Eltrombopag inhibits the proliferation of leukemia cells via reduction of intracellular iron and induction of differentiation

Short title: Leukemia-inhibitory effect of eltrombopag

Michael Roth¹,², Britta Will², Guillermo Simkin², Swathi Narayanagari², Laura Barreyro², Boris Bartholdy², Roni Tamar³, Constantine S. Mitsiades⁴,⁵, Amit Verma³,⁶, Ulrich Steidl²,³

¹ Children’s Hospital at Montefiore, Division of Pediatric Hematology/Oncology, Bronx, NY, USA
² Albert Einstein College of Medicine, Department of Cell Biology, and Albert Einstein Cancer Center, Bronx, New York, USA
³ Albert Einstein College of Medicine, Department of Medicine (Oncology), Bronx, NY, USA
⁴ Dana Farber Cancer Institute, Department of Medical Oncology, Boston, Massachusetts, USA
⁵ Harvard Medical School, Department of Medicine, Boston, Massachusetts, USA
⁶ Albert Einstein College of Medicine, Department of Developmental and Molecular Biology, and Albert Einstein Cancer Center, Bronx, New York, USA

Corresponding author: Ulrich Steidl, Dept. of Cell Biology, Chanin Bldg. #606, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

Email: ulrich.steidl@einstein.yu.edu    Tel: 718-430-3437    Fax: 718-430-8574

Scientific Category: Myeloid Neoplasia
Abstract

Eltrombopag is a small molecule, non-peptide thrombopoietin receptor (TPO-R) agonist and has recently been approved for the treatment of thrombocytopenia in patients with chronic ITP. Prior studies have shown eltrombopag stimulates megakaryopoiesis in bone marrow cells from patients with AML and MDS, and further suggested it may inhibit leukemia cell growth. Here, we studied the effects of eltrombopag on leukemic cell proliferation and the mechanism of its anti-proliferative effects. We found that eltrombopag leads to a decreased cell division rate, a block in G1 phase of cell cycle, and increased differentiation in human as well as murine leukemia cells. As eltrombopag is species-specific in that it can only bind the TPO-R in human and primate cells, these findings further suggested that the anti-leukemic effect is independent of the TPO-R. We found that treatment with eltrombopag leads to a reduction in free intracellular iron in leukemic cells in a dose-dependent manner. Experimental increase of intracellular iron abrogated the anti-proliferative and differentiation-inducing effects of eltrombopag, demonstrating that the anti-leukemic effects of eltrombopag are mediated through modulation of intracellular iron content. Finally, determination of eltrombopag’s anti-leukemic activity in vivo shows its ability to prolong survival in two mouse models of leukemia.
Introduction

Survival in acute myeloid leukemia (AML) and high risk myelodysplastic syndrome (MDS) has remained poor despite recent efforts to treat patients with novel therapeutic regimens.\textsuperscript{1-4} In addition, complications secondary to thrombocytopenia and bleeding occur frequently in AML and MDS, leading to significant morbidity and mortality.\textsuperscript{5,6} Given the median age of patients with AML is close to 70 years, novel anti-leukemia agents, with limited bone marrow toxicity, are needed to improve outcomes.

Eltrombopag (EP) is an oral, non-peptide, small molecule thrombopoietin receptor (TPO-R) agonist which has proven efficacy in treating chronic immune thrombocytopenic purpura (ITP) and hepatitis C related thrombocytopenia.\textsuperscript{7,8} Despite concerns that some leukemia blast cells express the TPO-R, we and others have previously reported that EP does not stimulate leukemia or MDS cell growth, but may rather lead to a modest inhibition, while continuing to stimulate normal megakaryopoiesis in bone marrow samples of patients with AML or MDS.\textsuperscript{9,10} One study utilizing a close chemical derivative of eltrombopag indeed found a toxic effect on myeloid leukemia cells, suggesting that the entire substance class including eltrombopag itself may possess anti-leukemic activity.\textsuperscript{11} Cell line studies further suggested that the growth inhibitory effect of eltrombopag is not related to expression levels of the TPO-R.\textsuperscript{12} However, this hypothesis has not yet been formally tested, and the mechanism through which EP exerts its potential anti-leukemic effect is not known. Of note, the concentrations at which EP inhibits leukemia cell proliferation in vitro are clinically achievable with few side effects, and have led to desirable increases in circulating platelet counts in healthy volunteers.\textsuperscript{13}

In the current study we examined cellular and molecular mechanisms by which EP exerts its anti-leukemic effect, utilizing both \textit{in vitro} and \textit{in vivo} models of acute myeloid leukemia. We show that EP has a profound leukemia-inhibitory effect independent of the TPO-R \textit{in vitro} and \textit{in}
*vivo*, that EP leads to an induction of myeloid differentiation, and that these effects are mediated by a reduction of intracellular iron levels by EP.

