Mithramycin induces fetal hemoglobin production in normal and thalassemic human erythroid precursor cells

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Running title: Induction of HbF by mithramycin.

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Abstract

We report in this paper that the DNA-binding drug mithramycin is a potent inducer of γ-globin mRNA accumulation and fetal hemoglobin (HbF) production in erythroid cells from human normal subjects and β-thalassemia patients. Erythroid precursors derived from peripheral blood were grown in two-phase liquid culture. In this procedure, early erythroid progenitors proliferate and differentiate during phase I (in the absence of erythropoietin) into late progenitors. In phase II, in the presence of erythropoietin, the latter cells continue their proliferation and mature into Hb-containing orthochromatic normoblasts. Compounds were added on day 4-5 of phase II (when cells started to synthesize Hb) and cells were harvested on day 12. Accumulation of mRNAs for γ-globin, β-globin, α-globin, GAPDH and β-actin were measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), induction of HbF was analyzed by high pressure liquid chromatography (HPLC) and, at cellular level, by flow cytometry. We demonstrated that mithramycin was able to upregulate preferentially γ-globin mRNA production and to increase HbF accumulation, percentage of HbF-containing cells and their HbF content. Mithramycin was more effective than hydroxyurea, being, in addition, not cytotoxic. This was shown by the lack of cytotoxicity on erythroid and myeloid in vitro primary cell cultures treated with mithramycin at concentrations effective for HbF induction. These results are of potential clinical significance as increase of HbF alleviates the symptoms underlying β-thalassemia and sickle cell anemia. The results of this report suggest that mithramycin and its analogues warrant further evaluation as potential therapeutic drugs.
Introduction

Pharmacologically-mediated regulation of the expression of the human \(\gamma\)-globin genes could be of interest as potential therapeutic approach for hematological disorders, including \(\beta\)-thalassemia and sickle cell anemia.\(^{1-8}\) It is well established, indeed, that increase of fetal hemoglobin (HbF) to 30% of the total hemoglobin (Hb) leads to a significant improvement of the clinical status of patients affected by these hematological disorders.\(^{1-3}\) Therefore, current research has been focused on screening of various agents, such as hormones, cytotoxic drugs, hemopoietic cytokines and short fatty acids as agents capable of augmenting HbF levels in humans.\(^{8-13}\)

In this respect, DNA-binding drugs appear to be of great interest.\(^{14,15}\) These agents are known to modify the formation of DNA/nuclear protein complexes and, thereby, control gene expression.\(^{16-22}\) Our research group has recently demonstrated that tallimustine\(^{16}\) and some cisplatin analogues,\(^{15}\) as well as the GC-rich binders chromomycin and mithramycin (MTH),\(^{14}\) are powerful inducers of erythroid differentiation of the human leukemic K562 cell line, suggesting that the pattern of erythroid differentiation and of \(\gamma\)-globin gene expression could be influenced by treatment with DNA-binding drugs. Interestingly, while chromomycin binding to DNA generates stable complexes, MTH/DNA complexes are highly unstable.\(^{23}\) This could explain the low toxicity of MTH as compared to chromomycin.\(^{24}\) For this reason, MTH was proposed as therapeutic agent in several neoplastic diseases (such as chronic myelogeneous leukemia and testicular cancer),\(^{25}\) in Paget's disease\(^{26}\) and in pathologies associated to hypercalcemia.\(^{27,28}\)

The main issue of the present paper was to test whether MTH is able to augment HbF production in erythroid precursor cells from human normal subjects as well as from \(\beta\)-thalassemia patients. This is a mandatory, preliminary step in order to evaluate MTH as a potential drug for the development of treatments of these diseases.
Materials and Methods

DNA-binding drugs and inducers. Mithramycin (MTH), hydroxyurea (HU) and cytosine arabinoside (ara-C) were from Sigma Chemical Co. (St. Louis, MO, USA).

K562 cells. Human erythroleukemia K562(S) cells were cultured in humidified atmosphere of 5% CO₂ in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS, Celbio s.r.l., Milano, Italy), 50 units/ml penicillin and 50 µg/ml streptomycin. Treatment of K562 cells with inducers was carried out by adding the appropriate drug concentrations at the beginning of the cultures (cells were seeded at 30,000 cells/ml). The medium was not changed during the induction period.

