

INTRODUCTION

Erythropoietin (EPO) acts on the homodimeric EPO receptor (EPOR) to stimulate proliferation of erythroid progenitor cells and induce their survival and differentiation into red blood cells. Various recombinant human EPO derivatives, also known as erythropoiesis-stimulating agents (ESAs), are marketed or in clinical development for the treatment of anemia due to renal failure or cancer chemotherapy.

However, ESA treatment is associated with an increased risk of adverse cardiovascular complications in patients with kidney disease, and may be related to an increase in mortality in cancer patients, when it is used to increase hemoglobin levels above 13.0 g/dl. We have identified a series of novel non-peptidyl small molecules, as represented by LG5640, that selectively activate EPOR function, which may provide a unique therapeutic opportunity in the treatment of anemia.

MATERIALS AND METHODS

Viability and Apoptosis assays

UT-7/epo or F-36E cells were treated with vehicle, EPO (Epogen) or LG5640 for 48 hours (apoptosis) to 72 hours (viability). Apoptosis was determined using the Cell Death Detection ELISA (Roche); viability was assayed by ViaLight Plus (Lonza).

Erythrocyte Differentiation and BFUe Colony-forming Assays

CD34-positive human bone marrow cells were cultured in media supplemented with human stem cell factor (hSCF, R&D Systems), with vehicle, EPO (R&D Systems) or LG5640. The cells were stained with anti-CD235a (BD Biosciences) and analyzed by FACS. Activity was measured as percent CD235a-positive cells and the data expressed as the percent maximal rEPO response.

Detection of phospho-PI3K, phospho-GATA-1

Cells cultured with vehicle, EPO or LG5640 were fixed in 2% (final) paraformaldehyde, permeabilized with buffered ice-cold methanol, and stained with anti-phosphoprotein antibodies (BD Bioscience) and analyzed by FACS.

siRNA Knockdown of EPOR, GATA-1

UT-7/epo cells were transfected with siRNA specific for human EPOR (Dharmacon), GATA-1 (Santa Cruz), a non-targeting RNA control (NT2), or with no siRNA (mock). The cells recovered 24 hours in 1 U/ml EPO and continued presence of siRNA. The cells were cultured overnight in EPO-free media, and then treated with vehicle, EPO or LG5640 for an additional 72 hours before being assayed for viability.

Detection of GATA-1 DNA-Binding Activity and Gene Expression

UT-7/epo cells were cultured 30 min in vehicle, EPO, or LG5640. Nuclear extracts were prepared for electrophoretic mobility shift assay (EMSA) of GATA-1 DNA binding. Gene expression was quantitated by real-time PCR on an ABI7900.

RESULTS

Figure 1: LG5640 increases viability in EPO-deprived UT-7/epo (A) and F-36E (B) cells. LG5640 did not increase viability in GM-CSF-deprived Mo7e cells that lack EPOR. Mean (duplicates) ± SD