**Material and Methods**

**Reagents**

Eltrombopag (SB-497115) was dissolved as a 1mg/mL stock solution in distilled water and stored light protected at room temperature for up to 2 weeks. Salicylaldehyde isonicotinoyl hydrazine (SIH) was a generous gift of Dr. Katherine Franz (Duke University). Deferoxamine Mesylate (DFO), Ferrous Ammonium Citrate, N-acetyl-L-cysteine (NAC) were obtained from Sigma Aldrich. Recombinant human thrombopoietin (TPO) and recombinant mouse TPO were obtained from Invitrogen.

**Cell Culture**

HL60, U937, and HS5 cells were cultured in RPMI (CellGro) with 10% fetal bovine serum (Gemini) (FBS) at 37°C. URE murine AML cells were obtained from PU.1-knockdown mice with targeted disruption of the distal enhancer (URE) -14kb upstream of the PU.1 gene. URE cells were maintained in M5300 media (Stem Cell Technologies) supplemented with 10% heat-inactivated FBS, 15% supernatant of WEHI-3B culture medium, 15% supernatant of BHK culture medium and penicillin/streptomycin.

**Cell proliferation assays**
For MTS assays, cells were plated into 96-well plates with 100μL culture medium. After 72 hours cells were incubated with 10μl of MTS reagent (CellTiter 96® AQueous One Solution Cell Proliferation Assay kit, Promega), OD490 and OD650 were detected by a microplate reader (Versa max, Molecular probe). MTS proliferative index was calculated by subtracting the background from raw values, defined as [OD490 - OD650] of a well with cells minus [OD490 - OD650] of a well with medium only. Manual cell counts were performed culturing 2x10^5 cells/mL in 12-well plates. Viable cells were counted using trypan blue exclusion. Doxorubicin and Cytarabine were obtained from Hospira, Inc.

**Intracellular iron detection assays**

HL60, URE, and U937 cells were harvested and resuspended in RPMI supplemented with 10% FBS and 250 nM calcein-AM (Invitrogen) and were incubated for 5 minutes at 37°C. Cells were washed and resuspended in pre-warmed culture media. Cells were subsequently left untreated, or were treated with EP, TPO, SIH, or DFO for 1 or 4 hours. Intracellular calcein fluorescence was measured by flow cytometry on a BD FACSARia II instrument (BD Bioscience) and mean fluorescence was calculated by Diva software (BD Bioscience). Transferrin receptor expression was measured by FACS analysis utilizing antibodies against human CD71 (11071973; eBioscience) and murine CD71 (557416; BD Pharmigen).

**Cell cycle assays**

The Click-iT™ EdU Flow Cytometry Assay system (Invitrogen) was used following the manufacturer’s instructions. Briefly, after culture of cells with EdU (10μM) for 2 hours, cells were fixed by 4% paraformaldehyde, treated with saponin containing buffer, and then incubated with
Alexa Fluor 647 dye azide. 4',6-diamidino-2-phenylindole (DAPI) was added directly before flow cytometric analysis.

**Cell Division Assays**

The Cell Tracker Orange Assay (Invitrogen) was used following the manufacturer’s instructions. Briefly, cells were labeled with cell tracker at a concentration of 10uM via incubation at 37° for 30 minutes. Cells were washed, maintained in culture and analyzed by fluorescence activated cell sorting FACS every 24 hours.

**Differentiation Assays**

Cell differentiation was assessed 72 hours after treatment. Morphology was examined after Diff Quik (IMEB) staining as well as FACS analysis utilizing antibodies directed against CD11b (555389; BD Pharmigen) and CD14 (MHCD1406; Invitrogen) for human cell lines, and CD11b (12011282; eBioscience) for murine cell lines.

**Intracellular Reactive Oxygen Species (ROS) Assays**

FACS analysis was used to determine the level of intracellular ROS in response to various treatments. Briefly, HL60, URE, and U937 cells were loaded with 5uM H2DCFDA (Invitrogen) for 30 minutes, washed and evaluated by flow cytometry.
**In vivo leukemia models**

All animal experiments were performed in compliance with institutional guidelines and approved by the Animal Institute Committee of the Albert Einstein College of Medicine (protocol #20110102).

NOD-SCID IL2Rγ null (NSG) mice were sub-lethally irradiated (200 rad) and were intravenously inoculated with 1x10⁶ URE cells. At day +3 mice were divided into 3 groups. The first group received untreated drinking water, the second group received drinking water with 0.4mg/mL EP, and the third group received 1.0mg/mL EP in the drinking water. The untreated and treated water was changed every 3 days. Mice were monitored for signs of leukemia and their bone marrow and spleens were harvested at death. For experiments with human leukemia cells, NSG mice were sub-lethally irradiated (200 rad) and were intravenously inoculated with 1x10⁷ HL60 cells. At day +3 mice were divided into 3 groups. The first group received untreated drinking water, the second group received drinking water with 1.0mg/ml EP, and the third group received 3.0mg/ml EP. The untreated and treated water was changed twice every week. Mice were monitored, assessed for engraftment, and their bone marrow and spleens were harvested at death. Engraftment was assessed by FACS analysis utilizing antibodies directed against murine CD45.1 (12045383; EBioscience) and human CD15 (11015973; EBioscience).

