DR. NATEE JEARAWIRIYAPAISARN (Orcid ID : 0000-0002-1290-5603)

Article type : Letters

High level induction of fetal haemoglobin by pomalidomide in β-thalassaemia/HbE erythroid progenitor cells

Running Title: High HbF induction by pomalidomide

Pinyaphat Khamphikham, [1,2] Tiwaporn Nualkaew, [1] Phitchapa Pongpaksupasin, [1,3] Woratree Kaewsakulthong, [1,3] Duantida Songdej, [4] Kittiphong Paiboonsukwong, [1] James Douglas Engel, [5] Suradej Hongeng, [4] Suthat Fucharoen, [1] Orapan Sripichai [6] and Natee Jearawiriyapaisarn [1]

[1]Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand

[2]Department of Forensic Science, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani, Thailand

[3]Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[4]Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

[5]Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

[6]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand

Correspondence: Natee Jearawiriyapaisarn,
Thalassemia Research Center, Institute of Molecular Biosciences,
Mahidol University,
25/25 Phuttamonthon 4 Road, Salaya, Nakhon Pathom 73170, Thailand.
E-mail: natee.jea@mahidol.edu
Tel. (66)2889-2557, (66)2889-2558 Fax. (66)28892559

Word count: 1,109

Number of figures: 2 (6 supplemental figures, 3 supplemental tables, supplemental methods and supplemental text)

Number of references: 17

TO THE EDITOR:

Studies have shown that increased expression of fetal haemoglobin (HbF; $\alpha_2\gamma_2$) can ameliorate red blood cell deficiencies in patients with β-thalassaemia and sickle cell disease (SCD) (Nuinoon, *et al* 2010, Sankaran and Orkin 2013, Uda, *et al* 2008). Pharmacological induction of HbF expression in β-thalassaemia has been investigated using several classes of small molecules (Musallam, *et al* 2013), including 5-azacytidine (Ley, *et al* 1982), decitabine (Olivieri, *et al* 2011), hydroxyurea (Fucharoen, *et al* 1996), LSD1 inhibitors (tranylcypromine and RN-1) (Cui, *et al* 2015, Shi, *et al* 2013), and short chain fatty acid derivatives (Fucharoen, *et al* 2013, Patthamalai, *et al* 2014). Among these molecules, hydroxyurea (HU) is the only U.S. Food and Drug Administration (FDA) currently approved drug for the treatment of SCD and/or β-thalassaemia. However, HU has shown modest and variable responses with potential myelosuppression in β-thalassaemia patients. Therefore, more robust and safer HbF therapeutics are highly desired.

Pomalidomide, an FDA-approved immunomodulatory drug for the treatment of multiple myeloma (Bartlett, *et al* 2004, Lacy and McCurdy 2013), stimulates γ-globin mRNA and HbF expression in erythroid progenitor cells by downregulating factors involved in γ-globin repression including BCL11A, SOX6, GATA1, KLF1, and LSD1 (Dulmovits, *et al* 2016, Meiler, *et al* 2011, Moutouh-de Parseval, *et al* 2008). In addition, treatment of a humanized mouse model of SCD with pomalidomide induced comparable HbF expression to hydroxyurea but without myelosuppressive effects (Meiler, *et al* 2011).

Here, we investigated the therapeutic potential of pomalidomide and its combinatorial effects with other HbF inducers, including hydroxyurea, decitabine and RN-1, in erythroid

progenitor cells from compound heterozygous β^0-thalassaemia/HbE (*HBB*:c.79G>A) patients (Table SI) using a 3-phase liquid culture system that supports terminal maturation of erythroid cells (Supplemental Methods, Text and Figure S1). Comparison of results using optimal conditions for each compound (Figures S2-S3) revealed that pomalidomide was much more effective in inducing HbF expression than hydroxyurea, decitabine or RN-1 (Fig 1A, B). The greatest increase in HbF percentage from baseline level was observed in pomalidomide-treated cells, achieving 25.6 ± 1.1% as determined by high performance liquid chromatography (HPLC) (Fig 1A, B). β^0-thalassaemia/HbE precursors from patients of different β^0-thalassemic mutations (Table SI) showed similarly increased levels of HbF induction in response to pomalidomide treatment. This result suggested that deficient progenitors, regardless of specific β^0-thalassemic mutation or baseline HbF level, are all susceptible to strong induction with pomalidomide (Fig 1A, B and Table SI and SII). The percentage of cells expressing HbF (F cells) increased from 49.8 ± 4.7% for DMSO controls to 60.6 ± 2.5% after pomalidomide treatment (Figure S4). By quantitative RT-PCR, we found that pomalidomide significantly increased γ-globin (*HBG*) mRNA expression, achieving a 2.3 ± 0.3-fold increase over control cells, with coincidentally diminished β-globin (*HBB*) expression without significant change in α-globin (*HBA*) expression (Fig 1C).