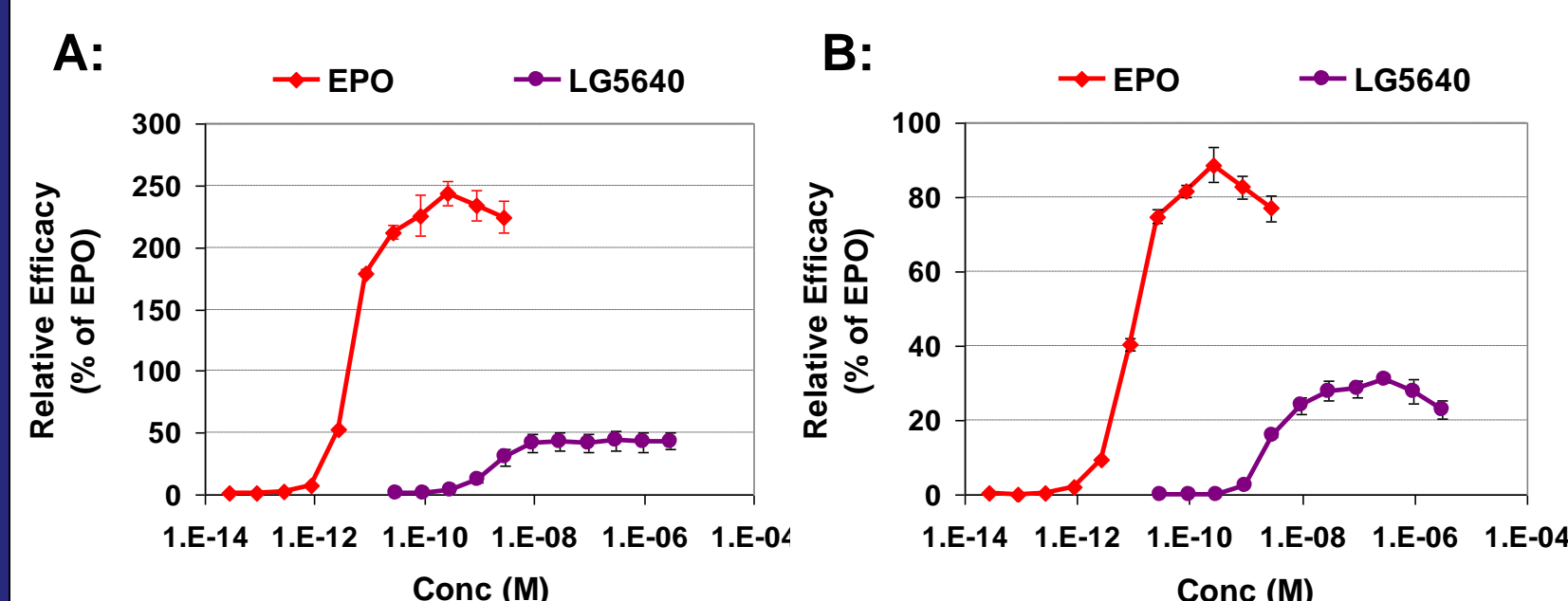


Figure 2: LG5640 inhibits apoptosis in EPO-deprived UT-7/epo (A) and F-36E (B) cells with efficacy equal to EPO. Mean (duplicates) ± SD