Erythroid cell cultures from normal donors and β-thalassemia patients. The two-phase liquid culture procedure was employed as previously described. Following obtaining informed consent, peripheral blood samples were drawn from normal donors and patients with β-thalassemia. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and seeded in alpha medium supplemented with 10% FBS (both from Biological Industries, Beit-Haemek, Israel), 1 µg/ml cyclosporine A (Sandoz, Basel, Switzerland), and 10% conditioned medium from the 5637 bladder carcinoma cell line. The cultures were incubated at 37°C, under an atmosphere of 5% CO₂ in air, with extra humidity. After 7-days incubation in this phase I culture, the nonadherent cells were harvested, washed, and recultured in fresh medium composed of alpha medium, 30% FBS, 1% deionized bovine serum albumin (BSA, Sigma), 10⁻⁵ mol/l β-mercaptoethanol, 2 mmoles/l glutamine, 10⁻⁶ moles/l dexamethasone and 1 U/ml human recombinant erythropoietin (EPO, Ortho Pharmaceutical, Raritan, NJ). This part of the culture is referred to as phase II. Compounds were added on day 4-5 of phase II. Cell samples were analyzed on days 12 or 13 of phase II. Hb-containing erythroid precursor cells were counted following staining by the benzidine:H₂O₂ procedure as previously described. The proportion of HbF out of the total Hb (%HbF) was determined by HPLC as elsewhere described.

Colony formation by normal erythroid and myeloid progenitors. Bone marrow derived mononuclear cells from normal donors were seeded in 35 mm culture dishes in premixed semi-solid culture medium containing 1.2% methylcellulose (Fisher Scientific, Fair Lawn, NJ) in alpha medium, 30% FBS, 1% BSA, 10⁻⁵ mol/l 2-mercaptoethanol, 2 mmoles/l L-glutamine in the presence of either 1 Unit/ml EPO or 10% 5637 cell conditioned medium. After 14 days of incubation, colonies of 50 or more cells were
counted under an inverted microscope. The erythroid nature of colonies was verified by staining with the benzidine:H₂O₂ procedure.

**Immunofluorescence staining for HbF-containing cultured cells.** Cells were washed with phosphate buffered saline (PBS), fixed for 15 min with 4% paraformaldehyde (Biolab, Jerusalem, Israel) and permeabilized with methanol:acetone 1:4 (v/v). After washing in PBS, the cells were stained with Phycoerythrin (PE)-conjugated anti-human HbF monoclonal antibodies (IQ products, Groningen, The Netherlands) for 1 hour at room temperature. PE-conjugated antibodies of the same isotype were used for control. Cell distribution with respect HbF was analyzed using the FACScalibur (Becton-Dickinson, Immunofluorometry systems, Mountain View, CA) flow cytometer. Cells are passed at a rate of 1000 cells/second using saline as the sheath fluid. A 488 nm argon laser beam served as the light source for excitation. Forward light scatter and fluorescence intensity of 10,000 cells were analyzed using the CellQuest® software.

**Real-time quantitative RT-PCR.** Reverse transcription-polymerase chain reaction (RT-PCR) was performed as recently described. After production of cDNA using 1 µg of total RNA, a control PCR for γ-globin gene expression was performed using the γ-globin mRNA-specific primers, 5′-ACT CGC TTC TGG AAC GTC TGA-3′ and 5′-AGT GCC CTG TCC TCC AGA TAC-3′. Quantitative real-time PCR assay of γ-globin mRNA, β-globin and α-globin transcripts were carried out using gene-specific double fluorescently labeled probes in a 7700 Sequence Detection System version 1.6.3 (Applied Biosystems, Warrington Cheshire, UK). The following primer and probe sequences were used for real-time PCR: γ-globin forward primer, 5′-TGG CAA GAA GGT GCT GAC TTC-3′; γ-globin reverse primer, 5′-TCA CTC AGC TGG GCA AAG G-3′; γ-globin probe, 5′-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3′; β-globin forward primer, 5′-CAA GAA AGT GCT CGG TGC CT-3′; β-globin reverse primer, 5′-GCA AAG GTG CCC TTG AGG T-3′; β-globin probe, 5′-FAM-TAG TGA TGG CCT GGC TCA CCT GGA C-TAMRA-3′; α-globin forward primer, 5′-TCC CCA CCA CCA AGA CCT AC-3′; α-globin reverse primer, 5′-CCT TAA CCT GGG CAG AGC G-3′; α-globin probe, 5′-FAM-TCC CGC ACT TCG ACC TGA GCC GGC A-TAMRA-3′. The fluorescent reporter and the quencher were: 6-carboxyfluorescein (FAM) and 6-carboxy-N,N,N’,N’-tetramethylrhodamine (TAMRA), respectively. For real-time PCR of the reference genes we used the endogenous control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin kits, where the probes were fluorescently-labeled with VIC™ (Applied Biosystems).
Statistical analysis. All the data were normally distributed and presented as mean ± S.D. Statistical differences between groups were compared using one-way ANOVA (ANalyses Of VAriance between groups) software. Statistical significance was assumed at P < 0.05.
Results

