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Inhibition of erythroblast growth and fetal hemoglobin production by ribofuranose-substituted adenosine derivatives.

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Abstract

In vivo, inhibition of fetal hemoglobin (HbF) expression in humans around the time of birth causes the clinical manifestation of sickle cell and beta-thalassemia syndromes. Inhibition of HbF among cultured cells was recently described by the adenosine derivative molecule named SQ22536. Here, a primary cell culture model was utilized to further explore the inhibition of HbF by adenosine derivative molecules. SQ22536 demonstrated down-regulation of growth and HbF expression among erythroblasts cultured from fetal and adult human blood. The effects upon HbF were noted in a majority of cells, and quantitative PCR analysis demonstrated a transcriptional mechanism. Screening assays demonstrated two additional molecules named 5'-deoxy adenosine and 2',3'-dideoxy adenosine had effects on HbF comparable to SQ22536. Other adenosine-derivative molecules, adenosine receptor binding ligands, and cAMP-signaling regulators failed to inhibit HbF in matched cultures. These results suggest structurally-related ribofuranose-substituted adenosine analogues act through an unknown mechanism to inhibit HbF expression in fetal and adult human erythroblasts.

Introduction

The regulation of erythroblast growth and differentiation represents the fundamental mechanism for the vertebrate response to environmental and tissue hypoxia as well as a central aspect of many disease states that are manifested as anemia or polycythemia. In a study of large primates, hypoxia-associated increases of erythroblast growth were further characterized by increased HbF expression through mechanisms that are not well defined [1]. Physiological and pharmacological elevations of fetal hemoglobin are associated with amelioration of symptoms in patients with hemoglobinopathies [2-5]. Cytotoxic drugs are used to elevate erythrocyte levels of HbF in patients with hemoglobinopathies [6]. Under some conditions of stressed erythropoiesis, either signal transduction or changes in the erythroid microenvironment are hypothesized to increase erythroblast growth and expression of HbF [1,7,8]. Several signal transduction pathways modulate HbF, including the mitogen-activated protein kinase (MAPK) [9,10] and cyclic nucleotide [11] cascades. Among *in vivo* assays for stressed erythropoiesis, the combination of erythropoietin (EPO), stem cell factor (SCF), and transforming growth factor beta (TGF- β), herein referred to as E+S+T, is particularly robust [12]. Pancellular expression of HbF to levels above 20% of the total hemoglobin produced is regularly achieved with this cytokine

combination using mobilized peripheral blood CD34+ cells from healthy adult donors.

Very recently, the adenosine derivative, CI-IB-MECA was shown to inhibit erythroblast growth and differentiation, mediated through G1/G0 cell cycle arrest [13]. Another adenosine derivative named SQ22536 was additionally shown to affect erythropoiesis by inhibiting HbF expression. Previously, SQ22536 was shown to inhibit the upregulation of gamma-globin gene promoter activity in erythroleukemia cells by hemin [11]. SQ22536 addition to primary erythroid cells also reversed the induction of gamma-globin mRNA and protein associated with hydroxyurea, sodium butyrate, and 5-azacytidine [14]. In this study, the effects of SQ22536 were further characterized in the context of erythroblast growth, and two additional adenosine derivatives were identified as HbF inhibitors.

Materials and methods

Primary erythroblast cultures and analyses.

Human CD34+ cells were isolated in high purity from the peripheral blood donated by normal human volunteers after Institutional Review Board approval. The cells were cultured in minimum Eagle's medium (Sigma, St. Louis, MO) containing 30% fetal bovine albumin, 1% deionized bovine serum albumin, 40mM

glutamine, 1U/ml penicillin-streptomycin, 100uM beta-mercaptoethanol, 1uM dexamethasone and 0.3 mg/ml holotransferrin, with 4U/ml of EPO alone versus EPO, SCF (50 ng/ml; R&D Systems, Minneapolis, MN) and TGF-B (1.25 ng/ml; R&D Systems). All reagents except fetal bovine albumin (Hyclone, Logan, UT), glutamine and penicillin-streptomycin (both from Biosource, Rockville, MD) were purchased from Sigma (St. Louis, MO).

Cells obtained from at least three separate donors were used for all assays. On day 14, a minimum of 30,000 erythroblasts (gated on size and granularity), were analyzed using an EPICS ELITE ESP flow cytometer (Beckman Coulter, Hialeah, FL) after immunostaining with antibodies directed against glycoporphin A (GPA), CD71 (Beckman Coulter, Miami, FL), HbF (Caltag Laboratories, Burlingame, CA) or HbA antibodies (Perkin Elmer Wallac, Norton, OH). Positive staining was defined by fluorescence at levels greater than two standard deviations above the isotypic controls. A Nucycl™ PI kit (Exalpha Biologicals, Watertown, MA) was used for cell cycle analyses on days 7 and 14 according to the manufacturer's protocol. Quantitative PCR for gamma- and beta-globin mRNA content and HPLC analyses were carried out as per conditions described previously [12]. Based upon the large number of CD34+ cells required for this study, cells from multiple human donors were utilized. Due to the predicted variability in

the baseline production of HbF in EPO and E+S+T between donors, matched controls containing no adenosine-related molecules were cultured simultaneously for statistical comparison of HbF in each experiment. Statistical significance for all experiments was determined by Student paired t-test analyses.

Adenosine signaling and cAMP assays.

The expression of adenosine receptors in the course of maturation of hematopoietic progenitors into erythroblasts was recently reported [13]. In order to investigate the effects of adenosine receptor agonists, antagonists and other adenosine derivatives (Supplemental Table I), CD34+ hematopoietic progenitor cells were cultured in EPO and E+S+T and titrated in 2-3 log concentration ranges. Doses were selected according to previously published reports. SQ22536, CI-IB-MECA, DPCPX and ZM were obtained from Tocris (Ellisville, MO, USA). The other compounds were purchased from Sigma (St.Louis, MO, USA). The Chemical Abstracts Service (CAS) registry or Molecular Design Limited (MDL) numbers are provided in the table for access.