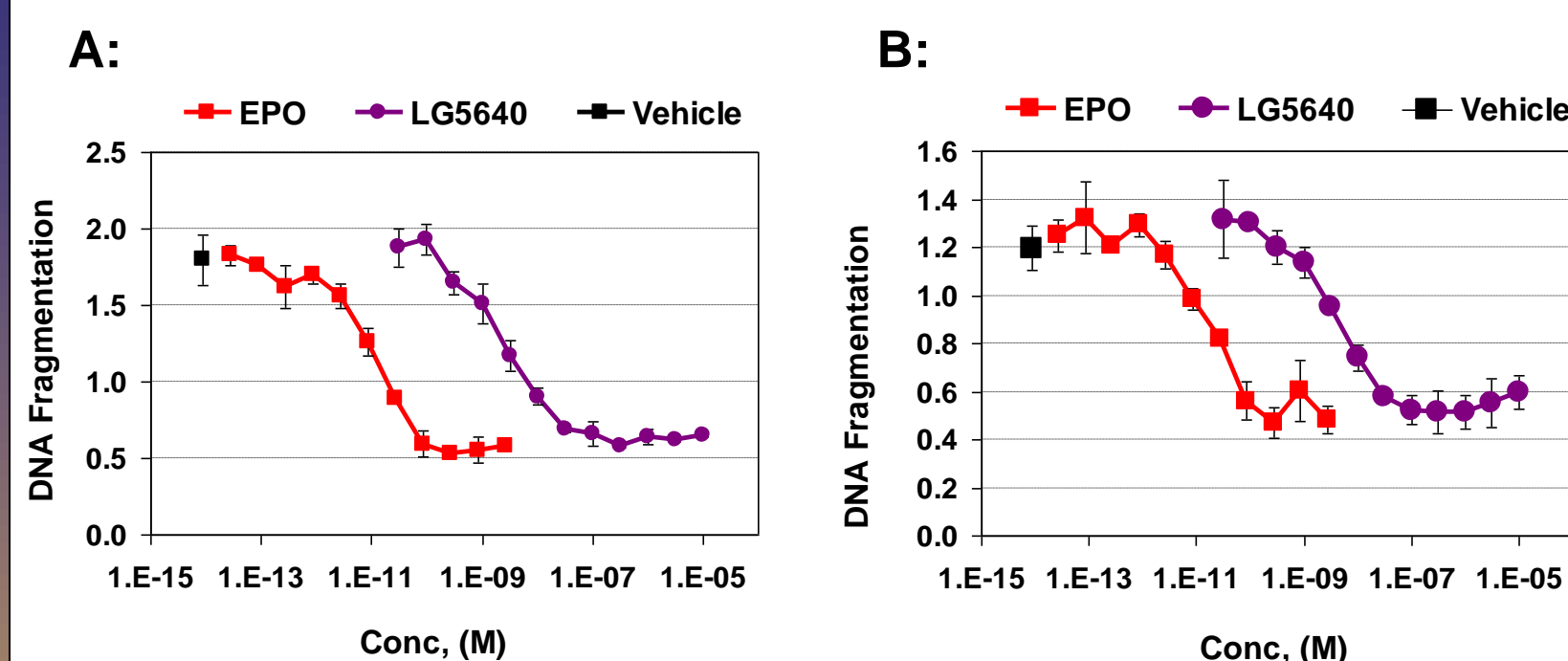


Figure 3: LG5640 induces the expression of the erythroid maturation marker CD235a (glycophorin A) and the development of blast-forming units-erythroid (BFU-Es) in CD34-positive, human bone marrow. LG5640 did not increase the percentage of cells positive for the megakaryocyte marker CD41 or the granulocyte marker CD15 (data not shown).

