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Study Title

# Effect of flumioxazin on heme synthesis in rat ES cell-derived embryonic erythroid cells.

Author

[redacted], M.Sc.

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Performing Laboratory

Environmental Health Science Laboratory,
Sumitomo Chemical Co., Ltd.
1-98, Kasugade-naka 3-chome, Konohana-ku, Osaka 554-8558, Japan

Laboratory Project ID

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## Signature

Author :

[redacted]

Date: Dec. 21. 2018

Environmental Health Science Laboratory,
Sumitomo Chemical Co., Ltd.

Study Director :

[redacted]

Date: Dec. 21. 2018

Environmental Health Science Laboratory,
Sumitomo Chemical Co., Ltd.

## Study location

Name : Environmental Health Science Laboratory,
Sumitomo Chemical Co., Ltd.
Address : 1-98, Kasugade-naka3-chome, Konohana-ku,
Osaka 554-8558, Japan

## Study schedule

Study initiate date : January 14, 2018
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## Personnel

Study director: [redacted], Ph.D.

Report preparation: [redacted], M. Sc.

Technical Staff
All Runs : [redacted]

Analysis Staff
All Runs : [redacted]

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# SUMMARY


Aim

To assess the relevance for humans of the developmental effects of flumioxazin observed in rats, comparative *in vitro* studies of the potential effect of flumioxazin on heme synthesis were carried out earlier on rat erythroid (REL) cells and human erythroid cells (K562 cell line, CD36+ cell line, and human induced pluripotent stem (hiPS) cell-derived embryonic erythroid cells). To evaluate the effects on rat primitive erythroids in a comparative manner to hiPS cell-derived primitive erythroids, we used rat embryonic stem (rES) cells to generate embryonic erythroid cells and evaluated the effects flumioxazin on heme synthesis, in comparison with a positive control, dihydroartemisinin (DHA).

Methods

rES cells were differentiated into primitive erythroid cells by using hematopoietic-inducing medium and rat erythropoietin, which is involved in erythroid differentiation. After 8 days in culture, they were exposed to vehicle only, flumioxazin, or a positive control, dihydroartemisinin (DHA). Heme synthesis, cell proliferation and protoporphyrin IX (PPIX; heme precursor) were measured for 4 days in rES cell-derived embryonic erythroid cells.

Results

The mRNA expression analysis and immunostaining analysis in rES cell-derived erythroid cells showed a high level of embryonic ε-globin expression, which is a characteristic of embryonic cells. Heme synthesis was reduced and accumulation of PPIX was observed in rat primitive erythroids when treated with flumioxazin in a dose-dependent manner. DHA, the positive control, caused a reduction in cell proliferation and a reduction in heme synthesis.

Conclusions

These results confirm that there is a clear qualitative difference between rat and human erythroid cells in their response to flumioxazin, in that there is no inhibition

of heme synthesis in hiPS cell-derived embryonic erythroid cells, like in the two types of human cells previously used, but there is a reduction in heme synthesis in newly obtained rES cell-derived embryonic erythroid cells, like in the REL cell line.

# Introduction

Fetal anemia from flumioxazin is caused by inhibition of heme synthesis resulting from inhibition of protoporphyrinogen oxidase (PPO), a key enzyme in heme synthesis. In teratology studies, flumioxazin induces developmental toxicity only in rats, not in rabbits[1].

To assess the relevance for humans of the developmental effects of flumioxazin observed in rats, comparative *in vitro* studies were carried out previously on human leukemic cell line K562 cells, human CD36+ cells derived from cord blood and rat erythroleukemia (REL) cell line to investigate the effects of flumioxazin on heme synthesis[2-4]. These showed no effect on heme synthesis in either of the human cell types, whereas heme synthesis in rat-derived REL cells was reduced in a dose-dependent manner by flumioxazin. Furthermore, we assessed the effects on human embryonic erythroid cells, which are equivalent to the cells produced in the rat during the critical period for flumioxazin's developmental toxicity in the rat. The human embryonic erythroid cells were derived from human induced pluripotent stem (hiPS) cells and there were no effects on heme content or cell proliferation, even when treated with 5 μM of flumioxazin, a concentration close to its water solubility limit[5].

In order to perform a comparable study using rat embryonic erythroid cells, we developed a differentiation method for embryonic erythroid cells from rat embryonic stem (rES) cells in this study. To evaluate the effect of flumioxazin on heme synthesis in rat primitive erythroids, rES cell-derived embryonic erythroid cells were exposed to flumioxazin or DHA (positive control).