To enhance the level of HbF induction, we investigated the effects of combinatorial treatment of pomalidomide either with or without other pharmacological HbF inducers. The combination of pomalidomide and decitabine had an additive effect on induction as shown by the differential HbF level (Δ%HbF = 36.7 ± 1.3) when compared to treatment with any single agent (Fig 1A, B). Hydroxyurea did not generate any additional increase in HbF when combined with pomalidomide. The combination of pomalidomide and RN-1 did increase the percentage of HbF (Fig 1A, B) and at the same time reduced *HBA*, *HBB*, and *HBG* mRNA expression (Fig 1C), suggesting that this combination negatively affected total globin mRNA expression. Taken together, these results suggest that pomalidomide and decitabine act through independent pathways to additively induce high level HbF expression, implying cooperative therapeutic potential for the treatment of β-thalassaemia.

We next determined the cytotoxicity of treatments and found that pomalidomide did not significantly affect erythroid cell proliferation (Figure S5A) or viability (Figure S5B). However, pomalidomide plus decitabine showed a reduction in cell proliferation at day 12 of culture without affecting cell viability. Erythroid cell proliferation and viability were significantly reduced in cells exposed to pomalidomide plus RN-1 (Figure S5A, B),

suggesting toxicity of the latter combination. Analysis of erythroid differentiation of cells treated with hydroxyurea or pomalidomide plus hydroxyurea was similar to that of DMSO-treated cells (Fig 2A, B), suggesting that these treatments did not affect erythroid terminal differentiation. We noted a trend toward increased differentiation of cells treated with pomalidomide, RN-1, and pomalidomide plus RN-1 compared with the controls. Interestingly, significantly accelerated erythroid differentiation was observed in decitabine alone and pomalidomide plus decitabine as evidenced by elevated $CD71^{medium}/GPA^{high}$ population and decreased $CD71^{high}/GPA^{high}$ cells (Fig 2A, B). Similarly, modified Giemsa-stained cytospins showed an increased number of late-stage erythroblasts in cells exposed to decitabine alone and pomalidomide plus decitabine when compared to control cells, indicating a shift toward normal erythroid cell maturation (Fig 2C and Figure S1). These results suggested that the differentiation of β^0-thalassaemia/HbE progenitor cells significantly improved after treatment with either decitabine alone or pomalidomide plus decitabine.

To investigate the effects of pomalidomide plus or minus these effectors on transcriptional regulation in β-thalassaemic erythroid progenitor cells, quantitative RT-PCR analyses revealed that one key γ-globin repressor mRNA, *BCL11A,* was only slightly reduced after treatment with pomalidomide or pomalidomide plus hydroxyurea. *BCL11A* was significantly downregulated (by 1.8- and 5.6-fold) after treatment with pomalidomide plus decitabine or pomalidomide plus RN-1, respectively (Fig 2D). Moreover, the expression of *SOX6*, *GATA1*, *HBS1L*, and *LRF* were modestly but significantly downregulated by pomalidomide, whereas other erythroid regulators were unaffected (Fig 2D and Figure S6). In addition, combined pomalidomide and decitabine treatment, which showed additive effects on HbF induction, reduced the expression of *KLF1*, *LSD1*, and *CHD4*. The combination of pomalidomide plus RN-1 significantly affected the expression of several key regulators, including *KLF1*, *SOX6*, *GATA1*, *HBS1L*, *DNMT1*, *LSD1*, *ID2*, *CHD4*, *FOXO3*, *NRF2*, and *MYB* (Fig 2D and Figure S6), consistent with the fact that this same combination significantly reduced cell proliferation and viability (Figure S5). Taken together, these results indicate that the mechanisms of action of pomalidomide and several coeffectors in induction of HbF expression partly involve transcriptional regulation of key HbF repressors and/or corepressors.

In summary, the present data show that pomalidomide is a potent HbF inducer and is more potent than hydroxyurea. The combination of pomalidomide and decitabine provide additive effects in inducing HbF expression in erythroid cells from β^0-thalassaemia/HbE patients.