**Microarray experiments and analysis**

RNA was extracted from HL60 cells after 12 hrs and 36 hrs of treatment with EP, TPO, or without treatment using Trizol Reagent (Invitrogen). After evaluation of the quality of RNA with an Agilent2100 Bioanalyzer, RNA was labeled with the GeneChip WT terminal labeling kit (Affymetrix), labeled cRNA of each individual sample was hybridized to Affymetrix Humane Gene 1.0ST microarrays (Affymetrix), stained, and scanned by GeneChip Scanner 3000 7G.
system (Affymetrix) according to standard protocols. The complete array data is deposited in the
gene expression omnibus (GEO) and are accessible through GEO series accession number
GSE37580.

Statistical Analysis

Results are reported as the mean plus or minus standard deviation. Student t test was utilized to
determine statistical significance in the in vitro experiments. P value less than 0.05 was
considered statistically significant. For in vivo experiments, log-rank test was utilized to
determine statistical significance of observed differences in overall survival.

Results

Eltrombopag leads to a decreased cell division rate and a block in G1 phase of cell cycle
in human and murine leukemia cells

We evaluated the dose of EP required to inhibit HL60 cell (human) and URE cell (murine)
proliferation. A murine cell line was evaluated in all experiments because EP is highly species-
specific and does not activate the TPO receptor pathway in murine cells.16 HL60 cells and URE
cells were incubated with increasing concentrations of EP for 72 hours. Manual cell counts and
MTS assays were performed every 24 hours. EP inhibits HL60 cells and URE cells (Figure 1A)
in a dose dependent manner at in vitro concentrations that are achievable in vivo.13,17 To
evaluate the effect of EP on the rate of cell division, HL60 cells and URE cells were labeled with
Cell Tracker Orange (Invitrogen) and cells were analyzed by FACS every 24 hours for 3 days.
EP slows cell division rate by 48 hours of treatment in both human HL60 cells and murine URE
cells (Figure 1B i-iv).
Given the effect seen on cell division, we next tested whether EP induced changes in the cell cycle that could contribute to slowed cell division. After 48 hours Eltrombopag treatment led to a block in G1 phase (increase from 30.1 to 54.0 %, p=0.02) with a subsequent decrease in S phase (64.5% (untreated) versus 29.5% (EP-treated) p=0.02) in both HL60 cells and URE cells (increase of cells in G1 from 50.0% to 69.1%, p=0.01, decrease of cells in S phase from 43.7% to 25.0%, p<0.001) (Figure 1C i-ii). Consistent with these findings, gene expression analysis by microarray of HL60 cells performed 36 hours after EP treatment showed the down-regulation of genes important for the transition from G1 to S phase compared to untreated cells. (Figure 1D, and supplemental Figure 1A-B). To confirm EP’s effect on cell division and cell cycling is consistent in other human cell lines, EP was tested in U937 cells. Supporting our findings, EP slowed cell division and led to a block in the cell cycle in G1 phase in U937 cells (supplemental Figure 2).

**Eltrombopag induces differentiation of leukemia cell lines**

Next we tested EP’s effect on differentiation. HL60 cells were incubated without EP, with 5 ug/ml of EP, or with thrombopoietin for 72 hours and cytopsins were performed followed by Diff Quik staining. HL60 cells treated with Eltrombopag showed clear signs of differentiation, significant changes in the organization of the nuclear contents, and an increase in the cytoplasm/nucleus ratio, while thrombopoietin did not induce significant morphologic changes (Figure 2A). FACS analysis confirmed EP differentiates HL60 cells with significant increases in mature myeloid markers, CD11b (2.98 fold, p<0.001) and CD14 (1.61 fold, p=0.003) (Figure 2B i-iv).

We evaluated whether EP induces differentiation of another human cell line (U937 cells), and murine URE leukemia cells. At 72 hours after EP treatment, both cell lines showed significant
differentiation-associated morphologic changes with increased cell size, and the formation of vacuoles within the cytoplasm of U937 cells (Figure 2C). EP caused an increase in CD11b (2.25 fold, \(p=0.007\)) consistent with a pre-macrophage state in U937 cells and also cause an increase in CD11b in URE cells (1.15 fold, \(p=0.04\)) (Figure 2D).