Mithramycin is a powerful inducer of γ-globin gene expression by human leukemic K562 cells and by normal human erythroid precursors: a real-time quantitative RT-PCR analysis

We first studied the expression of γ-globin mRNA by quantitative RT-PCR analysis performed in K562 cells. This rapid and sensitive technique allows precise quantitation of mRNA templates. The cells were induced to erythroid differentiation by 30 nmoles/l MTH and 0.5 μmoles/l ara-C, a well known inducer of K562 cells. cDNA was produced using total RNA as substrate. In agreement with our previous report, the results indicated that the kinetics of generation of γ-globin RT-PCR products is much faster using as substrate cDNA from MTH- and ara-C-treated cells as compared to control untreated cells. No major differences were observed in the kinetics of generation of GAPDH or β-actin RT-PCR products (data not shown). The data from four independent experiments were analyzed using the Sequence Detection Software System 1.6.3; the results indicated that in K562 cells treated with ara-C the fold induction of γ-globin mRNA compared to control untreated cells was 6.2 ± 1.6 (P < 0.001) using GAPDH mRNA as reference, and 7.1 ± 2.1 (P < 0.001) using β-actin mRNA as reference. In MTH treated K562 cells a significantly higher γ-globin mRNA content was found compared to untreated cells (14.5 ± 1.5 fold and a 12.5 ± 1.1 fold increase using as reference genes GAPDH and β-actin, respectively) (in both cases P < 0.001). In addition, the MTH induced increase in γ-globin mRNA content was found to be significantly higher even compared to ara-C treated cells (P < 0.002 and P < 0.02 using as reference genes GAPDH and β-actin, respectively). Taken together, these data give clear indication for a MTH-mediated induction of γ-globin gene expression in K562 cells.

We next studied the effects of MTH on γ-globin mRNA accumulation in human erythroid precursor cells cultured according to the two-phase liquid culture protocol. In this procedure, early erythroid committed progenitors derived from the peripheral blood proliferate and differentiate during phase I (in the absence of EPO) into late progenitors (CFUe). In phase II, in the presence of EPO, the latter cells continue their proliferation and mature into Hb-containing orthochromatic normoblasts. MTH (20 nmoles/l) and HU (150 μmoles/l), a known stimulator of HbF production in vitro and in vivo, were added on day 4-5 of phase II (when cells started to synthesize Hb) and the cells were harvested on day 12. The effects of MTH treatment on γ-globin, β-globin, α-globin and GAPDH mRNAs were studied by quantitative RT-PCR. Fig.1A shows a representative experiment indicating a significant higher γ-globin mRNA content in MTH- and HU-treated cells compared to untreated cells. No difference is observed among the cultures with respect to GAPDH mRNA content (Fig.1B). Fig.1
(C-E) shows a representative experiment outlining the fold increase in the content of γ-globin, β-globin and α-globin mRNAs in MTH- and HU-treated cultures compared to untreated cultures (in both cases P < 0.001). The results of four independent experiments are shown in Fig.1F and indicate a preferential increase in γ-globin mRNA compared to the other globin-mRNAs in MTH-treated cells (P < 0.001). In all the experiments, MTH proved to be a more efficient inducer of γ-globin mRNA than HU (data not shown).