# OBJECTIVE

In order to perform a comparable study using rat embryonic erythroid cells, we generated embryonic erythroid cells from rat embryonic stem (rES) cells which are capable of differentiating into every type of cell[6], including erythroids, and exposed rES cell-derived embryonic erythroid cells to flumioxazin or DHA (positive control). To examine the effect of flumioxazin on the heme synthetic pathway in rat primitive erythroids, the concentrations of heme and protoporphyrin IX (PPIX; heme precursor) were analyzed.

# MATERIALS AND METHODS

## 1. Materials

Dihydroartemisinin (DHA, *Lot No. 56MTB-FA*) was purchased from Tokyo Chemical Industry Co., Ltd. Flumioxazin (*Lot No. 080839G*) was obtained from Sumitomo Chemical Co., Ltd. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Co., Ltd.

## 2. Cells and culture conditions for REL cells

REL cells were provided by Institute of Molecular Genetics and Genetic Engineering (Prof. Popovic, Serbia). REL cells were cultured in Roswell Park Memorial Institute 1640 (RPMI) medium (*ThermoFisherScientific, 11875, Massachusetts, US*) supplemented with 40 % Fetal Bovine Serum (FBS, *Biosera, FB-1061/25, France*) and 100 IU/mL penicillin 100 μg/ml streptomycin (P/S; *Sigma-Aldrich, P0781-100ML, Missouri, US*) (*REL medium*) at 37 °C in a humidified chamber with 5 % $CO_2$ ($CO_2$ incubator). The cells were sub-cultured every other day.

REL cells were differentiated by culturing in REL medium containing 1.0 mM hexamethylene bisacetamide (HMBA; *Sigma-Aldrich, 224235-10G, Missouri, US*) for 8 days.

### 3. Rat ES cells maintenance and erythroid differentiation

Rat embryonic stem (rES) cell line (*named internally SMR01 line*) was provided by DS Pharma Biomedical Co., Ltd. (Japan). rES cells were maintained on mouse embryonic fibroblast cells (MEF*; reprocell, Japan*) in a seeded cell culture dish with StemMedium (*DS Pharma Biomedical, Japan*) supplemented with rat LIF (*Millipore, ESG2207, Massachusetts, US*) and undifferentiating factors, and passaged by dissociating into single cells using Accutase (*Innovative Cell Technologies, AT104, California, US)* twice a week.

Erythroid cells were obtained from rES cells using a differentiation medium A [redacted] for 3 days, followed by a differentiation medium B [redacted] for the next 5 days. Then, a differentiation medium C [redacted] was used from differentiation day 8 to day12.

### 4. Preparation of test solution

Flumioxazin and DHA were dissolved in DMSO to prepare a stock solution of 1,000 times higher concentration than the final test concentration. Differentiation medium C was used as base medium. Flumioxazin (1 or 5 mM) or DHA (0.5 mM) solution were mixed with differentiation medium C to prepare 1 μM and 5 μM flumioxazin medium, and 0.5 μM DHA medium. A single dose of 0.5 μM of DHA was adopted as the positive control because it was the highest dose within the acceptable level of the cell viability in the preliminary study and the number of erythroid cells was not sufficient to set 2 doses of DHA treatment. Differentiation medium C containing 0.1 % DMSO was used as a control.

### 5. Treatment of rES cell-derived erythroid cells with flumioxazin or DHA

The best time to start the exposure was determined to be from day 8, during a

preliminary study, based on the high expression of ε-globin and a satisfactory number of cells available. On differentiation day 8, rES cell-derived erythroid cells were collected and centrifuged at 1,000 rpm for 5 minutes, resuspended in differentiation medium C containing either 0.1% DMSO, flumioxazin (at 1 μM or 5 μM) or DHA (at 0.5 μM). Then, the cells were plated onto 6 well cell culture plates (*Corning, 353046, New York, US*) at $2.0 \times 10^5$ cells/1.5 mL/well, and were placed in a humidified incubator at 37 °C with 5 % $CO_2$.

On differentiation day 12, the floating cells were collected by pipetting. The heme synthesis and differentiation level were assessed within 4 days after treatment because rES cells-derived erythroid cells differentiate rapidly into β-globin expressing cells (Figure 3A).

Collected rES cell-derived erythroid cells were centrifuged at 1,000 rpm for 5 minutes and resuspended with phosphate buffered saline (*magnesium, calcium free*) (PBS(-); *ThermoFisherScientific, 10010-049*) to measure the cell densities. The number of living cells stained by trypan blue solution (*ThermoFisherScientific, T10282, Massachusetts, US*) was counted using a hemocytometer (*Erma, Japan*), and the cell viability was calculated.