**Eltrombopag reduces intracellular iron in leukemia cells**

Upon treatment with EP, gene expression arrays of HL60 cells showed an overexpression of the transferrin receptor (CD71) which is required for iron transport. This increase in CD71 expression was validated by FACS (see also Figure 3D). Given the overexpression of CD71 and the knowledge that EP shares structural similarities to well-described metal chelators, we hypothesized that some of the observed cell biological effects of EP could be mediated by reduction of intracellular iron (supplemental Figure 3). To address this hypothesis we evaluated the effect of EP on intracellular iron levels.\(^{18,19}\) HL60 cells were loaded with the intra-cellular iron binding compound calcein-AM (Invitrogen). When bound to \(\text{Fe}^{2+}\) calcein-AM’s fluorescent signal is quenched, however, upon release from Fe with addition of an iron chelator, calcein-AM’s fluorescent signal intensifies. Following calcein-AM loading, HL60 cells were treated with varying concentrations of EP, TPO (as a negative control), SIH (a highly potent iron chelator), or a high concentration of DFO (as another positive control). After 1 hour of treatment, concentrations 5\(\mu\)g/ml and 10\(\mu\)g/ml of EP significantly increased calcein-AM fluorescence by 4.48\% (\(p=0.03\)) and 8.03\% (\(p=0.004\)), respectively, indicating reductions of free intracellular iron in cells treated with EP (Figure 3A). After 4 hours of treatment, concentrations as low as 1\(\mu\)g/ml of EP increased the labile intracellular iron pool by 5.12\% +/- 0.79\% (Figure 3A). Interestingly, treatment with 10\(\mu\)g/ml of EP (pharmacologically achievable concentration) resulted in even greater intracellular iron reduction (23.5\%, \(p=0.003\)) than the highly potent chelator SIH (Figure
To determine whether reduction of intracellular iron is a consistent feature of EP across multiple leukemia cell lines, 5 additional cell lines were tested (4 human, 1 murine). All cell lines displayed significant intracellular iron reduction after treatment with 5ug/ml of EP (Figure 3B). Consistent with the finding that EP chelates intracellular iron, gene expression 12 and 36 hours after EP treatment showed the cells are responding to iron depletion by up-regulating the transferrin receptor (CD71) and down-regulating the ferritin light and heavy chains (Figure 3C). The transferrin receptor transports iron intra-cellularly while ferritin stores iron when it is in excess. In addition, we found CD71 surface antigen in both HL60 and URE cells to be overexpressed as early as 24 hours after treatment with EP (Figure 3D). To show CD71 expression is increased in response to EP induced iron deprivation, HL60 cells were pre-loaded with iron utilizing ferrous ammonium citrate (FAC) and subsequently treated with EP. Consistent with our hypothesis, cells loaded with iron and treated with EP showed decreased expression of CD71 compared to cells treated only with EP (Figure 3D). To assess whether iron depletion is characteristic of other TPO agonists, we assessed the ability of a peptidyl TPO agonist to deplete intracellular iron. Four hours after treatment with high dose Romiplostim no significant reduction in iron was observed (supplemental Figure 4).

**Inhibition of leukemia cell proliferation and induction of differentiation by EP is mediated by a reduction of intracellular iron**

The mechanism by which EP inhibits leukemia cell growth has not been well understood. Given that EP leads to a reduction of intracellular iron and iron chelators have been shown to have anti-proliferative effects in myeloid leukemia cells, we tested whether EP’s anti-leukemic effect is secondary to its ability to deplete intra-cellular iron. We performed iron rescue experiments by pre-loading HL60 cells with 500ug/ml FAC for 24 hours, which resulted in a 9.76% +/- 2.10%
increase in intracellular iron. HL60 cells were subsequently left untreated (control), treated with EP, TPO (negative control), or DFO (positive control). Pre-loading cells with iron resulted in a rescue from the anti-proliferative effects of EP as determined by cell count (95% growth increase, p<0.01) and MTS assay after 72 hours (Figure 4A). We made very similar observations in murine leukemia cells as iron-loaded URE cells were also rescued from the anti-proliferative effects of EP (84% growth increase, p<0.01) (Figure 4B).

To assess whether the inhibition of cell division rate by EP could also be rescued by increasing the intracellular iron content of HL60 cells, FAC was preloaded into cells for 24 hours followed by cell tracker labeling of HL60 cells. At 48 hours post EP treatment cell division was significantly greater in cells preloaded with iron and treated with EP compared to cells only treated with EP (Figure 4C). Similarly, EP’s ability to differentiate HL60 cells was reduced by preloading the cells with iron as portrayed by morphologic changes (Figure 4D) and cell surface expression of CD11b and CD14 (Figure 4E). The morphologic changes induced by EP in HL60 cells were similar to changes induced by treatment with DFO. These findings show that the leukemia-inhibitory and differentiation-inducing effects of EP are mediated by a reduction of intracellular iron.

Knowing that ROS levels vary with intracellular iron levels and that iron chelators have been shown to induce ROS formation, we investigated whether EP also induces ROS.\(^\text{20}\) We used H2DCFDA (Invitrogen) with flow cytometry to assess intracellular ROS levels. HL60, URE, and U937 cells were treated with 5µg/ml EP for 1 hour and were subsequently incubated with 5µM H2DCFDA. All cell lines showed significant increases in ROS formation after only one hour of treatment (supplemental Figure 5A-B). In order to determine whether ROS generation was involved in EP induced differentiation of HL60 cells, we incubated the cells with the anti-oxidant N-acetyl-L-cysteine during EP treatment, which resulted in a 92% rescue of ROS activity (supplemental Figure 5C). CD11b and CD14 expression (-22.1%, p= 0.045 and -23.6%,
p=0.01, respectively) both decreased in anti-oxidant treated cells treated with EP compared to cells treated with EP alone (supplemental Figure 5D).