For cAMP studies, CD34+ cells from three healthy donors were cultured in EPO for 6 days and SCF and TGF-B were added for 10 min. and immediately harvested and kept at 4°C. Cells were lysed and total

cellular cAMP was extracted according to the protocol of cAMP Biotrak Enzyme Immunoassay kit (Amersham Biosciences, Piscataway, NJ). Non-acetylated cAMP was assayed according to the manufacturer's instructions. For cAMP signaling studies, cultured cells were incubated for 14 days in increasing concentrations of activators of the adenylate cyclase pathway: forskolin (0.8 μ M – 20 μ M) activating adenylate cyclase, 8-pCPT-2Me cAMP (5 μ M - 500 μ M) for EPAC-specific Rap1 as well as 8-bromo cAMP (0.1 μ M – 100 μ M) for PKA activation. Inhibitors included MDL12330A (1 μ M – 100 μ M) for adenylate cyclase, H-89 (0.1 μ M - 1 μ M) and KT 5720 (4nM - 400nM) for protein kinase A (PKA) and rolipram (1 μ M - 100 μ M) for PDE4. All inhibitors were purchased from Calbiochem (La Jolla, CA).

Results

Inhibition of HbF expression by ribofuranose-substituted adenosine derivatives.

In this study, primary human erythroblast cultures were used to screen and compare effects of adenosine receptor agonists and antagonists, as well as adenosine derivatives that act primarily upon adenylate cyclase (Supplemental Table I) for effects upon cellular proliferation, maturation and expression of HbF. The majority of the screened adenosine derivatives had no significant effect on HbF or

erythropoiesis. Additionally, CI-IB MECA inhibited erythroblast growth but had no demonstrable effect upon HbF production (data not shown). MRS1220 and 3'-deoxy adenosine also inhibited erythroblast growth, but further analyses were confounded by cellular toxicity. Among the other screened molecules, 5'-deoxy adenosine, 2',3'-dideoxy adenosine, and SQ22536 inhibited erythroblast growth and were additionally associated with significant reductions in HbF (Table I). These three molecules are structurally related derivatives of adenosine. As shown in Fig. 1, 5'-deoxy adenosine lacks the primary hydroxyl group of the native ribose sugar, and 2',3'-dideoxy adenosine lacks both secondary hydroxyl groups. Interestingly, the structure of SQ22536 lacks all three hydroxyl groups and the 5' methylene moiety leaving only a tetrahydrofuran ring conjugated to adenine.

Combined with previous reports [11,14], these data suggest SQ22536 acts as a general inhibitor of HbF induction by cytokine signal transduction, hemin and cytotoxic drugs. Therefore, SQ22536 was examined in more detail to determine whether its effects are mediated by inhibition of adenylyate cyclase. Repeated titrations using cells from three separate donors with SQ22536 (0-600uM) produced similar results (Figs. 2A and 2B). At 300uM and 600uM, SQ22536 showed a significant inhibition of HbF with E+S+T. A significant reduction in cell growth was also detected at the highest dose of

600uM. Analysis of expression of the transferrin receptor (CD71), and glycoporphin A (GPA) using flow cytometry revealed decreased percentages of cells expressing glycoporphin A as a function of SQ22536 concentration (Fig. 2C) indicative of decreased maturation of these cells.

To determine whether the SQ22536 effects upon HbF were limited to cells from the adult stage of human development, additional assays were performed using progenitor cells cultured from human umbilical cord blood. It was previously shown that E+S+T augments HbF production among cord blood progenitor cells [15,16]. As shown in Fig. 3A, SQ22536 significantly downregulates HbF production in both EPO (from $22.8 \pm 1.2\%$ to $5.9 \pm 1.0\%$, $p=0.02$) and E+S+T (from $52.8 \pm 7.5\%$ to $31.8 \pm 1.8\%$, $p=0.02$). In the absence of SQ22536, proliferation of the cord blood-derived cells was more robust than cells from adults. With the addition of SQ22536, such proliferation was significantly reduced in EPO, with no significant reduction in E+S+T (Fig. 3B).

SQ22536 effects upon cellular maturation and the cell cycle.

Based upon the previously reported inhibition of erythropoiesis by Cl-IB-MECA [13], detailed analyses of erythroblast maturation were performed. Matched cultures of CD34+ cells in EPO and E+S+T from

three donors grown in the presence and absence of 300uM SQ22536 were examined on days 0, 2, 4, 6, 8, 10, 12 and 14 (Figs 4A, 4B). Reduced maturation was noted in the presence of 300uM SQ22536 during the second culture week as evidenced by an increase in the percentage of proerythroblasts (Fig. 4). Earlier, it was shown that the adenosine derivative, CI-IB-MECA inhibits erythropoiesis via a mechanism of G0/G1 cell cycle arrest [13]. Therefore, cell cycle assays were performed to determine if a similar mechanism of growth inhibition was mediated by SQ22536 (Fig. 5). Rather than accumulation in G0/G1, the percentage of cells in G0/G1 was reduced in SQ22536 under all culture conditions. On day 7, statistically significant increases ($p=0.01$) in apoptotic cells (sub G0/G1) and decreases ($p=0.04$) in G2/M cells were additionally noted.

SQ22536 inhibits gamma-globin gene expression.

To determine whether SQ22536 inhibited cytokine-signaled HbF production by reducing levels of gamma-globin mRNA, quantitative PCR assays were performed. Table II shows the average expression levels of gamma- and beta-globin genes from three separate donors. Significant reductions in the level of both gamma- and beta-globin mRNA with SQ22536 were detected in the EPO controls. As shown previously [12], cells grown in E+S+T demonstrated a highly

significant increase in the gamma-globin expression. The addition of SQ22536 to E+S+T significantly reduced the level of gamma-globin transcripts, but the beta-globin mRNA levels were reciprocally increased. As a result, SQ22536 was associated with significant decreases in the fraction of gamma globin mRNA (gamma/gamma+beta %) in both culture conditions.

The inhibitory effects of SQ22536 upon HbF expression.