The increase in the ratio of γ-globin mRNA/GAPDH mRNA was MTH dose-dependent; erythroid precursors treated with 100-200 nmoles/l MTH showed a 25.5 ± 3.5 fold increase with respect to control untreated cells (P < 0.001) in four different experiments. This was, however, associated with strong inhibition of cell proliferation; we, therefore, preferred to limit the MTH doses to low, non-cytotoxic concentrations (10-20 nmoles/l).

Mithramycin-mediated induction of HbF in normal human erythroid precursors: HPLC and flow cytometric analyses

Determination of the HbF content in normal erythroid cultures treated with either MTH or HU from day 4 to day 12 of phase II was performed by HPLC.\textsuperscript{10} The results (Fig.2A) are presented as percentage of HbF with respect to total Hb (%HbF). The results of 9 independent experiments demonstrate that HbF increases from 1.5 ± 0.5% in control cells to 4.7 ± 1.3% (P < 0.001) in HU-treated cells and to 7.4 ± 1.8% (P < 0.001) in MTH-treated cells. The different levels of HbF production in HU- and MTH treated cells was also significant (P < 0.005). The fold increase of HbF in MTH-treated cells (5.1 fold increase ± 0.5) fairly correlates with the increase of γ-globin mRNA content (5.3 fold ± 0.3). A similar correlation was found in HU-treated erythroid precursor cells (data not shown).

We next determined the effects of timing of MTH addition on HbF production (Fig.2B) and cell growth in normal erythroid cultures. MTH (at 0, 10 and 15 nmoles/l) was added on either the first or the fourth day of phase II. On day 13 cells were harvested, an aliquot was counted by benzidine staining, and the rest of the cells were analyzed for hemoglobins by HPLC. The % HbF (Fig.2B, left side of the panel) and the number/ml of erythroid cells (Fig.2B, right side of the panel) are presented. The results indicate that the day of MTH addition did not affect the number of erythroid cells in the cultures (P > 0.6). However, addition of MTH on day 1 stimulated higher proportion of HbF compared to when the addition was delayed until day 4 (P < 0.001), suggesting that, for maximal effect on HbF
production, MTH should be present at the onset of EPO-mediated differentiation of erythroid progenitors.

In order to determine the erythroid cell distribution with respect to HbF, a flow cytometric analysis was performed. Erythroid cells were cultured with or without 15 nmoles/l MTH from day 4 to day 13 of phase II. The cells were then harvested, washed, fixed, permeabilized and stained with anti-human HbF antibodies. The cells were analyzed by flow cytometry. Dot plots of forward light scatter (FSC) and phycoerythrin (PE) fluorescence of 10,000 cells are shown in Fig.2C. The horizontal lines show the level of fluorescence of cells stained with an isotype control antibody. The results of this experiment demonstrate a 2.4-fold increase in the percentage of HbF-containing cells and 1.8-fold increase in the intensity of the fluorescence (mean fluorescence channel) of MTH-treated cells as compared to untreated cells. In this experiment the percentage of Hb-containing cells was 1.6 % in the control and 4.1 % in MTH-treated cells. The results of 5 independent experiments of cultures obtained from different donors showed 3.1 ± 1.2 fold increase in the percentage of HbF-containing cells (P < 0.005) and 1.8 ± 0.4 fold increase in the mean fluorescence channel of MTH-treated cells vs. untreated cells (P < 0.01). These results indicate that both the number of HbF-containing cells and their level of HbF content is increased following MTH treatment.

Mithramycin-mediated induction of HbF in human erythroid precursors from homozygous β-thalassemic patients