**Eltrombopag prolongs survival in mouse models of leukemia**

Given the *in vitro* anti-leukemic effect of EP, we next evaluated the effect of EP in murine transplantation models of leukemia. Ten million HL60 cells were intravenously injected into NSG mice via the tail vein and mice were treated starting day +3 with 1.0mg/ml EP in the drinking water or untreated drinking water. Bone marrow aspirations at day +21 showed that mice treated with EP had significantly lower donor cell chimerism (5.6% vs. 21.1%, p=0.03) (Figure 5Ai). EP treatment significantly improved survival in these mice (p=0.01) (Figure 5Aii) and engrafted leukemia cells in EP-treated mice showed increased CD11b and CD14 expression (supplemental Figure 6), consistent with our observations in vitro. Given that high doses of EP have been well tolerated in humans we assessed the effect of higher dose EP in our HL60 leukemia mouse model. Mice treated with 3.0mg/ml EP in the drinking water showed significantly increased median survival compared with control mice and mice treated with 1.0mg/ml EP, respectively (p<0.01) (Figure 5Aii).

We next assessed the effect of EP in NSG mice transplanted with URE leukemia cells. One million URE cells were intravenously injected via the tail vein in NSG mice. Mice were then treated starting day +3 with 0.4mg/ml or 1.0mg/ml EP in the drinking water, or untreated drinking water. We have previously shown that treatment with 0.3mg/ml EP in the drinking water results in EP serum levels greater than 2ug/mL. EP showed a dose dependent, statistically significant (0.4mg/ml EP p=0.005, 1.0mg/ml EP p=0.002) effect on the survival of mice with URE cell leukemia (Figure 5B), once again demonstrating the leukemia-inhibitory effect of EP *in vivo*. 
We assessed the effect of EP given in combination with standard AML chemotherapeutic agents. HL60 cells were incubated with EP with or without Cytarabine or Doxorubicin. Manual cell counts at 72hrs showed the addition of EP significantly enhanced the anti-proliferative effects of Cytarabine and Doxorubicin, respectively (p=0.002; and p=0.005) (Figure 5Ci-ii).

Finally, given that stromal cells in multiply transfused patients are often iron overloaded and may mitigate the anti-leukemic effect of EP, we performed co-incubation experiments in vitro. Human HS5 stromal cells, were preloaded with iron or mock-treated for 72 hours and then co-incubated with HL60 cells with or without EP treatment. Neither untreated nor iron overloaded stromal cells alleviated the inhibitory effect of EP on HL60 cell proliferation (supplemental Figure 7).

Discussion

In this study we report the non-peptide, small molecule thrombopoietin receptor agonist eltrombopag inhibits leukemia cell growth by depletion of intracellular iron. Eltrombopag is currently being investigated in clinical trials for treatment for thrombocytopenia in patients with MDS and the new discovery that EP reduces intracellular iron provides support that eltrombopag may be a promising adjuvant agent for patients with AML and MDS for a number of reasons. First, the majority of patients with AML and MDS are over the age of 60 and thus many patients are not able to tolerate intensive chemotherapy and transplant conditioning regimens. Additionally, many patients with AML and MDS are transfusion dependent and suffer from iron overload. Finally, many patients endure significant morbidity and mortality related to thrombocytopenic bleeding in MDS. An agent such as Eltrombopag that has an anti-leukemic effect and does not induce thrombocytopenia, but rather stimulates megakaryopoeisis and
platelet production, has the potential to decrease clinically significant thrombocytopenic bleeding and improve survival.

Our in vitro studies demonstrate that EP inhibits leukemia cell growth through induction of differentiation, as well as slowed cell division mediated by inhibition of cell cycle transition from G1 to S phase. These observations are similar to and consistent with prior literature describing the anti-leukemic effects of known iron chelators and differentiating agents.\textsuperscript{20-26} Studies show that iron chelators have anti-cancer effects in a variety of malignancies including breast cancer, and melanoma, as shown in patients as well as solid tumor xenograft models.\textsuperscript{25,27,28} It is unclear which exact molecular pathways are mediating the anti-leukemic effects upon EP-induced reduction of intracellular iron. Our data suggest that down-regulation of cell cycle-promoting genes and/or an increase of ROS may play a role. However, near complete rescue of ROS with NAC resulted only in a partial rescue from the differentiating effects of EP, suggesting that ROS is not the sole pathway mediating differentiation. Further mechanistic studies will be required to understand the precise molecular and functional sequelae of EP treatment in leukemia cells. It will also be important to further understand how EP interacts with traditional chemotherapy agents, as clinically it would likely be given in conjunction with other cytotoxic therapies. Given EP’s ability to reduce intracellular iron and inhibit growth in leukemia cells, it also seems promising to evaluate the anti-proliferative or differentiating effects of EP in other malignancies.