After that, the cells were centrifuged again at 7,500 rpm for 3 minutes by tabletop centrifuge (*Centrifuge 5424; Eppendorf, Germany*) and approximately $0.3\text{-}1.0 \times 10^5$ cells were stored at -80 °C for the measurements of heme and PPIX.

For measurements of mRNA expression, approximately $0.8\text{-}1.0 \times 10^5$ cells were dissolved in RLT lysis buffer used with the RNeasy Micro Kit (*QIAGEN, 74004, Germany*) and stored at -80 °C at day 8, 10 and 12.

## 6. Immunohistochemistry

Immunocytochemistry was performed with the primary antibodies described below. The antibodies were used at the following dilutions: HBE1 (*GeneTex/GTX108396/rabbit/1:750*).

On differentiation days 8, 10 and 12, floating cells were collected with a pipette, and centrifuged at 7,500 rpm for 3 minutes by tabletop centrifuge (*Centrifuge 5424; Eppendorf, Germany*). Supernatant was removed, then cells were rinsed in PBS and fixed in 4% paraformaldehyde (*Wako, 163-20145, Japan*)/PBS for 15 min at room temperature (RT).

Next, the cells were rinsed in PBS and resuspended in PBS containing 2% Fetal

Bovine Serum (FBS; *Corning, 35-010-CV, Lot No. 35010118, New York, US*) to a concentration of approximately 6.7 x $10^4$ cells/ml. 300 µl of suspended cells were centrifuged onto a coated-glass slide (*ThermoFisherScientific, 5991056, Massachusetts, US*) using Cytospin4 (*ThermoFisherScientific, Massachusetts, US*) at 1,000 rpm for 3 min. These slides were dried at RT and stored at -80 °C.

For immunostaining of these slides, barriers were drawn around cells with ImmEdge Hydrophobic Barrier PAP Pen (*Vector Laboratories, H-4000, California, US*). The cells were permeabilized with 0.2% Triton X-100 (*Wako, 591-12191, Japan*)/ Tris-Buffered Saline (TBS; *Takara, T9141*) for 15 min at RT and were incubated with Superblock Blocking Buffer (*ThermoFisherScientific, 37581, Massachusetts, US*) for 30 min or more at RT. Primary antibodies were diluted with Antibody Diluent OP Quant (*ThermoFisherScientific, TA-125-ADQ, Massachusetts, US*) and applied on the slides. The slides were then incubated at 4 °C overnight.

Next day, the slides were washed three times with 0.05% Tween/TBS (*Takara, T9142, Japan*) for 5 min, and were incubated with donkey anti-rabbit IgG (H+L) Alexa Fluor 488 (*ThermoFisherScientific, A20206, Massachusetts, US*) for 1 hour at RT. Secondary antibody diluents were applied to centrifugal filters (*Ultrafree-MC, GV 0.22 µm; Millipore, UFC30GVNB, Massachusetts, US*) and centrifuged at 12,000 rpm for 1 min. 2 µg/ml Hoechst33342 (*DOJINDO, H342, Japan*) was added to the secondary antibody diluent for nuclear counterstain. The slides were again washed three times with 0.05% Tween/TBS for 5 min, and rinsed once with water. The slides were then mounted by cover glass with Prolong Diamond Antifade Mountant (*ThermoFisherScientific, P36970, Massachusetts, US*), and were dried in dark place at RT overnight.

Images were acquired with a fluorescence microscope (*Axioimager M2, Carl Zeiss, Germany*).

**7. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

rES cell-derived erythroid cells (days 10 and 12) and REL cells (days 0, 2, 4 and 8, same culture period used for the differentiation of REL cells into erythroid-like cells producing heme in the previous study[4]) were collected and mRNA expression levels of β-like globins, including ε-globin (Hbe1, embryonic), γ–globin (Hbg1, embryonic and fetal) and β-globin (Hbb, adult) [10] were evaluated. Total RNA was isolated using RNeasy Micro Kit (*QIAGEN, 74004, Germany*). Complementary DNA

(cDNA) was obtained using Superscript III First-Strand Synthesis System (*ThermoFisherScientific, 18080-085, Massachusetts, US*) in the Veriti 96-Well Thermal Cycler (*ThermoFisherScientific, Massachusetts, US*).