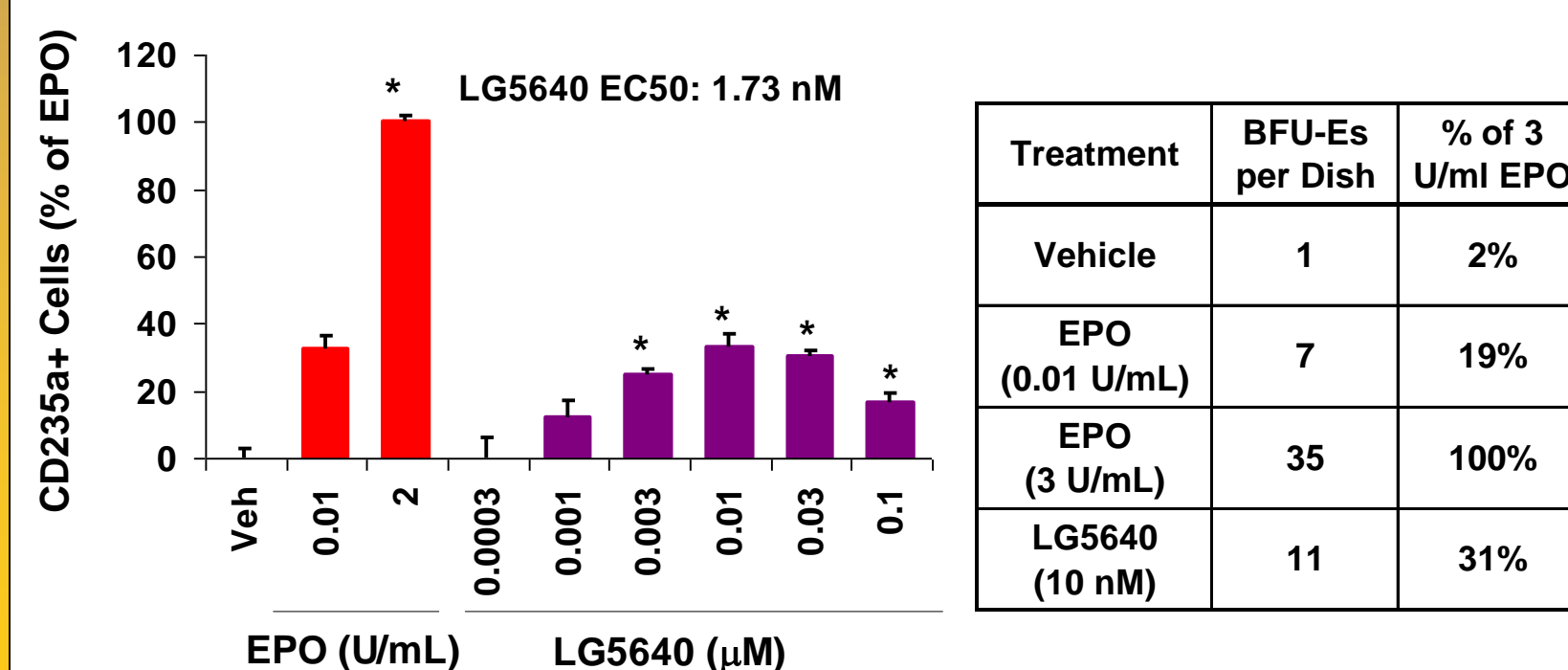


Figure 4: LG5640 enhances EPO activity in EPO-dependent cell lines (viability) and CD34-positive bone marrow cells (CD235a expression)

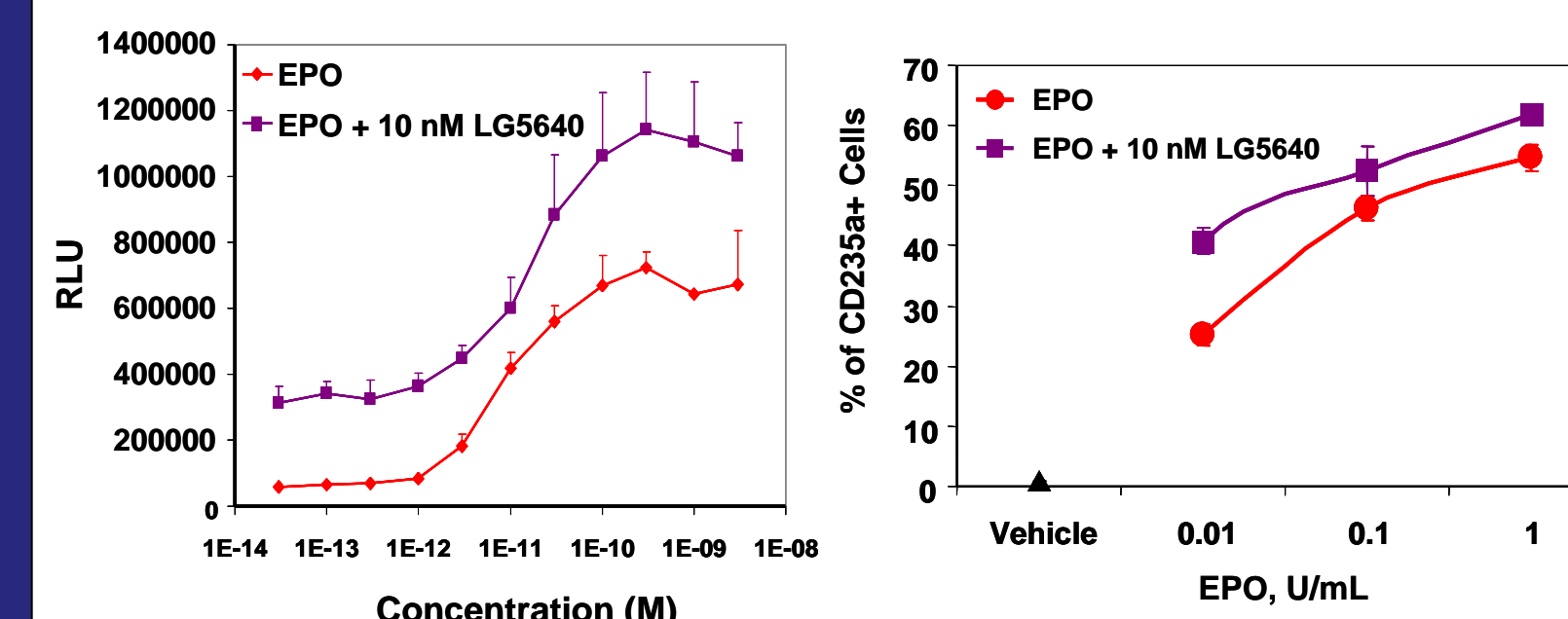


Figure 5: LG5640 induces the phosphorylation of PI3K and GATA-1 (A). The PI3K inhibitor LY294002 inhibits the activity of both EPO and LG5640 in the F-36E viability assay (B). LG5640 did not stimulate phosphorylation of STAT5 or ERK/MAPK (data not shown). Mean (duplicates) ± SD

