Identification and validation of a new class of HDAC inhibitors able to induce HbF in thalassemic erythroid precursors.

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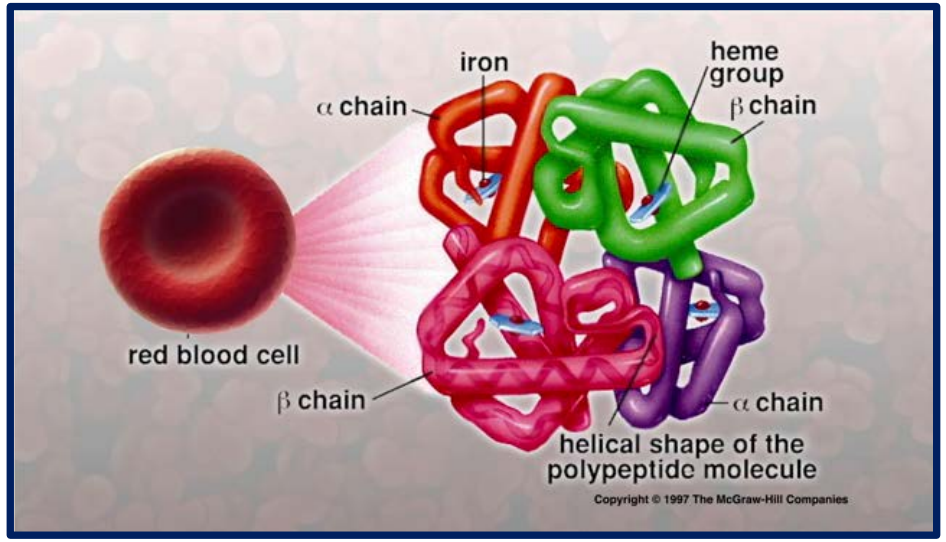
INTRODUCTION

The project THALAMOSS (**THALA**ssaemia **MO**dular **S**tratification **S**ystem for personalized therapy of β-thalassemia) aims at the identification of novel drugs and treatments specific for β-thalassaemia patients.

Thalassemia

Genetic blood disorder characterized by abnormal structure or underproduction of the normal form of hemoglobin molecule resulting in anemia. β-thalassemia occurs when there is a genetic deficiency in the synthesis of β-globin chains.

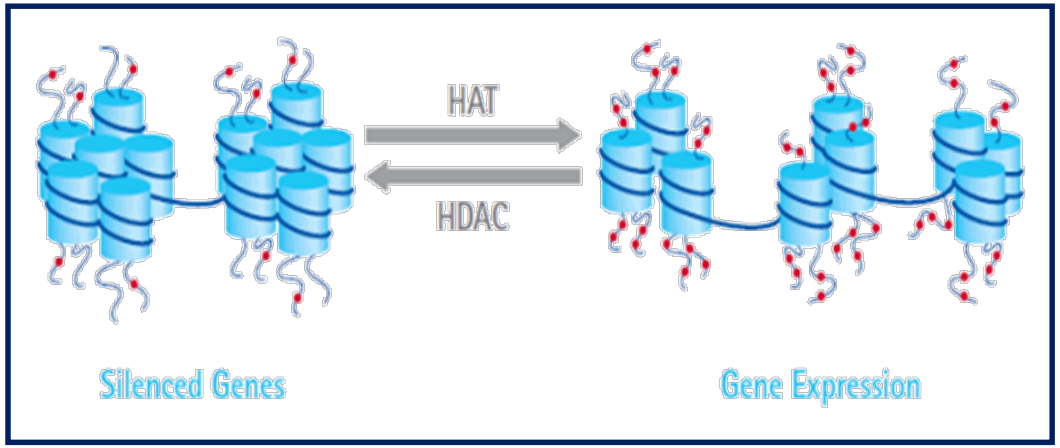


	Hemoglobin	Structural Formula	
Adult	Hb-A	$\alpha_2\beta_2$	97%
	Hb-A_2	$\alpha_2\delta_2$	1.5–3.2%
Fetal	Hb-F	$\alpha_2\gamma_2$	0.5–1%
	Hb-Bart's	γ_4	
Embryonic	Hb-Gower 1	$\zeta_2\varepsilon_2$	
	Hb-Gower 2	$\alpha_2\varepsilon_2$	
	Hb-Portland	$\zeta_2\gamma_2$	