Quantitative PCR assays were performed using gene specific Taqman Gene Expression Assays (*ThermoFisherScientific, Massachusetts, US*) to evaluate mRNA expression levels for ε-globin (*Hbe1, Rn01754780_g1*), γ–globin (*Hbg1, Rn00756487_mH*), and β-globin (*Hbb, Rn00583657_g1*) in the StepOnePlus Realtime PCR system (*ThermoFisherScientific, Massachusetts, US*).

To compare the expression level of each globin, absolute quantification was performed. The copy number of each mRNA was calculated with the standard curves obtained from the diluted amplicons which were amplified by PCR using primers containing the region of the target sequence of each Taqman assay primers. The following primers were used:

Hbe1 forward primer, 5'- ACTGCTGAGGAAAAGAGCCT -3';
Hbe1 reverse primer, 5'- TTGCCGAAGTGACTAGCCAG -3';
Hbg1 forward primer, 5'- GGTTCACTTCACAGCTGAGGA -3';
Hbg1 reverse primer, 5'- AACATGTTGCCCAGGAGCTT -3';
Hbb forward primer, 5'- CCATGGTGCACCTGACTGAT -3';
Hbb reverse primer, 5'- TGAATTCCTTGCCCAGGTGG -3'.

**8. Extraction and purification of PPIX and heme from the cells**

The PPIX and heme were extracted according to the previously described method[7,8]. The method was slightly improved by a solid phase extraction to improve the purity of the samples. After thawing cells at room temperature, 700 μL of basic methanol (methanol/0.1 M ammonia solution = 9/1, v/v) and internal standards were added to the tubes. As the internal standards for PPIX and heme, 10 ng/mL of protoporphyrin IX-$d_4$ (PPIX-$d_4$) and 500 ng/mL of deuteroporphyrin IX (DP) were used. Then, the tubes were vortexed for 30 seconds, sonicated for 30 seconds, vortexed again, and centrifuged at 15,000 rpm, 4 °C for 15 minutes.

Obtained supernatants were purified with an Oasis MAX μElution Plate (*Waters Corporation, Massachusetts, US*). Firstly, the purification plate was equilibrated with 400 μL of methanol and water using the positive pressure manifold. Then 700 μL of the supernatants were loaded into the wells, and the extracts were trapped to the solid phase. After being washed with 400 μL of 5%

ammonia solution, 200 μL of methanol and 200 μL of hexane, PPIX, heme and their internal standards were eluted with 100 μL of methanol containing 2% formic acid. The eluted samples were analyzed by LC/MS.

**9. Quantification of PPIX and heme in the cell extracts**

Concentrations of PPIX and heme in the purified samples were analyzed by LC/MS according to the previously described method[9], with minor modifications in the HPLC conditions, as follows:

<LC condition>

Pump: LC-20AD (*Shimadzu Co.*)

UV Detector: SPD-20A (*Shimadzu Co.*)

Autosampler: SIL-20AC HT (*Shimadzu Co.*)

Column: YMC-Pack Pro C4 (*3 μm, 3.0 mm × 50 mm, YMC Co., Ltd.*)

Mobile Phase: A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile

Gradient Conditions:

| Time (min) | A (%) | B (%) |
|---|---|---|
| 0.0 | 90 | 10 |
| 0.5 | 90 | 10 |
| 0.8 | 65 | 35 |
| 1.2 | 65 | 35 |
| 2.0 | 0 | 100 |
| 7.0 | 0 | 100 |

Flow Rate: 0.3 mL/min

UV Wavelength: 400 nm

Temperature: Ambient

<MS condition>

Mass Spectrometer: TSQ Vantage (*Thermo Fisher Scientific Inc.*)

Ionization: HESI (positive)

Scan mode: Selected reaction monitoring (SRM)

<Monitored peak>

PPIX : m/z = 563.3/504.4, CE = 34, Rt = 4.5 min

PPIX-$d_4$ : m/z = 567.3/507.9, CE = 35, Rt = 4.5 min

| | | | |
|---|---|---|---|
| Heme | : | UV, wavelength = 400 nm, | Rt = 3.6 min |
| DP | : | UV, wavelength = 400 nm, | Rt = 4.2 min |

PPIX and heme were analyzed and quantitated with MS and UV, respectively. As with the cell extract samples, calibration samples were also analyzed. These samples were prepared by adding a small volume of the serial dilutions of PPIX and heme into untreated cell extracts, and purifying in the same manner. After the LC/MS analysis, the peak areas of the analyses in the samples were computed and quantified using Thermo Xcalibur 2.2 (*Thermo Fisher Scientific Inc.*).

The concentration of PPIX and heme were calculated with the regression line obtained from the calibration samples.