Adult CD34+ cells from three donors maintained in EPO or E+S+T with or without 300uM SQ22536 were studied on day 14 (Fig. 6). Flow cytometric analysis of cells stained for HbF revealed reduced expression of HbF in the presence of SQ22536 in EPO and E+S+T. HbF-positive cells were significantly reduced, from $29.2 \pm 15\%$ in EPO to $5.0 \pm 4.3\%$ in EPO+SQ22536 ($p=0.03$). The percentage of HbF-positive cells was also reduced dramatically for E+S+T cultures supplemented with SQ22536 (E+S+T= $92.6 \pm 0.6\%$; E+S+T+SQ22536= $32.4 \pm 9.1\%$; $p=0.003$).

Lack of involvement of cAMP in HbF induction.

SQ22536 is a known inhibitor of adenylate cyclase in other cell culture models [17-19]. Therefore, we investigated if the inhibition of HbF in human erythroblasts by SQ22536 in this culture model involved

recognized elements of the adenylate cyclase pathway (Fig 7A). Exploring the cAMP-PKA pathway using inhibitors, H-89 and KT5270, we found that cytokine-induced HbF augmentation was not altered (Fig. 7B). Neither EPAC activation by 8-pCPT-2Me nor blocking the breakdown of cAMP by PDE4 using rolipram provided further modulation of HbF. MDL12330A is another adenylate cyclase inhibitor (structurally unrelated to SQ22536), but it failed to inhibit HbF expression. The effects of SQ22536 and forskolin on cAMP production were also examined in this experimental model (Fig. 8). As expected, there was a rapid and significant rise in cAMP after the addition of 20uM forskolin, as well as the significant decrease ($p < 0.03$) in cAMP concentration when SQ22536 was added with forskolin. Despite this clear effect upon cAMP in erythroblasts, forskolin slightly decreased HbF when added for the entire 14 day culture period in EPO or E+S+T. When SQ22536 and forskolin were both added to E+S+T cultures, a significant reduction in HbF was also detected (Fig. 8D).

Discussion

Recent reports demonstrated the potential for adenosine derivative molecules to regulate erythropoiesis and hemoglobin expression [11,13]. Here, screening assays were performed to show that three structurally-related adenosine derivatives act by inhibiting

erythroblast growth and HbF expression. Structure activity relationship analysis of ligands related to both SQ22536 and adenosine revealed a profile that can modestly be correlated to the degree of hydroxylation of the ribofuranose moiety of the adenosine chemical structure. While 5'-deoxy adenosine retains two of the native hydroxyl groups, 2',3'-dideoxy adenosine is devoid of two hydroxyl groups. On the other hand, SQ22536 lacks the hydroxyl groups and the 5' methylene moiety. The changes in HbF-related activity in relation to these hydroxyl groups may be relevant for further investigations of the underlying molecular mechanism as well as the identification of other structurally-related HbF inhibitors. The potent inhibition of HbF and absence of G0/G1 cell cycle arrest also suggests SQ22536 and CI-IB-MECA affects erythropoiesis through distinct mechanisms.

Surprisingly, SQ22536-mediated inhibition of HbF production occurred under all cytokine-supplemented culture conditions, and among erythroblasts from cord as well as adult blood. A statistically significant reduction of HbF was not detected by bulk assay (Table I) in EPO-supplemented cultures of adult cells, evidently due to the lower level of HbF in those cells at baseline. However, the reduced distribution of HbF-containing adult cells and the reduction in gamma-globin mRNA suggests the SQ22536 inhibition or silencing of HbF occurs even when the total amount of HbF in each cell is low. The

results further suggest that the SQ22536 effects are not limited to those experimental models, but extend also to cytokine-mediated HbF induction and HbF production in fetal erythroblasts (cord blood). Hence, these combined results suggest that SQ22536 is associated with inhibition of gamma-globin transcription and HbF expression regardless of the biological setting (hemin, cytotoxic drugs, cytokines and development).

The quantitative PCR assays further demonstrate that HbF inhibition occurs at the level of gamma-globin transcription. This result is consistent with the ability of SQ22536 to inhibit gamma-globin gene promoter activity in erythroleukemia cells [11]. SQ22536, 2',3'-dideoxy adenosine, and 5'-deoxy adenosine are all known inhibitors of adenylate cyclase [17]. SQ22536 affects the particulate, but not soluble isoform of the enzyme [19]. Despite the assumption that inhibition of HbF expression was likely being mediated by a mechanism involving adenylate cyclase, we were unable to demonstrate that cAMP itself or other elements of the cAMP-signaling pathway were effectors of HbF expression. Instead, dissection of the adenylate cyclase-cAMP-PKA pathway with multiple activating and inhibiting components of this signaling module suggested a lack of association between cAMP signaling and HbF. MDL12330A was also screened as a separate adenylate cyclase inhibitor predicted to lower intracellular cAMP [20],

but that molecule had no significant effect upon HbF expression. MDL12330A is not structurally related to adenosine. In addition, erythroblast cAMP was clearly increased by forskolin in the absence of any significant changes in HbF. Differences between the results reported here and elsewhere [21] may reflect the source of erythroid progenitor cells or culture conditions, and strongly suggest gamma-globin gene and protein expression may be influenced by more than one signal transduction pathway.

The clinical manifestation of all sickle cell and beta-thalassemia syndromes begins with HbF silencing during early postnatal life. Among all the physiological changes associated with birth, it is possible that inspired oxygen is the initial signal for decreased levels of erythropoiesis and silencing of fetal hemoglobin expression during post-natal human life. HbF down-regulation begins in the fetus, but silencing is almost completely restricted to extra-uterine life [22]. By the age of six months, silencing is nearly complete in healthy infants. Perinatal HbF expression is increased by hypoxia [23]. Several structurally related adenosine derivative molecules were shown here to inhibit HbF expression *ex vivo* through a mechanism that appears distinct from adenylate cyclase and cAMP signal transduction. As such, the data add support to the notion that small molecules may serve

some role in HbF silencing in vivo, and support further investigation in this area of research.

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Figure Legends

Figure 1. Structural formulas of adenosine, 5'-deoxy adenosine, 2',3'-dideoxy adenosine and 9-(tetrahydro-2-furyl)adenine (SQ22536).