Despite these promising results, it must be emphasized that the potential risks associated with the use of pomalidomide include developmental defects (if taken during pregnancy), thrombosis and pancytopenia (Miguel*, et al* 2013), which are similar to toxicities of parental drugs, lenalidomide and thalidomide. Development of pomalidomide structural refinements or analogues with similar biological effects may lead to future fully effective, reduced adverse effects and possible clinical application.

Acknowledgements

The authors would like to thank the patients and their families for their contributions to this study; and Thongperm Munkongdee, Nattrika Buasuwan, and Nurmeeha Hinna for their assistance with the DNA diagnosis for thalassaemia and haemoglobin analysis. The technical assistance of Greggory Myers is greatly appreciated. This work was supported by grants from Mahidol University, the Thailand Research Fund and the Office of the Higher Education Commission (MRG5680092) to N.J. P.P was supported by the Siriraj Graduate Scholarship.

Author contributions

Contribution: P.K., T.N., O.S., and N.J. designed the research; P.K., T.N., P.P., and W.K. performed experiments; P.K., T.N., and N.J. analyzed data; D.S., K.P., S.H., and S.F. provided samples and resources; P.K., J.D.E., and N.J. wrote the manuscript; J.D.E., S.H., S.F., O.S., and N.J. conceptualized the idea and supervised the project; and all authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

References

Bartlett, J.B., Dredge, K. & Dalgleish, A.G. (2004) The evolution of thalidomide and its IMiD derivatives as anticancer agents. *Nat Rev Cancer,* **4,** 314-322.

Cui, S., Lim, K.C., Shi, L., Lee, M., Jearawiriyapaisarn, N., Myers, G., Campbell, A., Harro, D., Iwase, S., Trievel, R.C., Rivers, A., DeSimone, J., Lavelle, D., Saunthararajah, Y. & Engel, J.D. (2015) The LSD1 inhibitor RN-1 induces fetal hemoglobin synthesis and reduces disease pathology in sickle cell mice. *Blood,* **126,** 386-396.

Dulmovits, B.M., Appiah-Kubi, A.O., Papoin, J., Hale, J., He, M., Al-Abed, Y., Didier, S., Gould, M., Husain-Krautter, S., Singh, S.A., Chan, K.W., Vlachos, A., Allen, S.L.,

Taylor, N., Marambaud, P., An, X., Gallagher, P.G., Mohandas, N., Lipton, J.M., Liu, J.M. & Blanc, L. (2016) Pomalidomide reverses gamma-globin silencing through the transcriptional reprogramming of adult hematopoietic progenitors. *Blood,* **127,** 1481-1492.

Fucharoen, S., Inati, A., Siritanaratku, N., Thein, S.L., Wargin, W.C., Koussa, S., Taher, A., Chaneim, N., Boosalis, M., Berenson, R. & Perrine, S.P. (2013) A randomized phase I/II trial of HQK-1001, an oral fetal globin gene inducer, in beta-thalassaemia intermedia and HbE/beta-thalassaemia. *Br J Haematol,* **161,** 587-593.

Fucharoen, S., Siritanaratkul, N., Winichagoon, P., Chowthaworn, J., Siriboon, W., Muangsup, W., Chaicharoen, S., Poolsup, N., Chindavijak, B., Pootrakul, P., Piankijagum, A., Schechter, A.N. & Rodgers, G.P. (1996) Hydroxyurea increases hemoglobin F levels and improves the effectiveness of erythropoiesis in beta-thalassemia/hemoglobin E disease. *Blood,* **87,** 887-892.

Lacy, M.Q. & McCurdy, A.R. (2013) Pomalidomide. *Blood,* **122,** 2305-2309.

Ley, T.J., DeSimone, J., Anagnou, N.P., Keller, G.H., Humphries, R.K., Turner, P.H., Young, N.S., Keller, P. & Nienhuis, A.W. (1982) 5-azacytidine selectively increases gamma-globin synthesis in a patient with beta+ thalassemia. *N Engl J Med,* **307,** 1469-1475.

Meiler, S.E., Wade, M., Kutlar, F., Yerigenahally, S.D., Xue, Y., Moutouh-de Parseval, L.A., Corral, L.G., Swerdlow, P.S. & Kutlar, A. (2011) Pomalidomide augments fetal hemoglobin production without the myelosuppressive effects of hydroxyurea in transgenic sickle cell mice. *Blood,* **118,** 1109-1112.