**10. Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 5 (*GraphPad Software, CA, USA*). The averages in various conditions were evaluated by Dunnett's test. Standard errors of the mean are shown as error bars in all figures. The sample sizes (n) were defined by biological replicates.

# RESULTS

**Erythroid differentiation from rat ES cells and analyses of globin type expressed in rES cell-derived erythroid cells (Figures 1, 2, and 3; Tables 1 and 2; Appendices 1 and 2)**

Rat ES cells were differentiated into erythroid cells by using hematopoietic inducing medium, rat erythropoietin and rat stem cell factor (SCF). Through the 8 days of differentiation, floating blood cells emerged. The pellets of produced cells showed dark red color, which indicates a high level of heme synthesis (Figure 1).

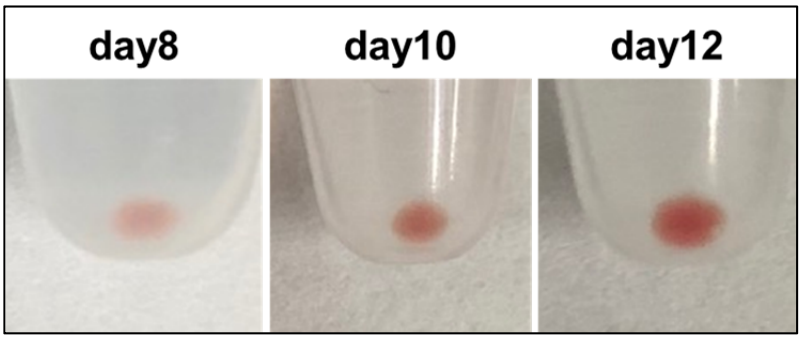


**Figure 1. Heme synthesis in rES cell-derived erythroid cells.**
Erythroid cells derived from rES cells were collected and centrifuged to pellet at days 8, 10 and 12. Increasing red color indicates a continuous production of high level of heme.

In addition, most floating blood cells expressed embryonic ε-globin, which is characteristic of embryonic erythroid cells[10], at days 8, 10 and 12 in immunostaining analyses (Figure 2).

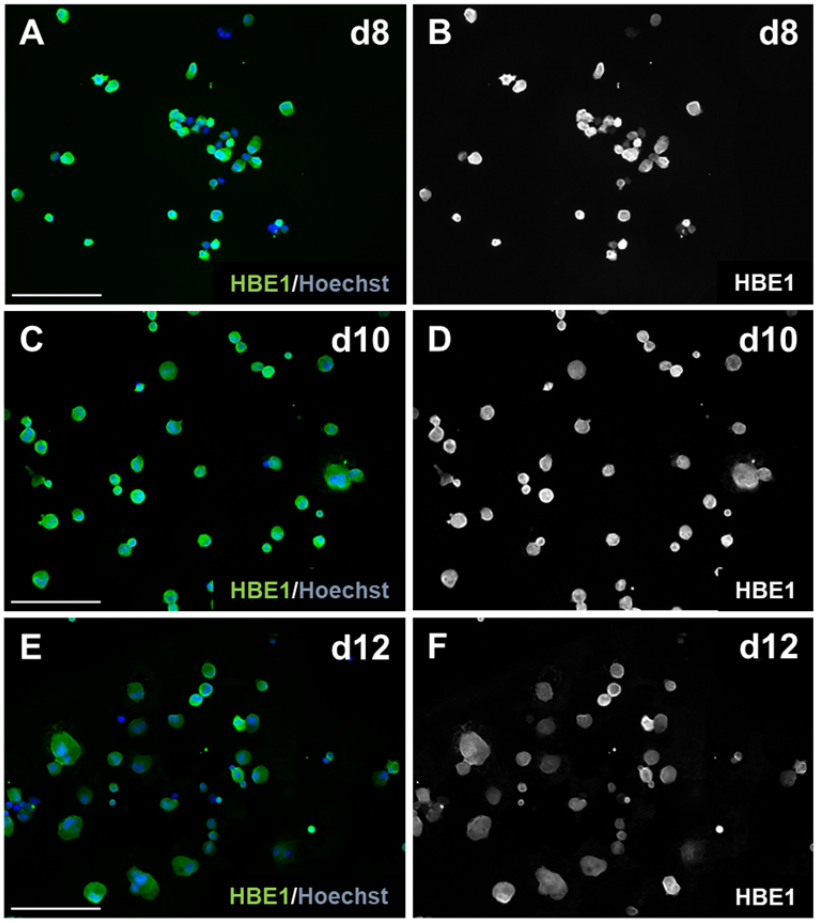


**Figure 2. Expression of the HBE1 in rES cell-derived erythroid cells.**
(A-F) Immunostaining of HBE1 in erythroid cells from differentiation days 8 (A, B), 10 (C, D), and 12 (E, F). Stains are for HBE1 (A, C, E; green) with nuclear staining with Hoechst 33342 (dark blue) and HBE1 only (B, D, F; white). Most cells expressed HBE1 from day 8 to day12. (Scale bar: 100 μm)