Figure 2. Effect of SQ22536 on fetal hemoglobin expression and erythroblast proliferation. (A) HbF expression (shown as HbF/HbF+HbA%) and (B) Cell counts ($\times 10^5$ per ml) of matched cultures in EPO (dashed line) and E+S+T (solid line) in the presence of 0, 75, 150, 300 and 600uM of SQ22536 analyzed on day 14. (C) Corresponding flow cytometric analysis of GPA (y-axis) and CD71 (x-axis) expression shown. The percentage of dual positive cells (GPA+CD71+) is provided in the top right corner of each panel. Values with standard deviation bars from day 14 cultures of three separate donors are shown. Asterisks (*) denote statistical significance of $p < 0.05$.

Figure 3. SQ22536 effects in cord blood derived erythroblasts. (A) HbF/HbF+HbA% and (B) Cell counts ($\times 10^5$ per ml) from matched cultures of cord blood CD34+ cells grown in EPO (open bars) versus E+S+T (solid bars), with (+) and without (-) 300uM SQ22536 for 14 days. Values with standard deviation bars from day 14 cultures of three separate donors are shown. Asterisks (*) denote statistical significance of $p < 0.05$.

Figure 4. SQ22536 effects on differentiation of cell types. CD34⁺ cells cultured in (A) EPO_±SQ22536 and (B) EST (E+S+T)_±SQ22536 as examined by microscopy for changes in morphology on culture days 0, 2, 4, 6, 8, 10, 12 and 14 (x-axis). On the y- axis, mean percentages of cells identified as undefined blasts (vertical lines), prepro/proerythroblasts (gray), basophilic normoblasts (slanted lines), polychromatic normoblasts (white), and orthochromatic normoblasts (black) are represented.

Figure 5. Cell cycle kinetics were analyzed in EPO_±SQ22536 and EST(E+S+T)_±SQ22536 on days 7 and 14. The percentage in each cell phase of the cycle defined by propidium iodide staining is shown on the y-axis. The cell cycle phases [sub G0-G1 (hatched), G0/G1 (solid), S (open), and G2/M (gray)] are represented with standard deviation bars using cells from three donors. Asterisks (*) denote statistical significance of $p < 0.05$.

Figure 6. Silencing of HbF expression in adult blood. CD34⁺ cells from adult blood were cultured in (A) EPO (B) EPO+SQ22536 (D) E+S+T and (E) E+S+T+SQ22536 for 14 days and the cellular distribution of HbF (%) was determined on day 14 by flow cytometric analysis. Representative scatter plots showing forward scatter (size; x-axis) versus HbF fluorescence (HbF %; y-axis). The horizontal lines in panels A,B,D, and E show the fluorescence level at two standard

deviations above the isotypic controls. The mean percentages of HbF-positive cells with standard deviations for three donors are shown in the upper right corner of each scatter plot. Histogram overlays of (C) EPO and (F) E+S+T show cell count (Counts, y-axis) versus HbF fluorescence (FU, x-axis) in the absence (open) versus presence (gray) of 300uM SQ22536.

Figure 7. SQ22536-related signal transduction. (A) Scheme showing known activator and inhibitors in cAMP pathway (B) Effect of adenylate cyclase activators, inhibitors and cAMP on HbF production. HbF production of cells cultured in EPO (dotted line) and E+S+T (solid line) were analyzed by HPLC. Dose-response curves of HbF/HbF+HbA% are represented for cAMP (brown), MDL (aquamarine), forskolin (purple), H-89 (orange), KT (violet), 8Me-cAMP (green) and rolipram (blue). The concentration ranges are described in the Materials and Methods section. Values with standard deviation bars from day 14 cultures of three separate donors are shown.

Figure 8. Combined effects of SQ22536 and forskolin upon cAMP and HbF in human CD34+ cells. (A) Cells were cultured in EPO for 6 days prior to the addition of SCF (50 ng/ml), TGF-B (1.25 ng/ml), SQ22536(300uM), and forskolin (20uM). cAMP (fmoles) was assayed using 10^8 cells at 0 (white), 10 (hatched) and 30 (gray) minutes. (B) Corresponding results in E+S+T shown. Data are mean values of

triplicate analyses with standard deviation bars. (C) For comparison, HbF levels were measured with cells cultured for 14 days in EPO, EPO supplemented with SQ22536(300uM) black bars; EPO, EPO supplemented with SQ22536(300uM) and forskolin(20uM) white bars. HbF levels were measured on day 14. (D) Corresponding HbF results in E+S+T cultures. Data are from triplicate donor experiments with mean values and standard deviation bars shown. Asterisks (*) denote statistical significance of $p < 0.05$. Abbreviations: SQ: SQ22536, EST: E+S+T. Forsk: forskolin.

Table I: Summary of adenosine derivatives with significant effects upon HbF expression.

Name (Concentration)	Cell count ($\times 10^6$ /ml)		HbF/HbF+HbA (%)	
	EPO	E+S+T	EPO	E+S+T
SQ22536				
0 μ M	1.3 \pm 0.0	1.3 \pm 0.1	0.3 \pm 0.2	29.5 \pm 4.7
30 μ M	1.1 \pm 0.0	0.7 \pm 0.1	0.1 \pm 0.0	26.3 \pm 7.4
150 μ M	0.9 \pm 0.0	0.7 \pm 0.0	0.3 \pm 0.2	12.3 \pm 2.2*
600 μ M	0.8 \pm 0.0	0.3 \pm 0.3	0.2 \pm 0.1	2.7 \pm 2.6*
5'-deoxy adenosine				
0 μ M	1.4 \pm 0.5	1.1 \pm 0.4	7.1 \pm 2.1	49.4 \pm 8.2
5 μ M	1.0 \pm 0.4	1.1 \pm 0.5	7.7 \pm 2.8	40 \pm 1.6
50 μ M	0.8 \pm 0.4	0.8 \pm 0.6	2.8 \pm 0.2*	21.4 \pm 4.3
500 μ M	0.3 \pm 0.3*	0.3 \pm 0.3*	1.7 \pm 0.3*	NA

2',3'-dideoxy**adenosine**

0 uM	1.4±0.5	1.1±0.4	7.1±2.1	49.4±8.2
0.03 uM	1.1±0.7	0.9±0.1	7.1±2.5	44±1.8
0.3 uM	1.0±0.5	0.6±0.1	6.2±3.1	45.5±12
3 uM	1.0±0.5	0.6±0.1*	4.8±2.2*	13.1±0.3*

Asterisk (*) denotes significance of $p < 0.05$ when compared to the controls for each screen. Screens were performed using cells from three separate donors, and adenosine derivatives were added on day 0. Proliferation and HbF production were determined on day 14. Differences in control levels of HbF production were due to the use of separate donor trios for each group of screened molecules.

