animals was observed, although the data were not statistically significant (Fig. 7).

Discussion

The present study demonstrates for the first time that HF has anti-leukemic properties, reducing tumor growth and inducing apoptosis in vitro and in vivo. A few studies have reported that HF inhibits angiogenesis in vitro [31] and T cell activation [32], with doses ranging from 5 to 400 ng/mL. Previous studies regarding
HF pharmacokinetics have suggested that intraperitoneal delivery of a 1.5-mg/kg dose in mice produced plasma concentrations between 173 and 209 ng/mL at 10 minutes after administration, with a 100% bioavailability and rapid distribution of the drug to all tissues, except the brain. In addition, HF doses greater than 1.5 mg/kg proved excessively toxic to mice [33]. In vivo studies using HF have reported significant anti-tumoral effects of the drug with intraperitoneal doses varying from 50 to 300 μg/kg with no

Figure 4. Halofuginone (HF) induces activation of TGFβ signaling in APL cell lines. (A–F) Real-time PCR analysis of TGFβ and its target genes in NB4 cells treated with increasing doses of HF, showing up-regulation of TGFβ (A), TGFβRI (B), SMAD3 (C), p15 (D), p21 (E), and repression of MYC (F).
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significant toxicity. Therefore, the doses we have chosen for both in vitro and in vivo assays were within the range previously reported to be effective and non-toxic. Importantly, HF has been previously shown to reduce solid tumor growth, but no published data exist on its actions in hematologic diseases.

HF exerted a potent anti-proliferative effect with an ED$_{50}$ of 18 ng/mL (95% CI: 15.5–20.8 ng/mL), and was as effective in NB4 as in NB4-R2 cells. HF-induced cell growth inhibition was associated with dose-dependent up-regulation of TGFβ target genes. Treatment resulted in an increase in the number of TGFβ

**Figure 5.** Halofuginone (HF) up-regulates the expression of TGFβ, TGFβ receptor II and Smad3 protein in the NB4 APL cell line. Western blot analysis shows that increasing doses of HF (6.25–200 ng/mL) induced the expression of TGFβ and its mediators after 72 hours of incubation. CTRL: control. doi:10.1371/journal.pone.0026713.g005

**Figure 6.** Halofuginone (HF) induces activation of TGFβ signaling in APL cells regardless of exogenous TGFβ addition. (A–D) Real-time PCR analysis in the presence or absence of exogenous TGFβ, showing similar effects of HF in NB4 cells: (A) TGFβRI (P = 0.699); (B) SMAD3 (P = 0.963); (C) MYC (P = 0.768), and (D) p21 (P = 0.295). doi:10.1371/journal.pone.0026713.g006
appears to be a very specific target of TGFβ-dependent kinase inhibitors induced by HF, our results showed up-regulation of the cyclin-
genes. Consistent with the activation of the TGFβ path-
way (including TGFβRI and SMAD3) were modulated by HF treatment. SMAD3, when
phosphorylated and released from the receptor complex by
SMAD3) were modulated by HF treatment. SMAD3, when
encoded proteins of the TGFβ pathway [34]. Moreover, the expression of other genes that
expressed effect in cell cycle regulation, which have shown that HF inhibits TGFβ-induced phosphorylation of
mRNA transcripts and TGFβ protein expression, which is consistent with the previously demonstrated positive feedback of
the pathway [34]. Moreover, the expression of other genes that
encode proteins of the TGFβ pathway (including TGFβRI and SMAD3) were modulated by HF treatment. SMAD3, when
phosphorylated and released from the receptor complex by
TGFβRI, forms a heterodimeric complex with SMAD4, which
then translocates to the nucleus to regulate transcription of target
genes. Consistent with the activation of the TGFβ pathway
induced by HF, our results showed up-regulation of the cyclin-
dependent kinase inhibitors p15 and p21 and down-regulation of
MYC, which may have contributed to TGFβ growth inhibition.
MYC appears to be a very specific target of TGFβ because its
transcription is mediated by SMAD3 binding to a responsive
element in the promoter of the gene [35,36]. In addition, our
findings agree with the evidence that, upon TGFβ stimulation,
down-regulation of MYC creates a positive feedback loop that
further amplifies p15 and p21 expression [1,2,37].

HF inhibited proliferation and induced apoptosis in the absence
of exogenous TGFβ, but addition of the latter potentiated the
effects of HF and resulted in a reduction of the ED50 concentration
by 45% and further up-regulated the expression of TGFβRI,
SMAD3 and p21. Of note, as shown in figure 6, the addition of TGFβ alone to NB4 cells was able to up-regulate its target genes.
This can be attributed to the TGFβ property of activating its own
mRNA expression and protein secretion. Therefore, one could
hypothesize that the up-regulation of TGFβ target genes by HF
results from a combination of increased secretion of TGFβ and a
direct effect of the drug on transcription.

The results obtained with HF treatment in a murine transplant
model of APL in NOD/SCID mice reinforced the potential anti-
leukemic effects of the drug. Mice transplanted with PML-RARα
cells and treated with HF presented hematological remission in
peripheral blood, bone marrow, and spleen as determined by
cell blood counts and cytological analysis. HF did not induce
differentiation of leukemic blasts, and the improvement of
 hematopoiesis resulted from the decrease of the leukemic burden.