Moreover, quantitative mRNA expression analysis revealed that the globin expression level in rES cell-derived erythroid cells showed a high percentage (>60%) of embryonic ε-globin thorough the 12 days differentiation (Figure 3A; Table 2; Appendix 2). In contrast, erythroid cells differentiated from REL cell line expressed virtually no ε-globin mRNA (Figure 3B; Table 1; Appendix 1).

The higher level of e-globin expression in erythroids derived from rES cells indicated that rES cell-derived erythroid cells could be considered as embryonic erythroid cells and are the closest to the rat primitive erythroid cells.

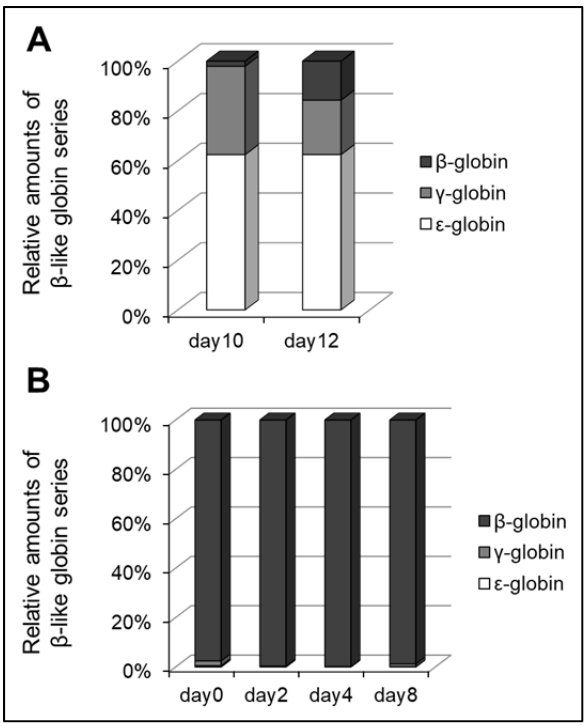


**Figure 3. Differentiation types of rES cell-derived erythroid cells and REL cells.**
(A, B) Relative RNA expression levels of ε-, γ-, and β-globin in rES cell-derived erythroid cells (A) and REL cells (B) analyzed by quantitative RT-PCR. (n = 3)

**Evaluation of effects of flumioxazin on heme synthesis in rES cell-derived embryonic erythroid cells (Figure 4; Table 3; Appendix 3)**

To evaluate effects of flumioxazin on heme synthesis in rat primitive erythroid cells, rES cell-derived embryonic erythroid cells were exposed to flumioxazin or DHA (positive control) for 4 days. As a result, heme synthesis was reduced and accumulation of PPIX was observed when treated with flumioxazin in a

dose-dependent manner. DHA, the positive control, caused a reduction in cell proliferation and a reduction in heme synthesis in rES cell-derived embryonic erythroid cells (Figure 4; Table 3; Appendix 3).