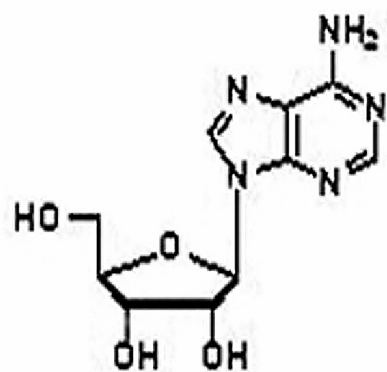
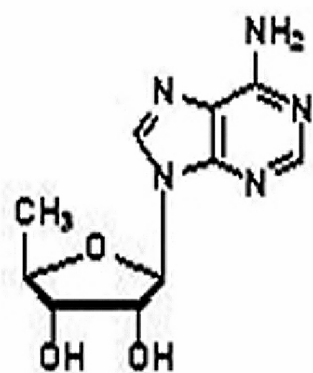
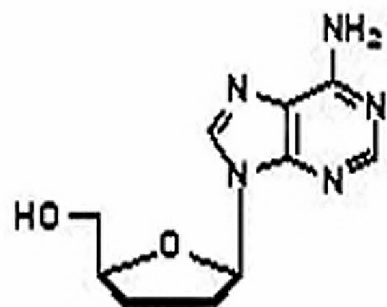
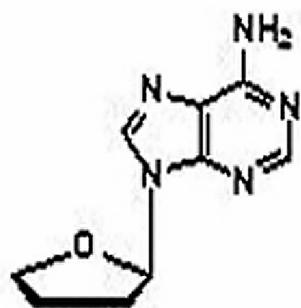
Table II. Effect of SQ22536 on gamma- and beta-globin mRNA expression in EPO and E+S+T.

Day 14	EPO	EPO+SQ22536	p value
gamma-globin mRNA	165 ± 22	1 ± 0.4	1.7E-08
beta-globin mRNA	4700 ± 710	777 ± 317	1.1E-07
gamma+beta mRNA	4870 ± 700	779 ± 317	6.9E-08
gamma/gamma+beta %	3.5 ± 0.9	0.2 ± 0.1	6.2E-06

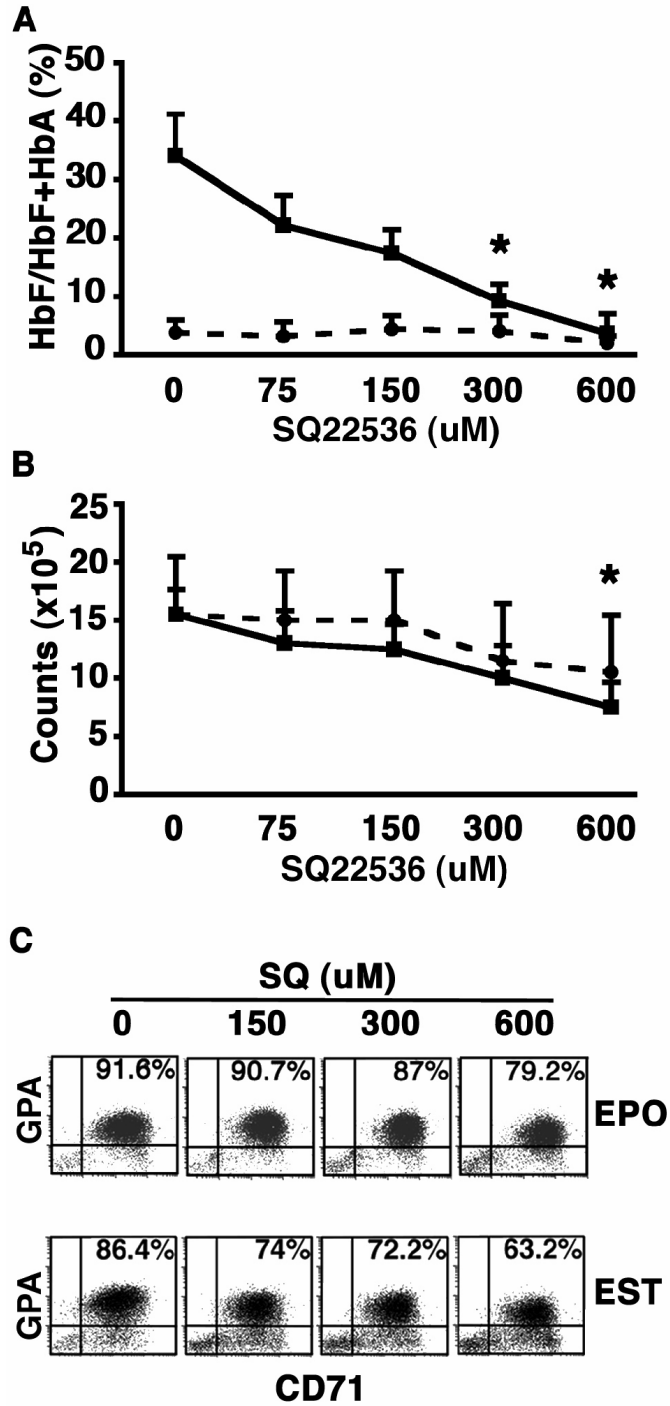
Day 14	E+S+T	E+S+T+SQ22536	p value
gamma-globin mRNA	5260 ± 2020	331 ± 245	3.4E-05
beta-globin mRNA	5040 ± 1070	8130 ± 5000	4.7E-02
gamma+beta mRNA	10300 ± 3050	8460 ± 5250	4.4E-02
gamma/gamma+beta %	49.9 ± 4.9	3.6 ± 0.6	1.4E-09

Using quantitative PCR, beta- and gamma-globin mRNA expression was examined in CD34+ cells cultured in EPO and E+S+T in the presence and absence of 300uM SQ22536 (added on day 0) for 14 days. The mean gamma-, beta- and gamma+beta-globin are shown as mRNA copies/cell along with %gamma/gamma+beta-globin. Experiments were performed in triplicate using cells from three separate donors. Mean values, standard deviations, and statistical significance (*p* values) are also shown for each culture condition.