Miguel, J.S., Weisel, K., Moreau, P., Lacy, M., Song, K., Delforge, M., Karlin, L., Goldschmidt, H., Banos, A., Oriol, A., Alegre, A., Chen, C., Cavo, M., Garderet, L., Ivanova, V., Martinez-Lopez, J., Belch, A., Palumbo, A., Schey, S., Sonneveld, P., Yu, X., Sternas, L., Jacques, C., Zaki, M. & Dimopoulos, M. (2013) Pomalidomide plus low-dose dexamethasone versus high-dose dexamethasone alone for patients with relapsed and refractory multiple myeloma (MM-003): a randomised, open-label, phase 3 trial. *Lancet Oncol,* **14,** 1055-1066.

Moutouh-de Parseval, L.A., Verhelle, D., Glezer, E., Jensen-Pergakes, K., Ferguson, G.D., Corral, L.G., Morris, C.L., Muller, G., Brady, H. & Chan, K. (2008) Pomalidomide and lenalidomide regulate erythropoiesis and fetal hemoglobin production in human CD34+ cells. *J Clin Invest,* **118,** 248-258.

Musallam, K.M., Taher, A.T., Cappellini, M.D. & Sankaran, V.G. (2013) Clinical experience with fetal hemoglobin induction therapy in patients with beta-thalassemia. *Blood,* **121,** 2199-2212; quiz 2372.

Nuinoon, M., Makarasara, W., Mushiroda, T., Setianingsih, I., Wahidiyat, P.A., Sripichai, O., Kumasaka, N., Takahashi, A., Svasti, S., Munkongdee, T., Mahasirimongkol, S., Peerapittayamongkol, C., Viprakasit, V., Kamatani, N., Winichagoon, P., Kubo, M., Nakamura, Y. & Fucharoen, S. (2010) A genome-wide association identified the common genetic variants influence disease severity in beta0-thalassemia/hemoglobin E. *Hum Genet,* **127,** 303-314.

Olivieri, N.F., Saunthararajah, Y., Thayalasuthan, V., Kwiatkowski, J., Ware, R.E., Kuypers, F.A., Kim, H.Y., Trachtenberg, F.L., Vichinsky, E.P. & Thalassemia Clinical Research, N. (2011) A pilot study of subcutaneous decitabine in beta-thalassemia intermedia. *Blood,* **118,** 2708-2711.

Patthamalai, P., Fuchareon, S., Chaneiam, N., Ghalie, R.G., Chui, D.H., Boosalis, M.S. & Perrine, S.P. (2014) A phase 2 trial of HQK-1001 in HbE-beta thalassemia demonstrates HbF induction and reduced anemia. *Blood,* **123,** 1956-1957.

Sankaran, V.G. & Orkin, S.H. (2013) The switch from fetal to adult hemoglobin. *Cold Spring Harb Perspect Med,* **3,** a011643.

Shi, L., Cui, S., Engel, J.D. & Tanabe, O. (2013) Lysine-specific demethylase 1 is a therapeutic target for fetal hemoglobin induction. *Nat Med,* **19,** 291-294.

Uda, M., Galanello, R., Sanna, S., Lettre, G., Sankaran, V.G., Chen, W., Usala, G., Busonero, F., Maschio, A., Albai, G., Piras, M.G., Sestu, N., Lai, S., Dei, M., Mulas, A., Crisponi, L., Naitza, S., Asunis, I., Deiana, M., Nagaraja, R., Perseu, L., Satta, S., Cipollina, M.D., Sollaino, C., Moi, P., Hirschhorn, J.N., Orkin, S.H., Abecasis, G.R., Schlessinger, D. & Cao, A. (2008) Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. *Proc Natl Acad Sci U S A,* **105,** 1620-1625.

Figure Legends

Fig 1. Robust HbF induction in β^0-thalassaemia/HbE erythroid cells by pomalidomide alone or in combination with other HbF inducers. β^0-Thalassaemia/HbE erythroblasts

were treated with 4.0 μM pomalidomide only (Pom, from day 4 to 14), 1.0 μM hydroxyurea only (HU, from day 8 to 14), 0.1 μM decitabine only (DAC, from day 8 to 14), 0.02 μM RN-1 only (from day 8 to 14), or in combination of pomalidomide with hydroxyurea, with decitabine, or with RN-1. (A) Representative HPLC chromatograms showing haemoglobin composition at day 14 of erythroid differentiation. (B) The percentage of HbF relative to total Hb (%HbF + %HbE) determined by HPLC at day 14 of erythroid differentiation. The increase in HbF percentage after treatment from the baseline level (DMSO control) was expressed as Δ%HbF (%HbF [compound treatment] - %HbF [DMSO control]). (mean ± standard error of the mean [SEM], n = 10 for HU, DAC and RN-1, n = 15 for Pom, Pom+HU and Pom+DAC, n = 13 for Pom+RN-1). (C) Quantitative RT-PCR analysis showing relative *HBA*, *HBB* and *HBG* mRNA expression levels normalized to β-actin (*ACTB*) at day 12 of erythroid differentiation. Data are presented as the mean of relative fold change of DMSO ± SEM. (n = 5) $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$; $^{****}P < 0.0001$.