In addition, consistent with the evidence of the downregulation
of TGFβ signaling in APL, TGFβ protein levels were decreased in
leukemic PML-RARα animals and HF increased TGFβ levels to
values very similar to those of control non-leukemic mice. The
partial hematological remission observed in HF-treated animals
may be associated with the direct induction of the cytokine TGFβ
itself, known to play a key role in the regulation of human
hematopoietic stem cell quiescence, proliferation and differen-
tiation [38]. Although the effect of TGFβ as a potent negative
regulator of hematopoiesis may contribute to tumor control, its
role in leukemogenesis has been more recently associated with the
findings of disruption in TGFβ signaling either by mutational
inactivation or down-regulation of its components [13]. Therefore,
we have attributed the TGFβ-dependent growth inhibitory effects
of HF to the indirect transcription regulation of cell cycle target
genes.

In contrast to our findings, previous studies of fibrosis models
have shown that HF inhibits TGFβ-induced phosphorylation of

Table 1. Peripheral blood cell counts, percentage of immature cells, and spleen/body weight ratio of wild-type control mice (WT; n = 3) and NOD/SCID leukemic mice that were treated with halofuginone (HF) (LEUK-HF; n = 5) or left untreated (LEUK; n = 5).

|       | WT    | LEUK | LEUK-HF |
|-------|-------|------|---------|
| Hb (g/dL) | 15.3 ± 0.6 | 9.6 ± 1.67 | 12.0 ± 1.40* |
| WBC (×10³/μL) | 2.33 ± 0.57 | 20.6 ± 21.9 | 4.2 ± 3.89** |
| Platelets (×10³/μL) | 1160.0 ± 215.9 | 552 ± 83.2 | 932.0 ± 122.5*** |
| PB blasts (%) | 3.6 ± 1.52 | 36.5 ± 21.04 | 15.0 ± 5.09*** |
| BM blasts (%) | - | 66.3 ± 17.9 | 27 ± 9.3 |
| Spleen/body weight ratio | 0.003 ± 0.0002 | 0.012 ± 0.004 | 0.006 ± 0.001 |

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Figure 7. TGFβ quantification in serum (A) and bone marrow (B) of leukemic NOD/SCID mice treated with halofuginone (HF). As determined by enzyme-linked immunosorbent assay, TGFβ serum levels were lower in leukemic transplanted mice (LEUK) compared with wild-type (WT) controls (P < 0.001), and HF increased TGFβ to levels similar to those in controls.

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significant differences in cell growth inhibition and apoptosis when hypoxia hypothesis of TGF\(\beta\) pathway may play a role in APL pathogenesis. PML-RAR proliferation in an expression, HF could reverse this blockade and prevent cell\(\beta\) complex, leading to blockade of TGF\(\beta\) interrupted the interaction of nuclear PML with the SMAD2/3/4 with those from healthy volunteers, suggesting that TH17 cells peripheral blood samples from AML patients when compared with those from healthy volunteers, suggesting that TH17 cells may play a role in leukemogenesis [42]. Although not dependent on TGF\(\beta\) regulation, this effect strengthens the potential of HF as an anti-leukemic drug.

Recent evidence has suggested that deregulation of the TGF\(\beta\) pathway may play a role in APL pathogenesis. PML-RAR\(\alpha\) may interrupt the interaction of nuclear PML with the SMAD2/3/4 complex, leading to blockade of TGF\(\beta\) transcriptional activity [13]. In this context, considering the effects on TGF\(\beta\) target gene expression, HF could reverse this blockade and prevent cell proliferation in an \textit{in vitro} model of APL. Consistent with the hypothesis of TGF\(\beta\) blockade in APL\(\alpha\), our results did not show significant differences in cell growth inhibition and apoptosis when NB4 and NB4 cells treated with TGF\(\beta\) were compared, suggesting that APL cells are resistant to the anti-proliferative effects of this cytokine.

We can hypothesize that although the disruption of the TGF\(\beta\) pathway itself is not sufficient to initiate malignant transformation, the loss of the TGF\(\beta\) response may be a critical second step that contributes to leukemia progression. In this context, the modulation of TGF\(\beta\) signaling may have therapeutic interest in APL.

Supporting Information

Figure S1 Cell cycle status of NB4 and NB4-R2 cells after treatment with increasing doses of halofuginone.

Cell cycle status according to BrdU incorporation by NB4 (upper graphic) and NB4-R2 (lower graphic) cells after treatment with increasing doses of halofuginone. The multivariate analysis using the mixed linear model confirmed that, in both cell lines, the drug inhibited cell proliferation (\(P<0.001\)) and caused apoptosis (\(P=0.002\)), although the pro-apoptotic effect in NB4 cells was visually less evident than the one observed in NB4-R2. (TIF)

Author Contributions

Conceived and designed the experiments: LLFP PAA BAASL RHJ RAP. Analyzed the data: LLFP PAA BAASL RJH RAP. Contributed reagents/materials/analysis tools: MAZ AN RPF EMR. Wrote the paper: LLFP EMR.

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