To date one potential approach to cure β-thalassemia seems to be the chemical induction of the γ-globin protein normally found in fetal hemoglobin.

HDAC INHIBITION

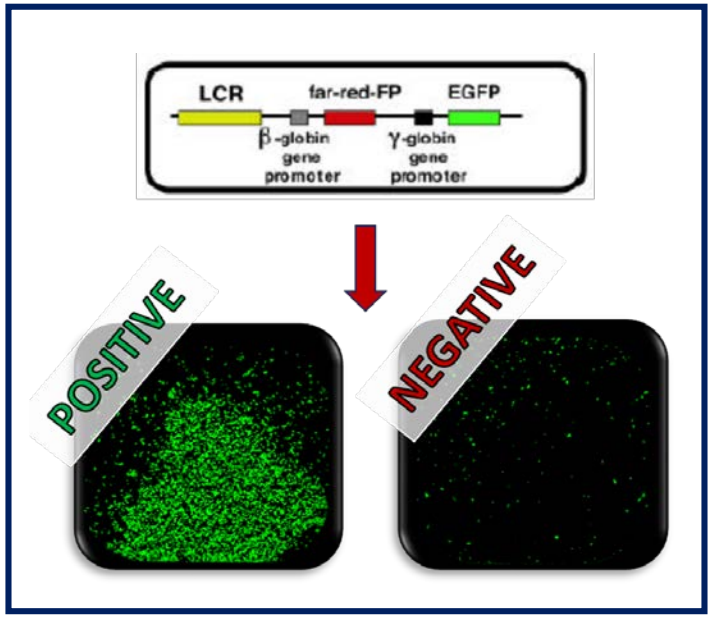
- Gene expression is controlled at the level of chromatin architecture by factors that alter nucleosome structure through a post-translational modification of histone tails.
- Histone deacetylases (HDACs), are chromatin-modifying enzymes that affect the acetylation status of histones and modulate gene expression.



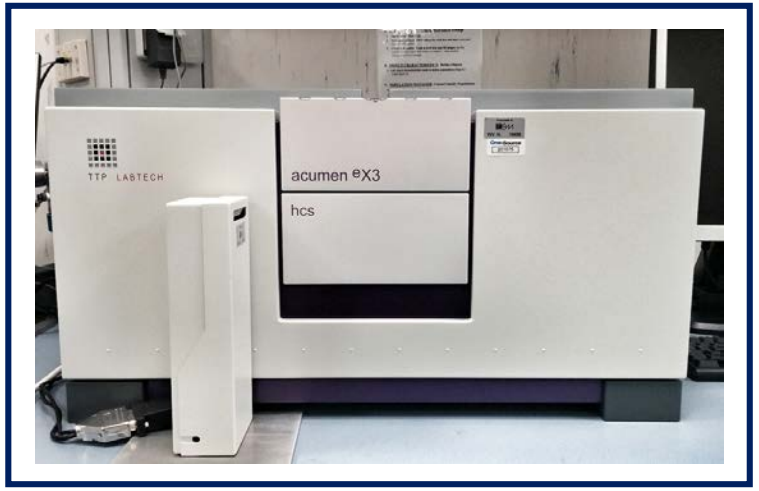
- Compounds that inhibit HDAC activity have the potential to induce the activation of repressed genes.
- It has been shown that inhibition of histone deacetylase activates γ globin gene expression.