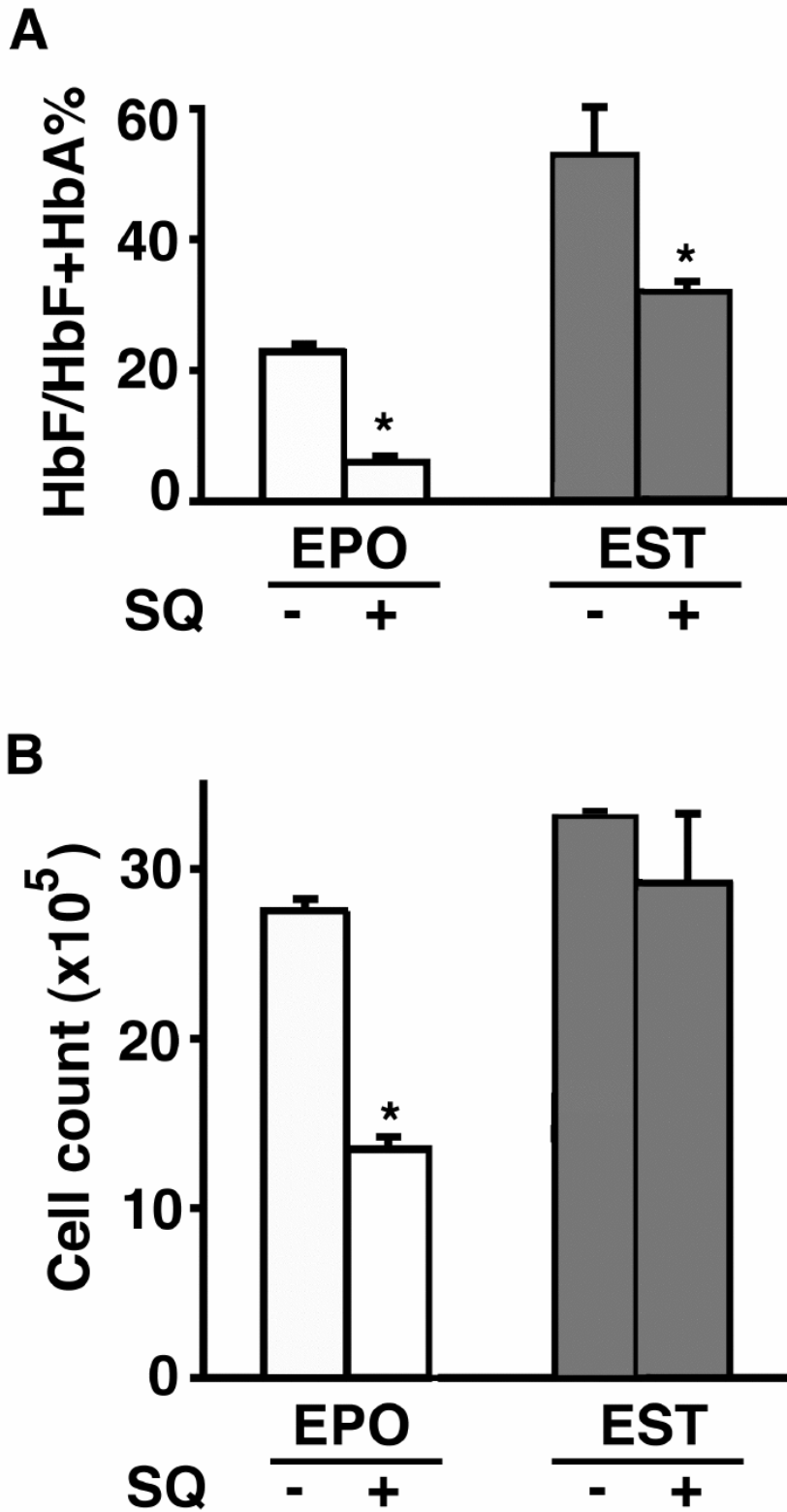
Bhanu et al., Figure 1

**Adenosine****5'-deoxy adenosine****2',3'-dideoxy adenosine****SQ22536**

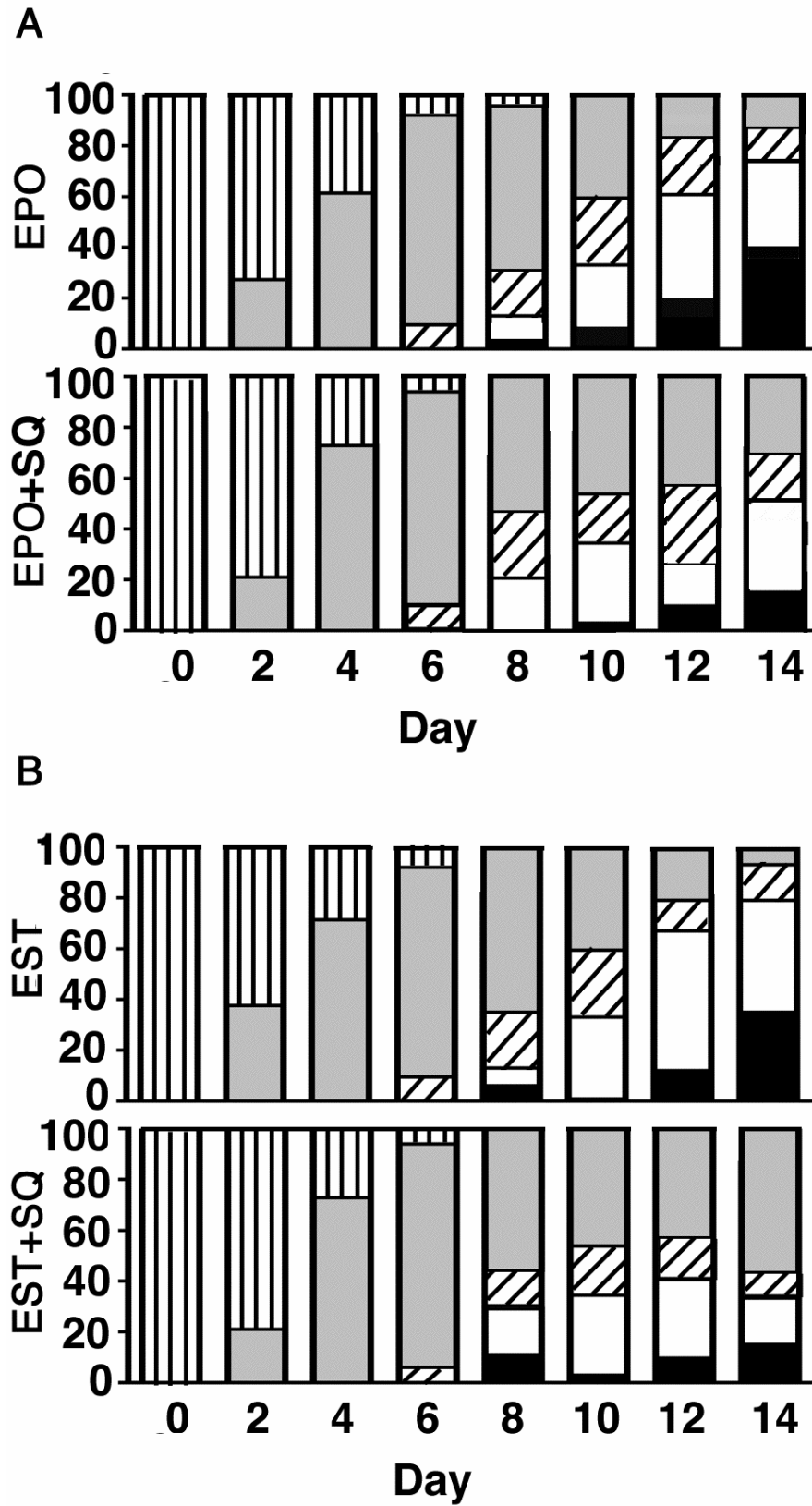