We next compared the effects of MTH and HU on cell growth and HbF production by thalassemic erythroid precursors. Table 1 shows the results obtained in cultures from nine β-thalassemia patients listed in order of increasing proportion of constitutive (in untreated cultures) production of HbF. The % of HbF in untreated cultures varied from 3.6% (pat. 1) to 79.8% (the HPFH pat. 9). This variability was due to a different level of expression of both β-globin and γ-globin genes. In any case, MTH was able to increase HbF production in all cases, while HU was not effective in two cases (pats. 3 and 6) and was toxic in one (pat. 4). In some cases (pats. 1, 2 and 8) the activity of MTH was similar to that of HU; in the majority of cases (pat. 3, 5, 6, 7 and 9) the activity of MTH was higher than HU. In all cases, HU was found to strongly inhibit cell proliferation, while, at concentrations able to induce HbF production, MTH was found to exert minimal effect on cell growth, in agreement with the results shown in Fig.2B.
In an attempt to have a general idea on the differential effects of HU and MTH on HbF production in erythroid precursors from β-thalassemia patients, we calculated the gain index as \[
\frac{\% \text{ of HbF (MTH)} - \% \text{ of HbF (control)}}{\% \text{ of HbF (HU)} - \% \text{ of HbF (control)}}
\]. Results from pats. 3, 4 and 6 were not considered, as no induction of HbF production was observed after HU treatment (pats. 3 and 6) or HU was highly cytotoxic (pat. 4). For the remaining patients, the average gain index value obtained was \(1.73 \pm 0.66\) (\(P < 0.03\)), indicating more than 1.5 fold higher activity of MTH with respect of HU.

As far as the effect on cell growth is concerned, the cell number/ml of HU-treated cell for patients 1, 2, 5 and 7-9 is \(24.4\% \pm 8.8\%\) (\(P < 0.02\)) of control untreated cultures, while that of MTH-treated cells (exhibiting the highest induction of HbF) was found to be \(75.2\% \pm 16.6\%\) (\(P > 0.2\)) of control cells, indicating that, compared to HU, MTH is about three fold less inhibitory to β-thalassemia erythroid cell growth in the second-phase of the culture protocol. The differential effects of HU and MTH on cell growth of erythroid precursors were found to be significant (\(P < 0.001\)).

**Effects of mithramycin on proliferation of normal human erythroid and myeloid precursors cells**

When MTH, at HbF-stimulating concentrations, was added to erythroid cultures from day 4 to day 13 of phase II it did not inhibit cell growth, in contrast to HU (Fig.2B and Table 1), in agreement with a previous report on HU.41 This period of the culture encompasses relative late stages of erythroid cell development (from proerythroblasts to orthochromatic normoblasts). In order to evaluate the effect of MTH on the entire erythroid, as well as the myeloid development processes, bone marrow cells were cloned in semi-solid medium in the presence of increasing concentrations of HU (Fig.3A) or MTH (Fig.3B), added at the onset of the cell culture period. In this system early committed progenitors proliferate and differentiate to develop by day 14 into either erythroid colonies (containing mainly orthochromatic normoblasts) in the presence of EPO or myeloid colonies (containing neutrophils or monocytes) in the presence of 5637 cell conditioned medium. The results indicated that in this system HU, at HbF-stimulating concentrations (100-150 µmoles/l), completely abolished the development of erythroid and myeloid colonies. In contrast, MTH, at HbF-stimulating concentrations (10-25 nmoles/l), had no effect on colony development. These results indicate that HbF stimulation can be induced by MTH under conditions that do not inhibit cell proliferation.
Discussion

Mithramycin is a DNA-binding drug able to reversibly interact with GC-rich target DNA sequences.\textsuperscript{20,21,23,42} In virtue to this feature, MTH has been proposed as a gene expression modulator and is actually used, as Plicamycin\textsuperscript{TM} and Mithracin\textsuperscript{TM},\textsuperscript{25,26} for treatment of human pathologies.\textsuperscript{25-28,43-46}

We have previously reported that MTH is a potent inducer of erythroid differentiation in K562 cells.\textsuperscript{14} Differentiation was found to be associated with increase in the synthesis of $\gamma$-globin mRNA and production of mostly Hb Portland, suggesting that this DNA-binding drug could be proposed as inducer of human $\gamma$-globin genes.