Previous studies have shown the TPO receptor is present on a significant percentage of AML cells, and have demonstrated TPO may stimulate blast cells expressing TPO-R.\textsuperscript{29-31} Recent studies have postulated the anti-leukemic effect of EP is independent of the TPO-R pathway by demonstrating the effect of EP in leukemia and lymphoma cells lines was not related to the level of TPO-R expression.\textsuperscript{12} In addition, a peptibody that stimulates platelet production through the TPO-R may stimulate blast production and the transformation form MDS to AML in patients with low-risk MDS.\textsuperscript{32} We demonstrate EP’s ability to reduce intracellular iron is not a mechanism
shared by the peptibody TPO-R agonist (supplemental Figure 3). Here, we show for the first time, the anti-leukemic effect of EP is not related to the TPO-R pathway. It has been previously shown that EP interacts with the transmembrane domain of the TPO-R, and that this interaction and consecutive TPO-R downstream signaling is highly species-specific and only occurs in humans and primates, but not in murine cells. Our finding of growth inhibition and differentiation of murine leukemia by EP in vitro and in vivo demonstrates that these effects are TPO-R-independent.

The design of small molecule, non-peptidyl thrombopoietin mimics was originally based on the premise these molecules have three main features: a lipophilic end, an acidic end, and a chelator backbone (supplemental Figure 2). The structure of Eltrombopag has these three elements and supports EP’s ability to reduce intracellular iron and potentially other polyvalent cations. Given that Eltrombopag has a chelator backbone that is not iron specific, it is likely that EP chelates other polyvalent cations such as zinc and copper. These cations are required for the function of a variety of important regulatory proteins and have been shown to play a role in cancer growth. Further studies are warranted to determine their contribution to EP induced inhibition of leukemia cells, and potentially other malignancies.

A recent double blind, placebo controlled randomized dose escalation study showed maximum serum concentrations of EP of over 20ug/ml are clinically achievable with minimal toxicity. These levels are equal or higher than the levels at which EP reduces intracellular iron and exhibits potent anti-leukemia effects in our in-vitro experiments and in-vivo mouse models. Interestingly, bone marrow suppression and anemia has not been reported in patients receiving eltrombopag. This supports the selective anti-cancer effect of iron chelators and the ability of normal hematopoietic cells to better tolerate iron depletion compared to malignant cells.
The properties of eltrombopag may prove important as both an adjuvant anti-cancer therapy, as well as a supportive care agent, potentially improving platelet counts and platelet recovery in patient’s receiving marrow suppressive treatments as well as reducing excess iron in multiply-transfused patients. Our study provides the basis to further study EP in MDS and AML patients as well as to consider exploring its effectiveness in other cancers.

Acknowledgements

We are grateful to the Einstein Stem Cell FACS and Xenotransplantation Facility (supported by NYSTEM, C024172). We thank the Albert Einstein Genomics Core Facility. U.S. is the recipient of a Howard Temin Award of the National Cancer Institute (R00CA131503) and a Medical Research Award of the Gabrielle’s Angel Foundation for Cancer Research. This work was supported by an American Cancer Society – J.T.Tai. & Company, Inc. postdoctoral fellowship (B.W.). U.S. is the Diane and Arthur B. Belfer Faculty Scholar in Cancer Research of the Albert Einstein College of Medicine.

Authorship Contributions

M.R. performed most of the experiments, analyzed data, designed the experiments, and wrote the manuscript; B.W. performed experiments, contributed to the design of experiments, analyzed data, and reviewed the manuscript, G.S. performed experiments and analyzed data, S.R. performed experiments, L.B. designed experiments and reviewed the manuscript; B.B. analyzed data, wrote the manuscript, and reviewed the manuscript, R.T. performed experiments and analyzed data; C.M. and A.V. contributed to the design of experiments, analyzed data, and reviewed the manuscript; and U.S. designed the research, supervised the study, analyzed data, and wrote the manuscript.
Disclosure of Conflicts of Interest

U.S. and A.V. are the recipients of funding from GlaxoSmithKline for research different from the research reported in this manuscript. The remaining authors declare no competing financial interests.