CELL SYSTEM

- Chronic myelogenous leukemia derived cells K562 have been used as model to study the hemoglobin switch in vitro as they are able to undergo erythroid differentiation and express fetal globin gene when stimulated with a variety of inducers.
- K562 cells were stably transfected with a gene encoding EGFP placed under the control of the simplify/modify β-cluster.
- A plate based cytometry screening was carried out with the Acumen plate based cytometer by using 384-well plates to identify compounds inducing the accumulation of EGFP.
- Stained nuclei (Hoecths) were used to assess the cell proliferation/death and to normalize the total EGFP/RFP intensity.



Acumen plate-based Cytometer



ASSAY OPTIMIZATION

- A known HDAC inhibitor, Vorinostat (SAHA), currently used in clinical trials for treatment of Sickle Cell Disease (Phase II) was chosen as control inducer. Its optimal concentration, that gives a clear induction and minimal cytotoxicity, was initially determined by flow cytometry (Fig.1).

- 1 µM SAHA and an incubation time of 64 hours were chosen as the best conditions; a clear EFGP induction is in fact observed mostly after 72 hours of compound incubation (Fig.2).

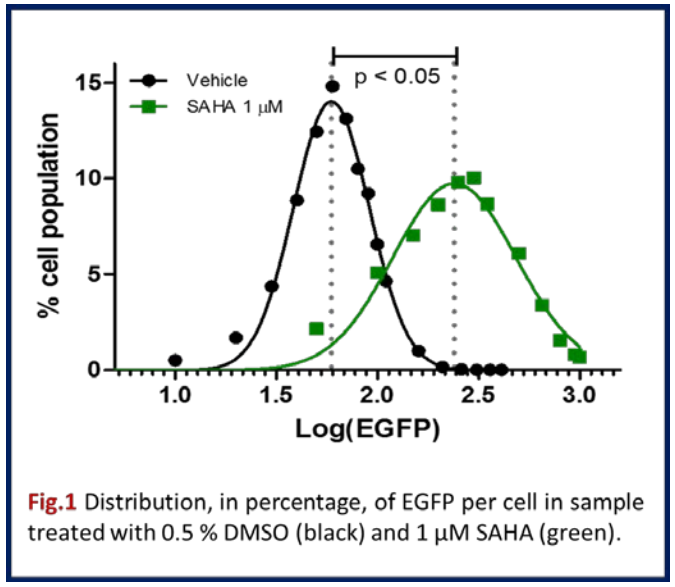


Fig.1 Distribution, in percentage, of EGFP per cell in sample treated with 0.5 % DMSO (black) and 1 µM SAHA (green).

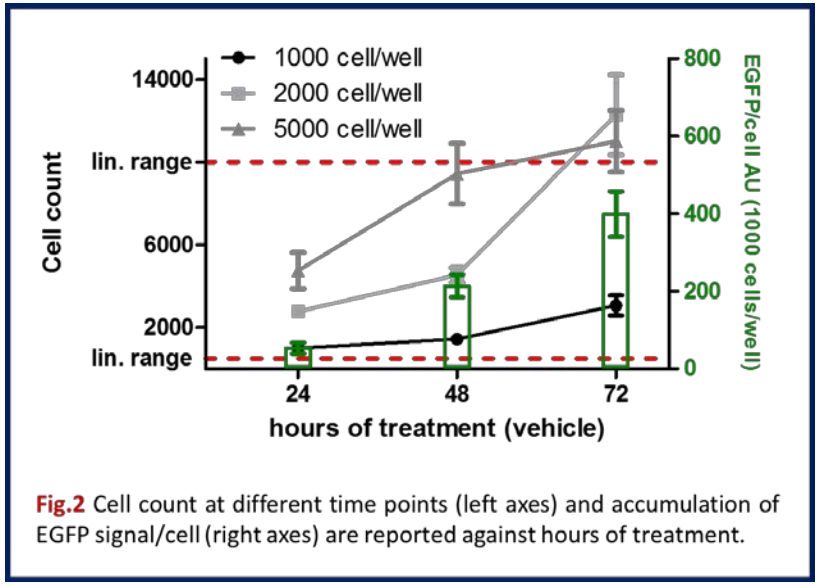


Fig.2 Cell count at different time points (left axes) and accumulation of EGFP signal/cell (right axes) are reported against hours of treatment.