In the present report we evaluated the effects of MTH on HbF production in human normal and thalassemic cultured erythroid precursor cells. In several independent experiments, using peripheral blood from different normal donors, we reproducibly found an increase in HbF production (from $1.4 \pm 0.4$ % of control cells in 20 independent experiments to $7.4 \pm 1.8$% of MTH-treated cells in 9 independent experiments) ($P < 0.001$). This increase was found to be consistently higher than that induced by HU (HbF production = $3.5 \pm 1.32$% in 18 independent experiments) ($P < 0.05$), a potent inducer of HbF both \textit{in vitro} and \textit{in vivo}.\textsuperscript{10} These data were fully in agreement with quantitative RT-PCR analysis, showing a preferential increase of $\gamma$-globin mRNA accumulation in MTH-treated erythroid precursors in comparison to $\alpha$-globin and $\beta$-globin mRNA. In addition, the data demonstrate that MTH is a powerful inducer of HbF production in erythroid progenitors from $\beta$-thalassemia patients (both HPFH and non-HPFH). Unlike HU, the effect of MTH was not associated with inhibition of cell growth.

Therefore, our data strongly support possible clinical application of MTH for induction of HbF in patients affected by $\beta$-thalassemia or sickle cell disease, since it has been already demonstrated that increase of HbF to 30% of total hemoglobins leads to a significant improvement of the clinical status of patients affected by these diseases. Thalassemic patients vary considerably with respect to HbF production. Our results suggest that those patients expressing low levels of HbF should benefit the most from such treatment. On the other hand, patients with high HbF (HPFH) are less likely to benefit from the treatment, since our results demonstrated lower increase in HbF in erythroid precursors expressing constitutive high levels of HbF (Table I). However, such HPFH patients are usually in a good clinical status.\textsuperscript{11-13}

It should be underlined, however, that as with other DNA-binding drugs, mutagenic and carcinogenetic effects of MTH cannot be ruled out. We have, however, demonstrated,\textsuperscript{23} by Surface
Plasmon Resonance analysis, that the MTH/DNA interaction is highly unstable, unlike many other DNA-binding drugs, even those that are very similar in structure to MTH (such as chromomycin). This explains the finding that MTH is much less mutagenic than chromomycin.24

Clinical data with a MTH -based drug (plicamycin) demonstrated a variety of side effects, including immediate effects (such as vein irritation, nausea and vomiting, hypocalcemia, fever), early effects (such as depression of clotting factors, alterations of WBC, RBC and platelets count, skin problems, azotemia, headache) and delayed effects (such as acute necrosis of the liver and tubular necrosis of the kidney).43 - 47 Serious side effects are present also during treatment with HU.48 Therefore, it is imperative to show that the concentrations of MTH and HU proven effective in vitro are attainable in vivo. Accordingly, HU at 100 µM which demonstrated effectiveness in vitro was maintained in vivo following continuous infusion of a therapeutic dose of 20 µg/kg HU.48 As far as MTH, the concentrations which were found in the present study to increase HbF production (10-25 nmoles/l, Table I) were lower than those found in vivo following treatment with plicamycin. Patients receiving a 2-hours continuous infusion of 25 µg/kg, reached peak MTH plasma levels of 300-350 nmoles/l. However, the rate of the plasma t½ decay (clearance) of MTH was rapid - 10.6 hours⁴⁹ - indicating that protocols for MTH-based drug infusion should be carefully designed in order to maintain effective drug concentration in the hematopoietic sites. Efficient methods for pharmacokinetic analysis of plicamycin may be useful in this respect.⁴⁹,⁵⁰

We underline that these studies should be performed before treatment can be considered. However, since MTH is already used in therapy (for chronic myelogeneous leukemia and testicular cancer, in Paget's disease and in pathologies associated with hypercalcemia), studying such patients (e.g., with respect to HbF) could provide valuable, yet not conclusive, information on MTH activity in vivo, which could be related to data obtained in experimental animals. This latter strategy is highly warranted, as for other HbF inducers.⁵¹

In conclusion, we hereby report the activity of MTH as HbF inducer. Since many analogues of MTH have been recently described,⁵²,⁵³ these and structurally-related agents should be screened in order to identify analogues exhibiting the lowest cytotoxic/mutagenic effects, the most favourable pharmacokinetics and the highest ability to induce HbF. Further experiments, including in vivo tests on experimental animals, are still necessary to conclusively determine the potential use of such agents in the treatment of severe hematological diseases, such as non HPFH-β-thalassemia and sickle cell anemia.
Acknowledgments