Fig 2. Effect of pomalidomide and its combinations on erythroid differentiation and mRNA expression of HbF regulators in cultured erythroid cells from β^0-thalassaemia/HbE patients. β^0-Thalassaemia/HbE erythroblasts were treated with 4.0 μM pomalidomide only (Pom, from day 4 to 14), 1.0 μM hydroxyurea only (HU, from day 8 to 14), 0.1 μM decitabine only (DAC, from day 8 to 14), 0.02 μM RN-1 only (from day 8 to 14), the combination of pomalidomide with hydroxyurea, with decitabine, or with RN-1. (A) Representative flow cytometry dot plots for erythroid differentiation analysis on day 12 of culture. Erythroid cells were gated into R1 to R4 populations according to the expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a). (B) The histogram represents the quantitation of erythroid subpopulations analyzed by flow cytometry. (mean ± SEM, n = 3). $^{*}P < 0.05$; $^{**}P < 0.005$, relative to DMSO control. (C) Representative modified Giemsa-stained cytospins at day 12 of culture showing erythroid morphology after DMSO or compound treatments. Scale bar = 10 μm. (D) Relative mRNA abundance of known HbF regulators normalized to β-actin (*ACTB*) determined by quantitative RT-PCR at day 12 of erythroid cell culture. Gene names are shown at the top of each histogram. Data are presented as the mean of relative fold change of DMSO ± SEM. (n = 5) $^{*}P < 0.05$; $^{**}P < 0.005$; $^{****}P < 0.0001$.