FOCUS SCREENING:
HDAC inhibitors

- A collection of 2200 small molecules endowed with potent HDAC inhibitory activity identified at the IRBM Science Park was tested at a fixed 1 μM concentration. The results of the screening were calculated as percentage of activation normalized between vehicle treated cells (0%) and 1 μM SAHA treated cells (100%). A frequency distribution histogram is shown in Fig.3.

- Active and not active compounds were finally correlated with their HDAC inhibition potency (EC50) in a cell based assay (Fig.4). The most active molecules in modulating the expression of γ-globin genes in the human K562 cell line were also the most potent HDAC inhibitors of the collection tested (lower EC50).

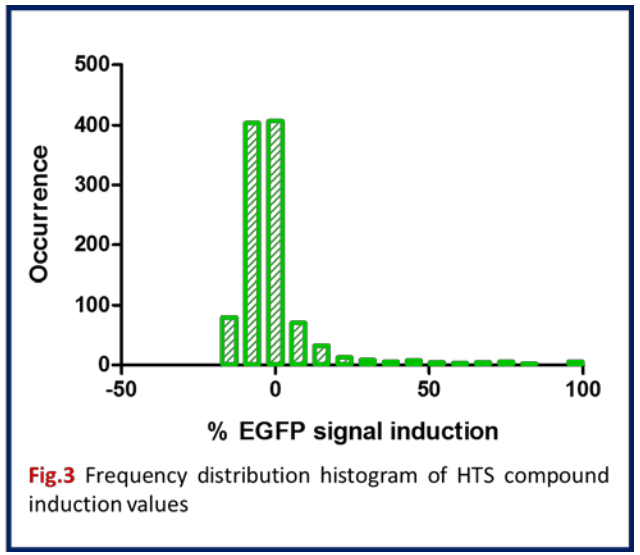


Fig.3 Frequency distribution histogram of HTS compound induction values

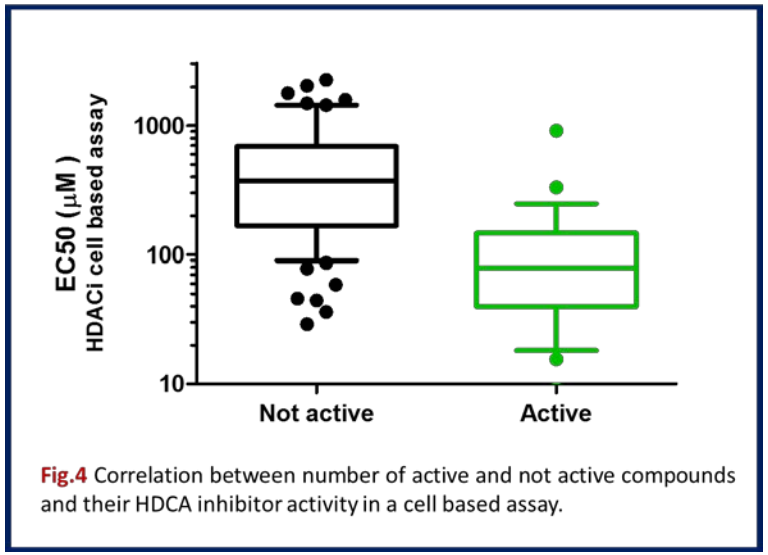


Fig.4 Correlation between number of active and not active compounds and their HDCA inhibitor activity in a cell based assay.