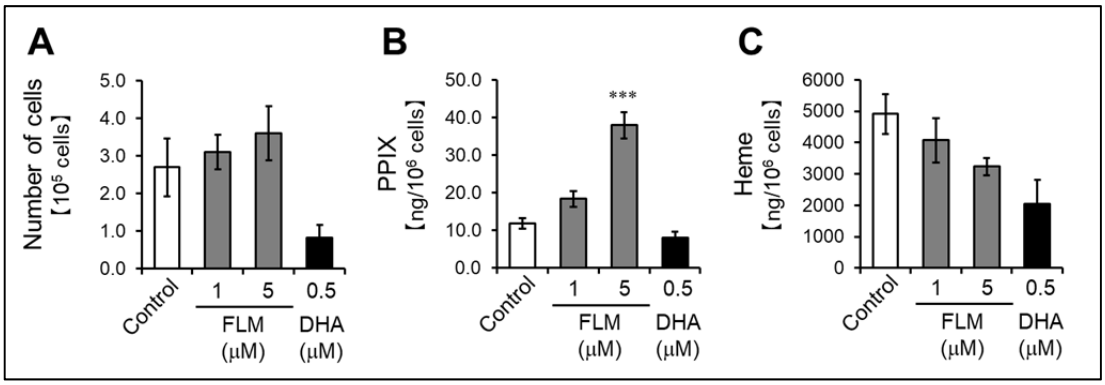


**Figure 4. Evaluation of effects of flumioxazin on heme synthesis in rES cell-derived embryonic erythroid cells.**
rES cell-derived erythroid cells were cultured in differentiation medium C from differentiation days 8. Erythroid cells were exposed to flumioxazin (1 or 5 μM), DHA (0.5 μM) or 0.1% DMSO (Control). Erythroid cells were sampled at day 12 and the number of living cells were counted (A). Then, the cells were analyzed by LC-MS for heme synthetic pathway products, PPIX (B) and heme (C), as described in the Materials and Methods. The concentration of PPIX and heme are shown per $10^6$ erythroid cells. (n = 3).
Values significantly different from control (DMSO only treated) are: ***$p<0.001$.
Abbreviations: DMSO, dimethyl sulfoxide, DHA, dihydroartemisinin, FLM, flumioxazin, PPIX, protoporphyrin IX.

# DISCUSSION

In this study, rat ES cells were differentiated into erythroid cells to perform a comparable study to that using human primitive erythroid cells[5]. The cells obtained showed dark red color which indicates a high level of heme synthesis (Figure 1). In addition, most cells derived from rES cells expressed embryonic ε-globin, which is characteristic of embryonic erythroid cells[10], in immunostaining analysis (Figure 2). Furthermore, their β-like globin expression level showed a high percentage (>60%) of embryonic ε-globin on days 10 and 12 (Figure 3). These results indicate that rat ES cell-derived erythroid cells are predominantly embryonic erythroid cells at least by differentiation day 12.

From the heme synthesis assay using rES cell-derived embryonic erythroid cells, heme synthesis was reduced and accumulation of PPIX was observed when treated

flumioxazin in a dose-dependent manner. DHA, the positive control, caused a reduction in cell proliferation and a reduction in heme synthesis in rat embryonic erythroid cells (Figure 4). Every experiment showed a reduction of heme synthesis after treatment with flumioxazin or DHA, compared with the control (Appendix 3), although the effect on heme synthesis was not statistically significant due to the limited number of biological replicates (n = 3).

These results, showing inhibition of heme synthesis in rES cell-derived embryonic erythroid cells treated with flumioxazin, are consistent with those previously reported using REL cells[4]. In human origin K562 cells, CD36+ cells and human induced pluripotent stem cell-derived embryonic erythroid cells, on the contrary, there were no effects on heme synthesis or cell proliferation, even when treated with 5 μM of flumioxazin, a concentration close to its water solubility limit[2-5].

Taken together, these results show a clear qualitative difference between human and rat erythroid cells in their response to flumioxazin, with no inhibition of heme synthesis in three different types of human erythroid cells but a reduction in heme synthesis in rat erythroid cells.

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# TABLES

**Table 1 Relative RNA expression levels of β-like globins in REL cells.**

| Genes | Days of differentiation | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| ε-globin(Hbe1) | 0.01 ± 0.001 (3) | 0.00 ± 0.000 (3) | 0.00 ± 0.000 (3) | 0.00 ± 0.000 (3) |
| γ-globin(Hbg1) | 0.02 ± 0.002 (3) | 0.00 ± 0.000 (3) | 0.00 ± 0.000 (3) | 0.01 ± 0.001 (3) |
| β-globin(Hbb) | 0.98 ± 0.002 (3) | 1.00 ± 0.000 (3) | 1.00 ± 0.000 (3) | 0.99 ± 0.002 (3) |

The copy number of each mRNA was calculated with the standard curves obtained from the diluted amplicons.
Relative amounts were calculated by dividing each expression level by the total β-like globin expression.

**Table 2 Relative RNA expression levels of β-like globins in rES cell-derived erythroid cells.**

| Genes | Days of differentiation | |
|---|---|---|
| | 10 | 12 |
| ε-globin(Hbe1) | 0.62 ± 0.016<br>(3) | 0.62 ± 0.034<br>(3) |
| γ-globin(Hbg1) | 0.36 ± 0.014<br>(3) | 0.22 ± 0.010<br>(3) |
| β-globin(Hbb) | 0.02 ± 0.005<br>(3) | 0.16 ± 0.024<br>(3) |

The copy number of each mRNA was calculated with the standard curves obtained from the diluted amplicons.
Relative amounts were calculated by dividing each expression level by the total β-like globin expression.