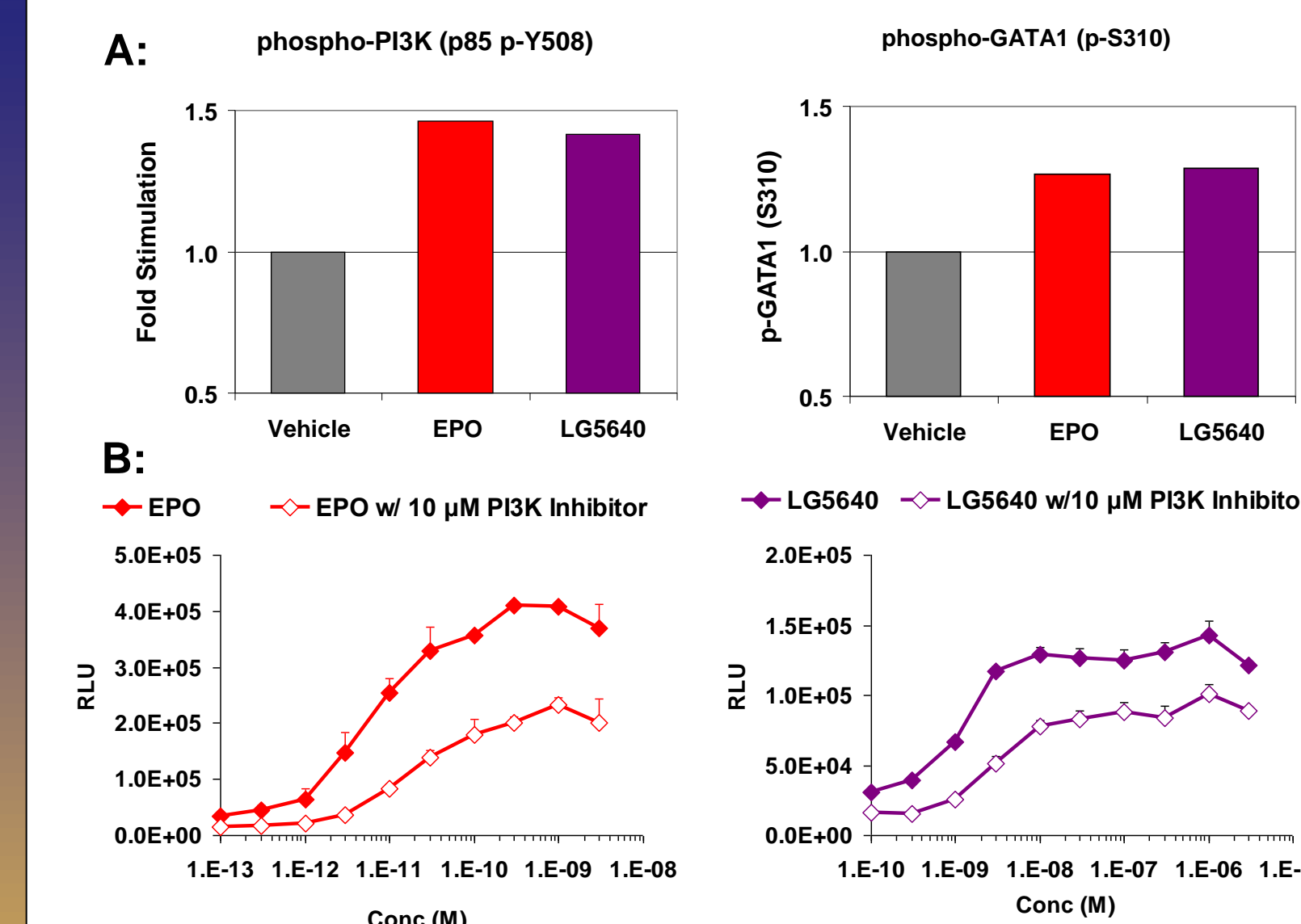


Figure 6: LG5640 requires the expression of EPOR and GATA-1 for activity. siRNA knockdown of EPOR or GATA-1 eliminates the