LEAD CHARACTERIZATION

The activity was afterwards confirmed in dose-response experiments on K562-EFGP cells and the EC50 for HbF activation calculated. A compound with promising potency was finally identified: **IRBM-Lead** (Tab.1).

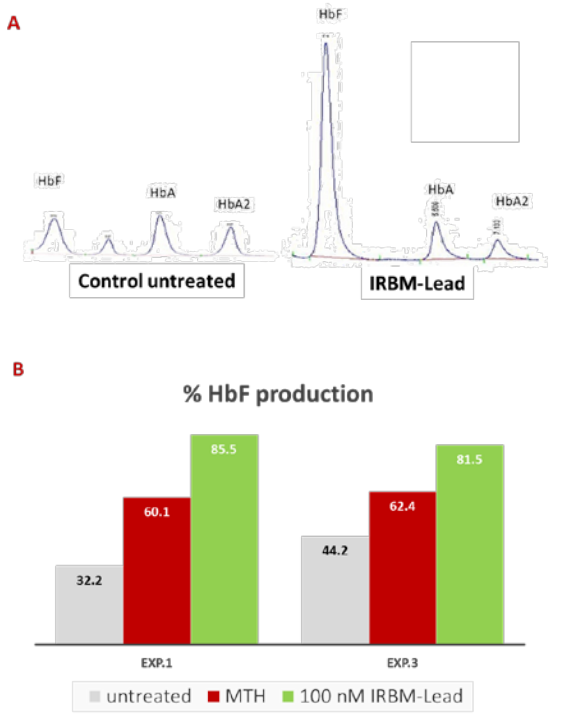


Fig.5 HbF production and globin chains evaluated by HPLC in erythroid precursor cells from β-thalassemia patients (A). Comparison of HbF induction after treatment with Mithramycin (MTH) and 100 nM IRBM-Lead (B).

- Its capacity to modulate the expression of fetal hemoglobin protein in human erythroid precursors (ErPC) was confirmed by cation-exchange HPLC and its activity compared to the one of the most efficient HbF inducers (Fig.5).
- Pharmacokinetic properties of IRBM-Lead were also investigated in mice (Fig.6). IRBM-Lead concentration in plasma was always higher than the one shown to be efficacious in vitro for HbF accumulation (100 nM).
- IRBM-Lead was found bioavailable with favorable PK properties.

Tab.1	IRBM-Lead
hHDAC inCell EC50 (nM)	50.6 ± 16.5
hHDAC-1 IC50 (nM)	1.77 ± 0.86
Mouse PK	
CLp (mL/min/kg)	12.9
$AUC_{0-\infty}$ (uM.h) (PO 20 mpk)	37.0
F %	61.5 %
HbF activation EC50	172.10 ± 2.03

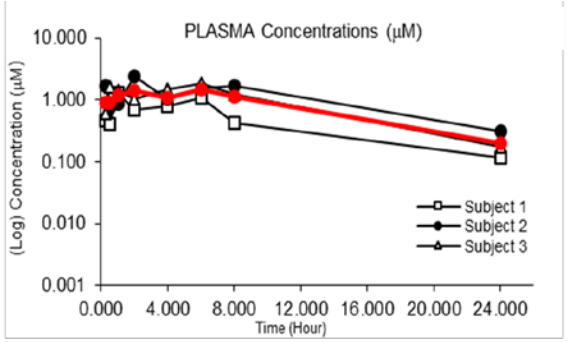


Fig.6 PLASMA concentration (µM) of IRBM-Lead in mice at different times (PO: 20 mpk).

CONCLUSIONS

- ✓ We identified a small potent HDAC inhibitor compound, **IRBM-Lead,** able to induce erythroid differentiation and human γ-globin gene expression in vitro with EC50 in the order of high nanomolar.

- ✓ IRBM-Lead showed high oral exposure and favorable PK properties when administered to preclinical species, which is promising for an improved therapeutic window.

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