**Table 3 Effects of flumioxazin on rES cell-derived embryonic erythroid cells after 4 days treatment (Mean).**

| Group and dose | Number of cells ($\times 10^5$ cells) (n) | PPIX concentration (ng/$10^6$ cells) (n) | Heme concentration (ng/$10^6$ cells) (n) | Relative cell viability (% of Control) (n) |
|---|---|---|---|---|
| Control (DMSO 0.1%) | 2.7 ± 0.77 (3) | 11.8 ± 1.35 (3) | 4908 ± 625.7 (3) | 1.0 ± 0.00 (3) |
| Flumioxazin 1 μM | 3.1 ± 0.46 (3) | 18.3 ± 2.17 (3) | 4069 ± 704.7 (3) | 1.2 ± 0.11 (3) |
| Flumioxazin 5 μM | 3.6 ± 0.72 (3) | 37.9 ± 3.54 *** (3) | 3231 ± 283.4 (3) | 1.2 ± 0.12 (3) |
| Dihydroartemisinin 0.5 μM | 0.8 ± 0.33 (3) | 7.9 ± 1.73 (3) | 2057 ± 744.3 (3) | 0.7 ± 0.18 (3) |

Values significantly different from control (DMSO only treated) are: ***$p<0.001$.

# APPENDICES

**Appendix 1　Relative RNA expression levels of β-like globins in REL cells.**

| Genes | Run No. | Days of differentiation | | | |
|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 8 |
| ε-globin(Hbe1) | 1 | 0.01 | 0.00 | 0.00 | 0.00 |
| | 2 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 3 | 0.01 | 0.00 | 0.00 | 0.00 |
| γ-globin(Hbg1) | 1 | 0.02 | 0.00 | 0.00 | 0.01 |
| | 2 | 0.02 | 0.00 | 0.00 | 0.01 |
| | 3 | 0.02 | 0.00 | 0.00 | 0.01 |
| β-globin(Hbb) | 1 | 0.97 | 1.00 | 1.00 | 0.99 |
| | 2 | 0.98 | 1.00 | 1.00 | 0.99 |
| | 3 | 0.98 | 1.00 | 1.00 | 0.99 |

The copy number of each mRNA was calculated with the standard curves obtained from the diluted amplicons.
Relative amounts were calculated by dividing each expression level by the total β-like globin expression.

**Appendix 2　Relative RNA expression levels of β-like globins in rES cell-derived erythroid cells**

| Genes | Run No. | Days of differentiation | |
|---|---|---|---|
| | | 10 | 12 |
| ε-globin(Hbe1) | 1 | 0.65 | 0.65 |
| | 2 | 0.60 | 0.56 |
| | 3 | 0.62 | 0.66 |
| γ-globin(Hbg1) | 1 | 0.33 | 0.21 |
| | 2 | 0.38 | 0.24 |
| | 3 | 0.36 | 0.21 |
| β-globin(Hbb) | 1 | 0.02 | 0.14 |
| | 2 | 0.03 | 0.20 |
| | 3 | 0.01 | 0.13 |

The copy number of each mRNA was calculated with the standard curves obtained from the diluted amplicons.
Relative amounts were calculated by dividing each expression level by the total β-like globin expression.

**Appendix 3 Effects of flumioxazin on rES cell-derived embryonic erythroid cells after 4 days treatment.**

| Group and dose | Run No. | Number of cells ($\times 10^5$ cells) | PPIX concentration (ng/$10^6$ cells) | Heme concentration (ng/$10^6$ cells) | Relative cell viability (% of Control) |
|---|---|---|---|---|---|
| Control (DMSO 0.1%) | 1 | 2.8 | 11.8 | 5231 | 1.0 |
| | 2 | 4.0 | 9.5 | 3699 | 1.0 |
| | 3 | 1.4 | 14.1 | 5792 | 1.0 |
| Flumioxazin 1 μM | 1 | 2.8 | 21.6 | 5479 | 1.1 |
| | 2 | 4.0 | 19.1 | 3389 | 1.1 |
| | 3 | 2.5 | 14.2 | 3341 | 1.4 |
| Flumioxazin 5 μM | 1 | 3.6 | 37.1 | 3757 | 1.0 |
| | 2 | 4.9 | 44.4 | 2785 | 1.1 |
| | 3 | 2.4 | 32.2 | 3151 | 1.4 |
| Dihydroartemisinin 0.5 μM | 1 | 0.4 | 11.3 | 3517 | 0.3 |
| | 2 | 1.5 | 5.8 | 1074 | 0.8 |
| | 3 | 0.7 | 6.6 | 1580 | 0.9 |