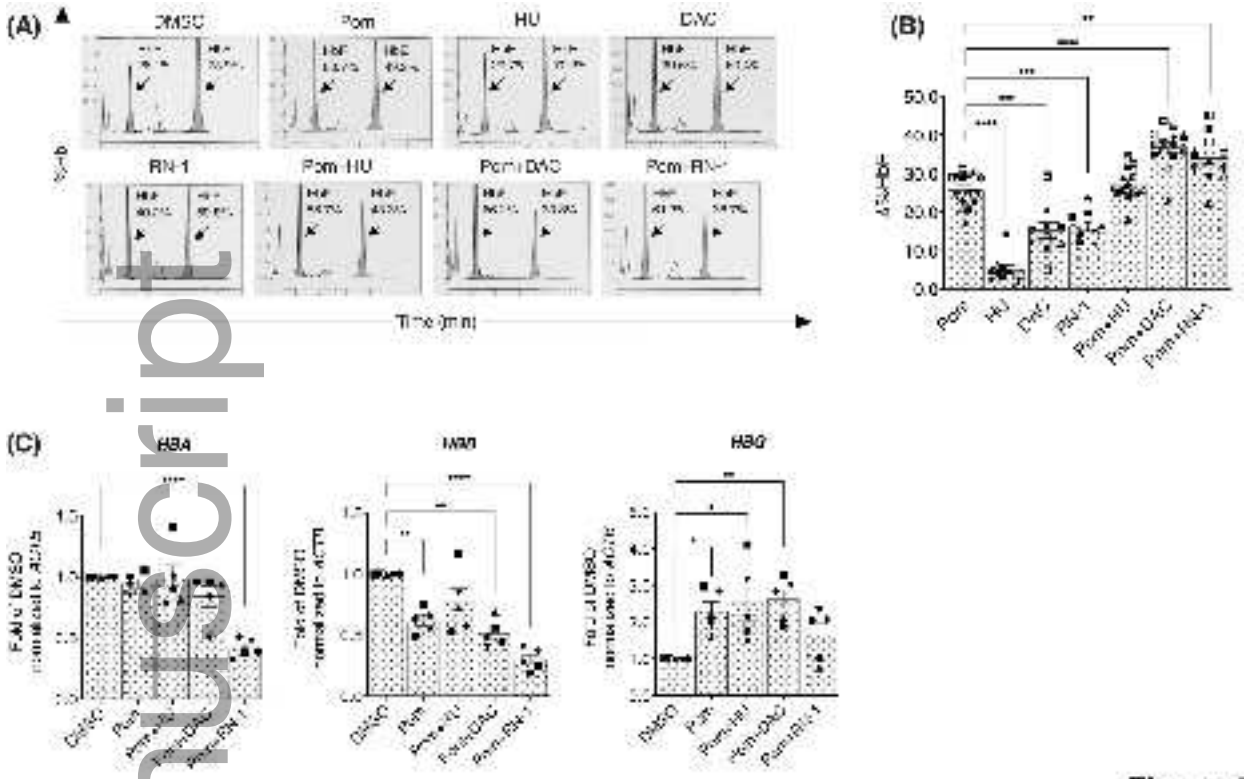

Figure 1

bjh_16670_f1.jpg

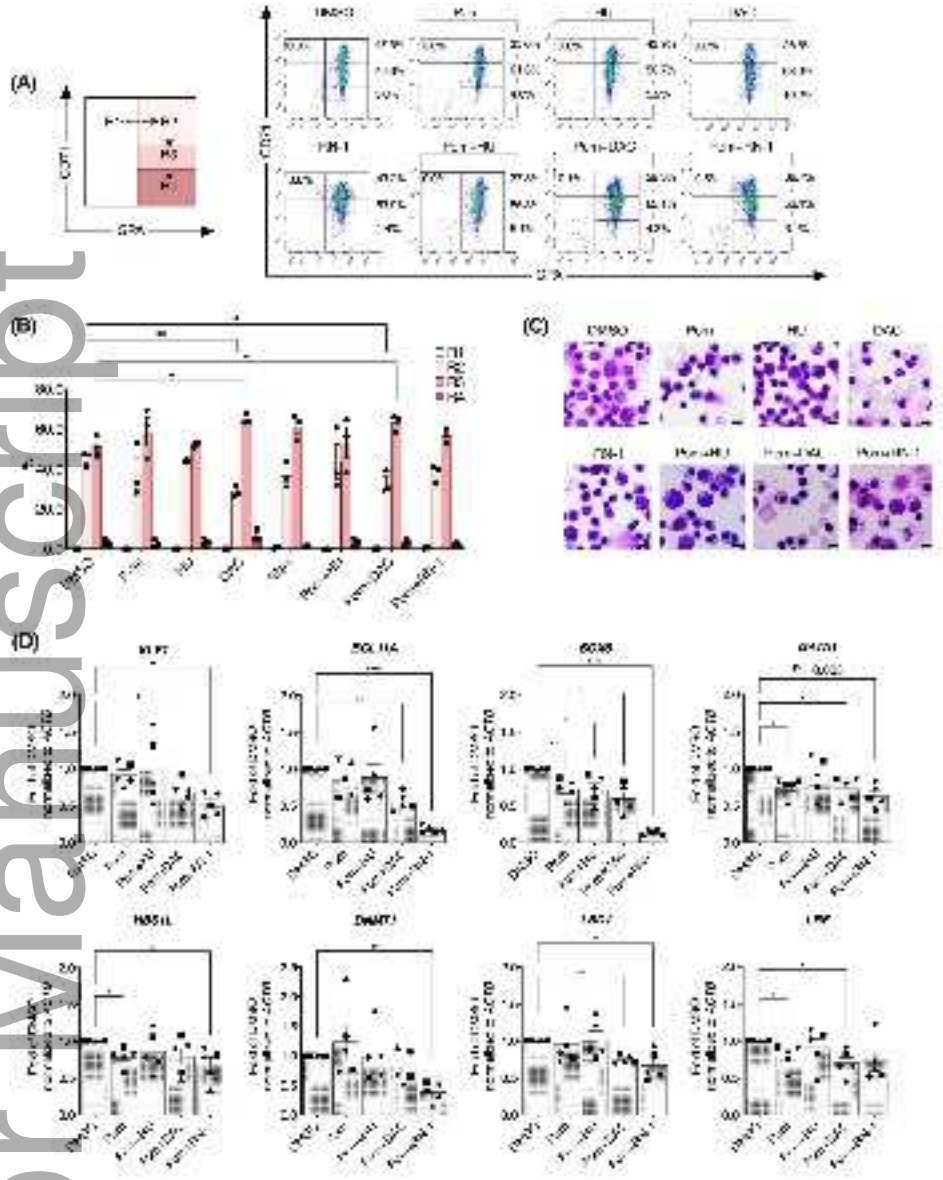

Figure 2

bjh_16670_f2.jpg