References


Figure Legends

Figure 1. Eltrombopag inhibits cell cycling and leads to a block in G1 phase. (A) HL60 cells and URE cells were treated with increasing concentrations of EP. Cell viability was measured by cell counts with trypan blue exclusion (top panel) and MTS assays (bottom) were performed every 24hrs for 72hrs. Data represents the mean +/- SD of viable cells performed in triplicate and MTS proliferative index (*p<0.05, **p<0.01). (B) HL60 Cells (i) and URE cells (ii) were incubated in 10uM Cell Tracker Orange for 30 minutes, washed and analyzed by FACS (red line= hr 0). Cells were treated without EP (blue line) or with 5ug/ml EP (orange line) and FACS analysis was performed every 24hrs for 3 days. Lower mean fluorescence indicates increased cell division. Fold change of HL60 cells (iii) and URE cells (iv) of FACS mean fluorescence intensity (MFI) +/- SD (n=3) of cell tracker orange labeled HL60 cells treated with 5ug/ml EP relative to untreated cells (*p<0.05, **p<0.01, ***p<0.001). EP slows cell division in HL60 and URE cells as higher MFI represents slower cell division. (C) Cell cycle analysis of HL60 cells (i) and URE cells (ii) with or without 5ug/ml or 10ug/ml EP for 48hrs. EP induces a cell cycle block in G1 phase with a subsequent decrease in S phase. (D) Fold change variation of gene expression by microarray in HL60 cells treated with 3ug/ml EP for 36 hours relative to untreated cells. EP down regulated genes necessary for the transition from G1 to S phase.

Figure 2. Eltrombopag induces differentiation of leukemia cell lines. (A) Representative morphology of HL60 cells treated for 72 hrs under the above conditions shown at 20x (left panel) and 63x (right panel). Cells treated with EP portray reorganization of the nuclear contents compared to untreated cells (red arrows) and cells treated with TPO. (B) Fold change of FACS mean fluorescence intensity (MFI) +/- SD (n=3) of CD11B expression (i) and CD14 (ii) in HL60 cells treated with the above conditions relative to untreated cells for 72hrs (*p<0.05, **p<0.01, ***p<0.001). FACS analysis of CD11B expression (iii) in untreated HL60 cells (blue line) versus cells treated with 5ug/ml EP (orange line) for 72hrs. FACS analysis of CD14 expression (iv) in untreated HL60 cells (green line) versus cells treated with 5ug/ml EP (orange line) for 72hrs. CD11B and CD14 are over expressed with EP treatment. (C) Representative morphology of U937 cells and URE cells treated for 72 hrs under the above conditions. U937 cells treated with EP demonstrate increased size with increased vacuoles in the cytoplasm. (D) Fold change of FACS mean fluorescence intensity (MFI) +/- SD (n=3) of CD11B expression in U937 cells and URE cells treated with or without EP for 72 hrs (*p<0.05, **p<0.01).

Figure 3. Eltrombopag depletes intracellular iron in leukemia cell lines. (A) HL60 Cells were labeled with 0.25uM intracellular iron-chelating dye calcein-AM for 5 minutes. Cells were washed then treated with 0ug/ml EP, 5ug/ml EP, 10ug/ml EP, 25ng/ml TPO (- control), 100uM SIH (+ control), or 100uM DFO (+ control) for 1 hour (left panel) or 4 hours (right panel) at 37°C. Cells were analyzed by FACs. Data represents the change in the mean fluorescence index (MFI) +/- SD (n=3) compared to untreated HL60 cells *p<0.05, **p<0.01, ***p<0.001. (B) Five leukemia/lymphoma cell lines were labeled with calcein-AM then treated with 5ug/ml EP for 4 hours. Cells were analyzed by FACs. Data represents the change in the mean fluorescence index (MFI) +/- SD (n=3) compared to untreated HL60 cells. (C) Fold change variation of gene expression in HL60 cells treated with 3ug/ml EP or 100ng/ml TPO for 12 hours (left panel) or 36 hours (right panel) relative to untreated cells. EP up-regulated the transferrin receptor (TFRC)
and down-regulated the ferritin light chain (FTL) and heavy chain (FTH1). (D) FACS analysis of CD71 expression in untreated HL60 cells (blue line) versus cells treated with 5ug/ml EP (orange line) for 24hrs (left panel). FACS analysis of CD71 expression in HL60 cells treated with 5ug/ml EP (orange line) versus cells preloaded with 500ug/ml ferric ammonium citrate (FAC) for 24 hours then treated with 5ug/ml EP (blue line) for 24hrs (middle panel). FACS mean fluorescence intensity (MFI) +/- SD (n=3) of CD71 expression in HL60 cells and URE cells untreated, treated with 5ug/ml EP, or preloaded with 500ug/ml FAC followed by treatment with 5ug/ml EP for 24hrs (right panel). P value (t-test) represents difference in MFI between treated and untreated cells *p<0.05, **p<0.01, ***p<0.001. CD71 is overexpressed in response to EP treatment and expression is decreased when cells are preloaded with iron.