response to both EPO and LG5640 in viability assays. Mean of triplicate samples ± SD

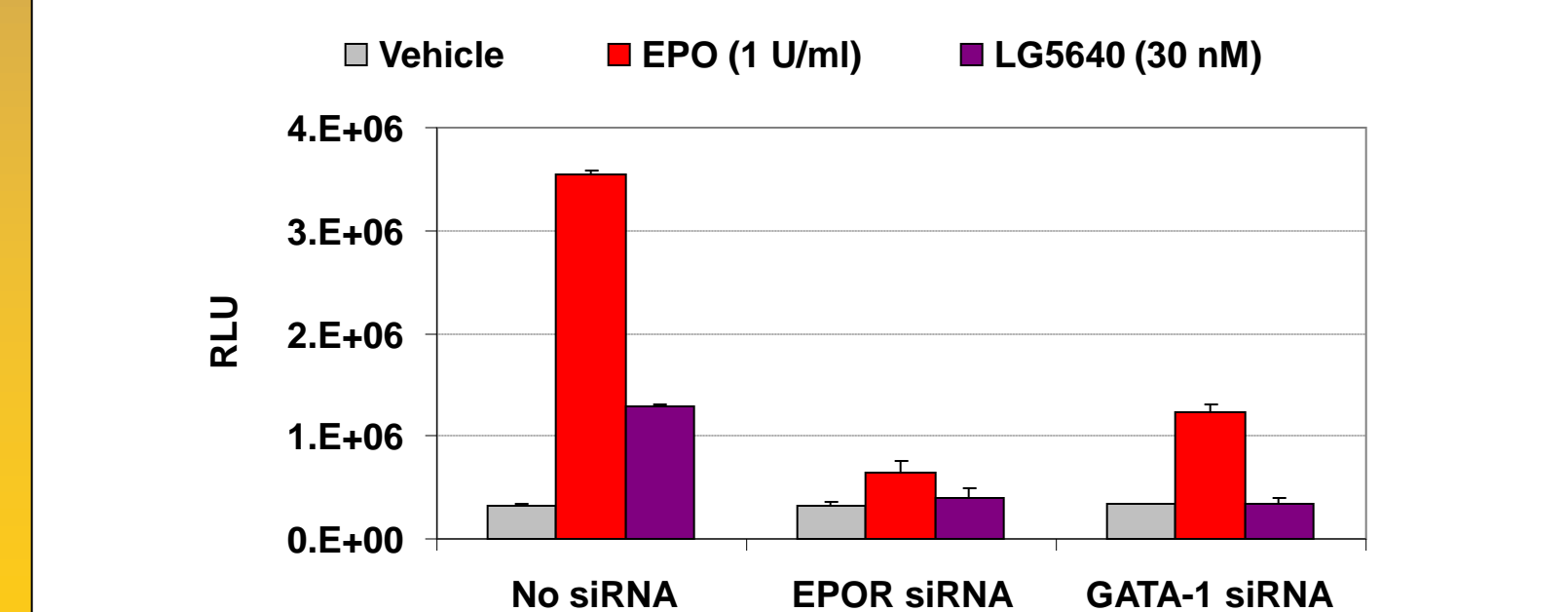
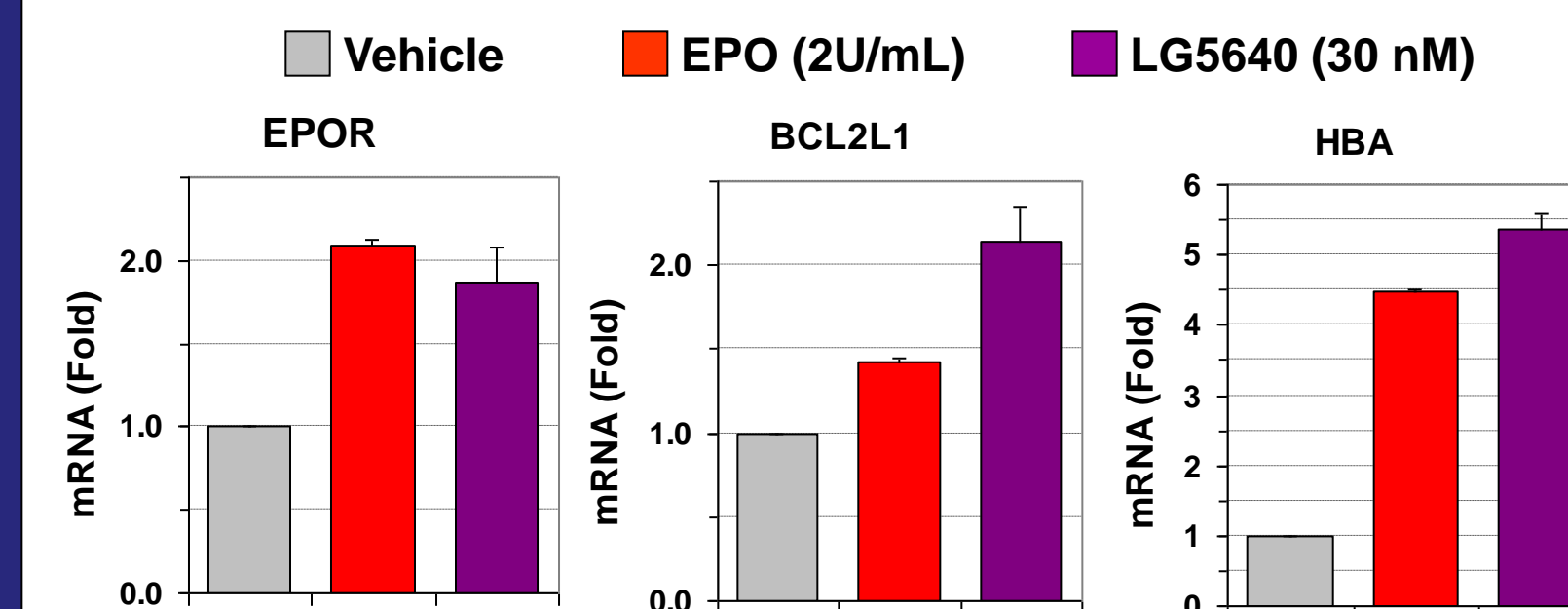