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Legends to Figures

**Fig.1.** Quantitative RT-PCR analysis of globin mRNAs from normal human erythroid cultures.
A, B. Normal erythroid precursors were harvested from untreated control cultures, or cultures treated with either 150 μmoles/l HU or 20 nmoles/l MTH. Total RNA was reverse transcribed and 50 ng used for PCR amplification. For each sample, the ΔRn for γ-globin (A) or GAPDH (B) are plotted against the cycle number (open squares: RNA from control untreated cultures; open circles: RNA from HU-treated cultures; closed squares: RNA from MTH-treated cultures). C-E. The data obtained were analyzed using the Sequence Detection Software System 1.6.3 and the fold-increase of γ-globin (C), β-globin (D) and α-globin (E) mRNA in cultures treated with HU or MTH compared to untreated cultures (taken as 1) was calculated. Data were derived from quantitative RT-PCR plots using GAPDH mRNA as reference and expressed as average ± SD of four independent RT-PCR analyses on a same donor sample (P < 0.001 when data from panel C of MTH-treated cells are compared to those of HU-treated or untreated control cells). F. Fold-increase of γ-globin, β-globin and α-globin mRNA in cultures treated with MTH compared to untreated cultures. Data were derived from quantitative RT-PCR plots using GAPDH mRNA as reference and expressed as average ± SD of five independent experiments using different human donors (P < 0.001 when data of γ-globin mRNA content are compared to either β-globin mRNA or α-globin mRNA content).

**Fig.2.** HbF in normal erythroid cultures: HPLC and FACS analyses. A. HPLC analysis. Cultures of cells derived from normal individuals were either untreated (white box) or treated with 150 μmol/l HU (grey box) or 20 nmoles/l MTH (black box). The drugs were added to the cultures on day 4 of phase II and the cells harvested on day 12. The cells were washed, lysed and the hemolysate analyzed for hemoglobins by HPLC. The results present the % HbF (mean values ± SD of independent induction experiments performed on precursors from five normal individuals) (P < 0.001 when data from MTH or HU-treated cell sample are compared to control; P < 0.005 when MTH-treated and HU-treated samples are compared). B. Effects of timing of addition of MTH on cell growth and HbF production. MTH (at 0, 10 and 15 nmoles/l) was added to erythroid cultures on either the first (black boxes) or the fourth (grey boxes) day of phase II. White boxes = control untreated cells. On day 13, cells were harvested, an aliquot was counted by benzidine staining, and the rest of the cells were washed, lysed and analyzed for hemoglobins by HPLC. Left side of the panel: %HbF out of the total Hb produced. Right side of the panel: number of erythroid cells per ml (x 10⁶). The results represent mean values ± SD of three independent experiments. C. FACS analysis. A culture aliquot was permeabilized, stained with anti-human HbF antibodies and analyzed by flow cytometry. Dot plots of forward light scatter.
(FSC) and phycoerythrin (PE) fluorescence of 10,000 cells are shown. The horizontal lines denote the level of fluorescence of cells stained with an isotype control antibody.

**Fig.3. Effects MTH on growth of erythroid and myeloid cultures.** Mononuclear cells derived from normal bone marrow were cultured in semi-solid medium in the presence of the indicated concentrations of HU (µmoles/l) (A) or MTH (nmoles/l) (B). The medium contained either 1 U/ml EPO for development of erythroid colonies (open circles) or 10% (v/v) 5637 cell conditioned medium for development of myeloid colonies (closed circles). Colonies of 50 or more cells were counted after 14 days of incubation under an inverted microscope. The results represent number of colonies/plate (average ± SD of three independent experiments).
Table 1. Induction of HbF in erythroid precursors from thalassemia patients

<table>
<thead>
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<th>Pat.</th>
<th>Untreated</th>
<th>HU (µmoles/l)</th>
<th>Mithramycin (nmoles/l)</th>
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<td></td>
<td></td>
<td>125</td>
<td>10</td>
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<td>1</td>
<td>Cells</td>
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<td></td>
<td>HbF</td>
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<td></td>
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<td>HbF</td>
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a = cell number /ml culture x 10^-6  
b = HbF (%)  
n.d. = not done
Mithramycin induces fetal hemoglobin production in normal and thalassemic human erythroid precursor cells

Eitan Fibach, Nicoletta Bianchi, Monica Borgatti, Eugenia Prus and Roberto Gambari