**Figure 4. Eltrombopag induced cell death is iron dependent.** (A) HL60 cells were untreated, treated with 5ug/ml, 25ng/ml TPO, or 100u MDFO for 72hrs +/- preloaded with 500ug/ml FAC for 24hrs. Cell viability was measured by cell counts with trypan blue exclusion (top panel) and MTS assays (bottom panel) performed at 72hrs. Data represents the mean +/- SD of viable cells performed in triplicate. P-values (*p<0.05, **p<0.01) represent the difference between iron-loaded and non-iron loaded cells. (B) URE^-/- cells were untreated, treated with 5ug/ml, or 10ug/ml EP for 72hrs +/- preloaded with 500ug/ml FAC for 24hrs. Cell viability was measured by cell counts with trypan blue exclusion (top panel) and MTS assays (bottom panel) performed at 72hrs. Data represents the mean +/- SD of viable cells performed in triplicate. P-values (*p<0.05, **p<0.01, ***p<0.001) represent the difference between iron-loaded and non-iron loaded cells. (C) HL60 Cells were incubated in 10uM Cell Tracker Orange for 30 minutes, washed and analyzed by FACS. Cells were treated without EP, with 5ug/ml EP, or were preloaded with 500ug/ml FAC then treated with/without 5ug/ml EP. FACS analysis was performed at 72hrs. Percent change in MFI of FACS mean fluorescence intensity (MFI) +/- SD (n=3) of cell tracker orange labeled HL60 cells treated with 5ug/ml EP +/- iron preload relative to untreated cells, corrected for hr 0 MFI. P-value represents the difference between the MFI of the iron preloaded cells treated with EP and the cells treated with EP without iron preload (*p<0.05, **p<0.01). EP slows cell division in HL60 cells and cell division is rescued by preloading cells with iron. (D) Representative morphology of HL60 cells treated for 72 hrs under the above conditions shown at 20x (left panel) and 63x (right panel). HL60 cells pre-loaded with iron and subsequently treated with EP display less segmented nuclei than EP treated cells without iron (cells with increased nuclear segmentation are indicated by arrows). (E) FACS analysis of CD11b expression (i) in HL60 cells treated with 5ug/ml EP (orange line) versus cells preloaded with 500ug/ml ferric ammonium citrate (FAC) for 24 hours then treated with 5ug/ml EP (blue line) for 72hrs. FACS analysis of CD14 expression (ii) in HL60 cells treated with 5ug/ml EP (orange line) versus cells preloaded with 500ug/ml ferric ammonium citrate (FAC) for 24 hours then treated with 5ug/ml EP (blue line) for 72hrs CD11b and CD14 are over expressed with EP treatment and is rescued by preloading cells with iron. Fold change of FACS mean fluorescence intensity (MFI) +/- SD (n=3) of CD11b expression (iii) and CD14 (iv) in HL60 cells preloaded with 500ug/ml FAC and then treated with 5ug/ml EP compared to cells treated with 5ug/ml EP without iron load. P-values (*p<0.05, **p<0.01) represent the difference between iron-loaded and non-iron loaded cells.
Figure 5. Eltrombopag prolongs survival in mouse models of leukemia. (A) Ten million HL60 cells were transplanted into the tail vein of NSG mice (n=10) 4 hours after sub-lethal irradiation. Mice were divided into two groups, one receiving untreated drinking water, and the other receiving 1mg/ml of EP in the drinking water starting day +3. Bone marrow aspirates were performed on day +21 and FACS analysis was performed assessing donor cell chimerism (CD15+, Ly5.1-) (i). Kaplan Meier survival curve of HL60 transplanted mice (ii) treated with 1mg/ml EP or 3.0mg/ml EP in the drinking water versus mice with untreated drinking water (*p<0.05, **p<0.01). (B) One million URE cells were transplanted into the tail vein of NSG mice 4 hours after sub-lethal irradiation. Mice were divided into three groups, one receiving untreated drinking water (n=7), one group receiving 0.4mg/ml of EP in the drinking water (n=5), and the other receiving 1mg/ml of EP in the drinking water (n=4) starting day +3. Kaplan Meier survival curve of URE transplanted mice treated with 0.4mg/ml EP or 1.0mg/ml in the drinking water versus mice with untreated drinking water (**p<0.01). (C) HL60 cells were treated with or without 20nM Cytarabine (i), with or without 24ng/ml Doxorubicin (ii) +/- 5ug/ml EP for 72 hrs. Cell viability was measured by cell counts with trypan blue exclusion. Data represents the mean +/- SD of viable cells performed in triplicate (**p<0.01, ***p<0.001).
Figure 1
Figure 2

A

HL60

20X

63X

0 EP

5 EP

25 TPO

B

CD11b

HL60

i

Fold Change MFI

***

**

*

0 EP

5 ug/ml EP

25ng/ml TPO

ii

CD14

Fold Change MFI

0 EP

5 ug/ml EP

25ng/ml TPO

iii

Count

Unstained

0 ug/ml EP

5 ug/ml EP

iv

Count

Unstained

0 ug/ml EP

5 ug/ml EP

C

U937

20X

63X

0 EP

5 EP

D

CD11b

Fold Change MFI

**

* U937

URE

0 EP

5 EP

10 EP
Figure 3
Figure 4
Figure 5
Eltrombopag inhibits the proliferation of leukemia cells via reduction of intracellular iron and induction of differentiation

Michael Roth, Britta Will, Guillermo Simkin, Swathi Narayanagari, Laura Barreyro, Boris Bartholdy, Roni Tamari, Constantine S. Mitsiades, Amit Verma and Ulrich Steidl