Figure 7: LG5640 induces the expression of genes regulated by GATA-1 during erythroid maturation in CD34-positive human bone marrow cells. EPOR, BCL2L1 and hemoglobin A (HBA) gene expression were determined by real time PCR. LG5640 induced GATA-1 DNA-binding as detected by EMSA (data not shown).



SUMMARY

- LG5640 is a novel small molecule EPOR agonist that increases viability of EPO-dependent cells
- LG5640 promotes the differentiation of BM-HCs into erythrocytes, increasing expression of CD235a and inducing the erythroid formation (BFU-Es)
- LG5640 is dependent on the expression of EPOR
- LG5640 selectively activates the EPOR/PI3K/GATA1 signal transduction pathway, induces GATA-1 DNA-binding and the expression of GATA-1 target genes

Conclusions

- A series of small molecule, selective EPOR agonists have been discovered that display partial efficacy in several models of EPO-induced erythropoiesis vs. the maximal effect of EPO
- Based on the novel profile of the series, several lead compounds have been identified as potential preclinical development candidates
- These lead compounds promote erythroid maturation in CD34 positive BM-HCs with nanomolar potency, and display oral bioavailability in the rat and monkey
- EPOR agonists are expected to lack the excessive erythropoietic stimulation that may possibly contribute to the adverse effects of ESAs and thus may provide a unique therapeutic opportunity in the treatment of anemia