Bhanu et al., Figure 2



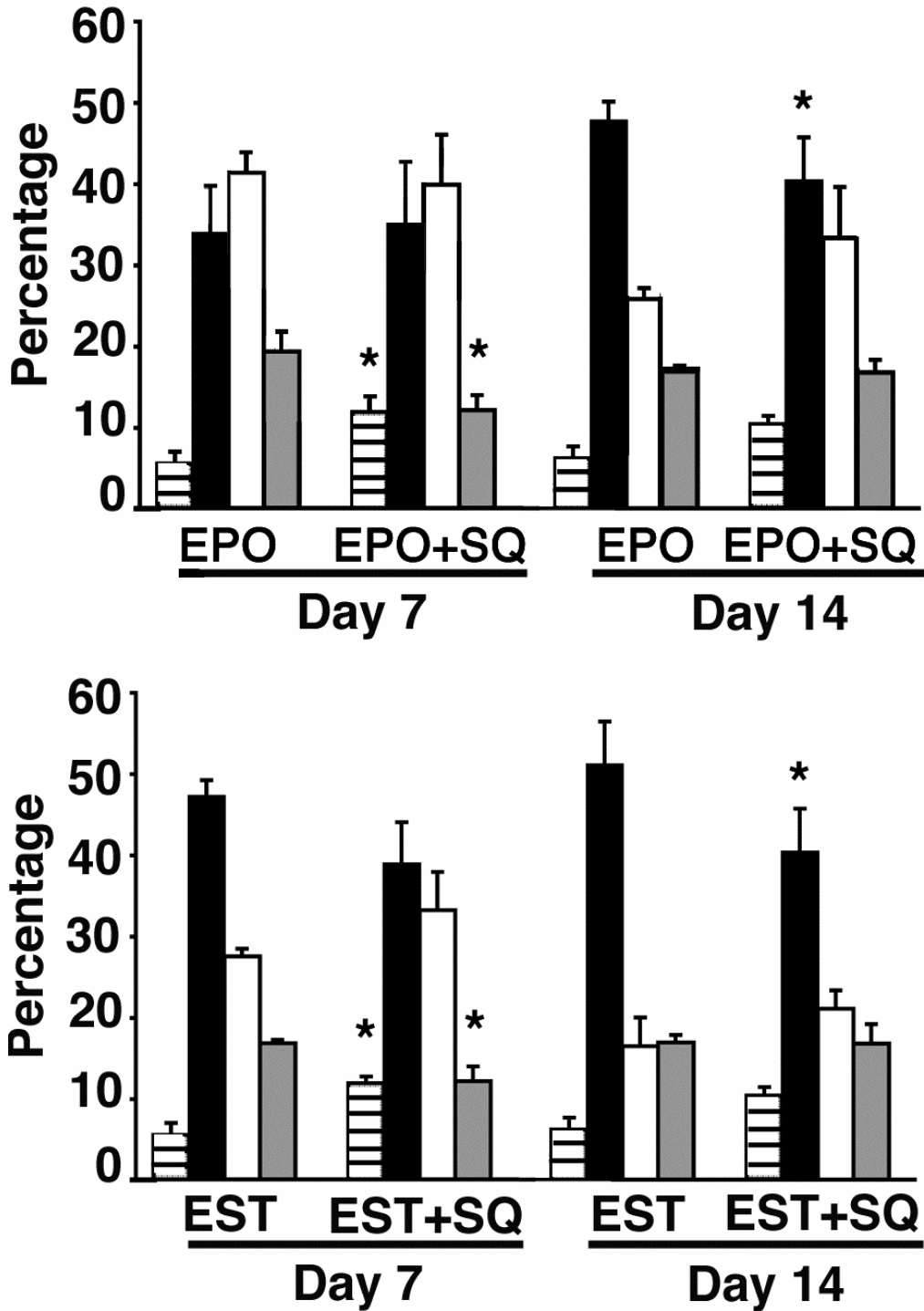
Bhanu et al., Figure 3



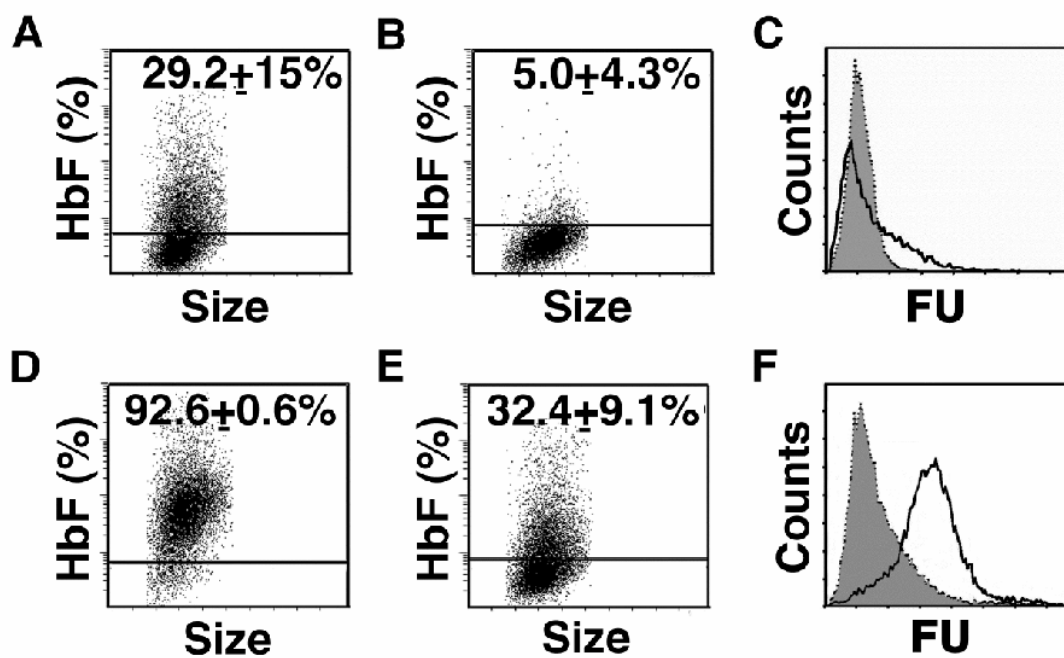
Bhanu et al., Figure 4



Bhanu et al., Figure 5

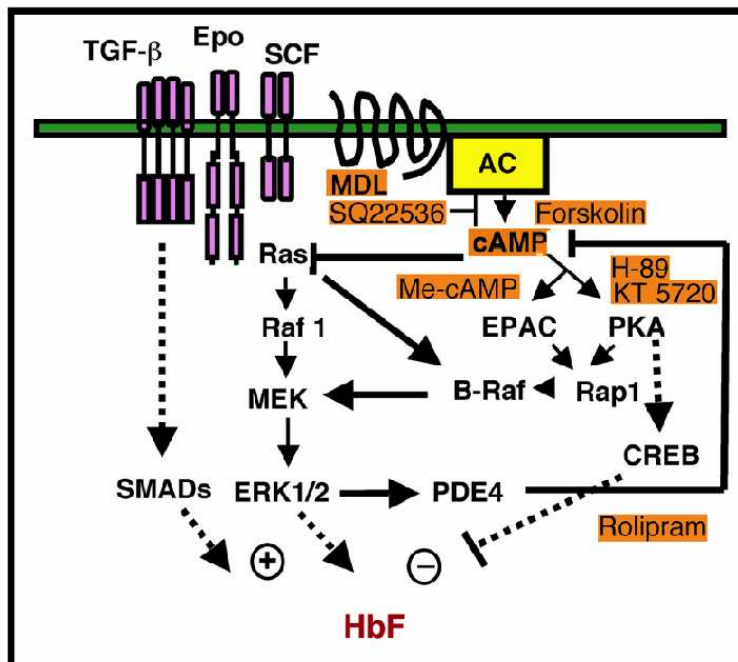


Bhanu et al., Figure 6

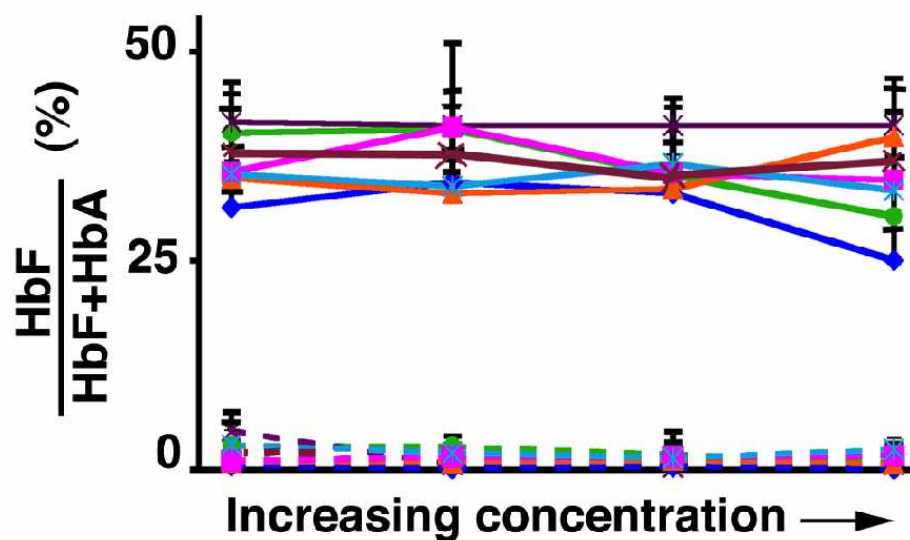


Bhanu et al., Figure 7

A



B



Bhanu et al., Figure 8

