Erymethionase, Methioninase entrapped in Red Blood Cells:
An innovative treatment approach for classical homocystinuria

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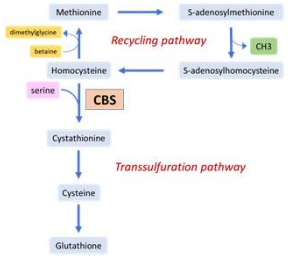
1 - Background

Classical Homocystinuria results from cystathionine beta-synthase (CBS) deficiency. Biochemical hallmarks of this disorder are elevated levels of homocysteine (Hcy) and methionine (Met).



Patients symptoms include intellectual disabilities, thromboembolism, ocular lens dislocation and osteoporosis.
Current treatments for non-responsive Vitamin B6 patients are methionine/protein restricted diets supplemented with betaine.
As an alternative treatment approach to reduce the toxic accumulation of Hcy and Met, Erytech envisioned an innovative enzyme therapy: **Erymethionase**, Methionine γ-Lyase (MGL) entrapped in red blood cells.

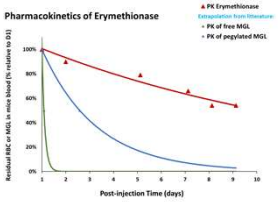
2 - Erymethionase product presentation

Erymethionase, Methionine γ-Lyase (MGL; EC number 4.4.1.11) entrapped in red blood cells is developed using Erytech's proprietary ERYCAPS technology platform.
MGL is a pyridoxal 5'-phosphate (PLP)-dependent enzyme and displays enzymatic activities with both Hcy and Met with similar Kms and a higher Vmax for Hcy (Ito *et al*, J Biochem vol 79, p 1263 and personal data):

L-homocysteine ⇌ H2S + NH3 + 2-oxobutanoate
L-methionine + H2O ⇌ methanethiol + NH3 + 2-oxobutanoate

MGL needs PLP cofactor to be active. Erythrocytes are a natural pool of PLP and exhibit the whole machinery to synthesize PLP from pyridoxine (vitamin B6).

Pharmacokinetics of Erymethionase



PK of **Erymethionase** was assessed in healthy CD1 mice.
CFSE-labeled RBC encapsulating MGL (CFSE-labeled **Erymethionase**) was intravenously injected at a volume of 8 mL/kg.
Half-life of labeled Red Blood Cells in blood circulation is estimated around 18 days. Free MGL and PEG-MGL are also represented.

Considerations for Homocysteine level measurement

Plasma Homocysteine level in healthy mice is very low (< 10-15 µM).
It is therefore complicated to explore the pharmacodynamics of **Erymethionase** with regard to Hcy in such animals.
Homocysteine molecule is present under different forms in plasma:

Total plasma Homocysteine (tHcy)	
Reduced form	
Homocysteine (Hcy-SH)	< 1%
Oxidized Forms	
Homocystine (Hcy-S-S-Hcy)	5-10 %
Mixed disulfide Hcy-S-S-Cys	5-10%
Protein-Cys-S-S-Hcy	70-80%

In humans

To assess total plasma homocysteine level, serum samples are treated with dithiothreitol (DTT) to reduce oxidized forms of homocysteine.

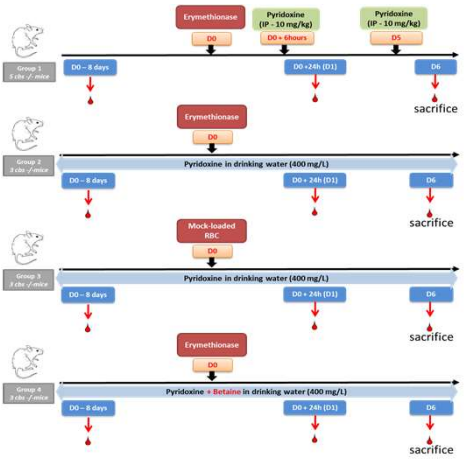
3 – CBS-deficient mouse model

The most common mutation found in human CBS-deficient patients is I278T (~25% of all the reported mutations found in CBS-deficient patients). I278T human CBS retains only 2.4% of the enzyme activity of wild-type human CBS. This human mutant CBS gene was inserted into the genome of C57BL/6J background strain mice. Expression of this transgene is under the control of a zinc-inducible metallothionein promoter (Wang *et al.* , 2005, Hum. Mol. Genet, 14, 2201).

The CBS knockout mice have extremely elevated plasma homocysteine, elevated plasma methionine and reduced total cysteine. It has well-characterized phenotypes including osteoporosis, decreased mean survival, decreased weight gain, low percentage of body fat and facial alopecia (Gupta *et al.*, 2014, FASEB J, 28, 781).

Homocysteine being solely derived from methionine, it has been demonstrated that a low-methionine diet can reverse all the phenotypes of CBS-deficient mice (Gupta *et al.*, 2014, FASEB J, 28, 781).

4 – *In vivo* study Design with CBS-deficient mice



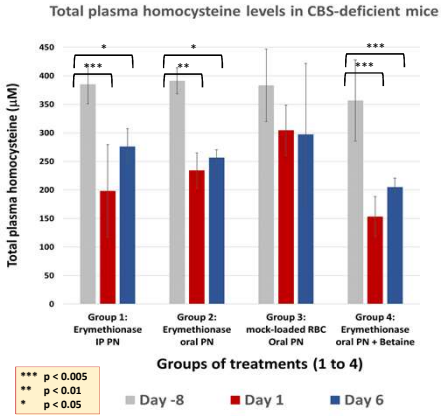
Four (4) groups of 3 to 5 CBS-deficient mice were designed to address the Erymethionase efficacy on total plasma homocysteine and plasma methionine levels.

Erymethionase product (140 µL / 120 µg) was intravenously injected at Day 0. To allow MGL enzymatic activity in blood circulation, pyridoxine was given under two different routes : intraperitoneal (10 mg/kg - group 1) or dissolved in the drinking water (400 mg/L - group 2).

As a control (group 3), mock-loaded RBC (140 µL) were intravenously injected to mice given pyridoxine in the drinking water (400 mg/L). Mice from group 4 were injected with **Erymethionase** and given pyridoxine and betaine dissolved in the drinking water (400 mg/L and 2% respectively).

Blood sampling was performed on each mouse, 8 days before Erymethionase or mock-loaded RBC administrations, the following day (day 1) and 6 days after the injections (day 6). Mice were sacrificed at day 6.
The fourteen (14) blood samples were centrifuged to isolate plasmas and treated with DTT to measure total plasma homocysteine and plasma methionine levels using an amino acid analyzer (Biochrom 30, Cambridge, UK).

5 – Results



	Total plasma Homocysteine concentrations (µM)			
Time points	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Day -8	385 ± 34	391 ± 23	383 ± 63	357 ± 71
Day 1	199 ± 81	234 ± 31	305 ± 44	153 ± 35
Day 6	276 ± 31	257 ± 14	297 ± 124	205 ± 15

Eight (8) days before **Erymethionase** treatments, total plasma homocysteine baseline is superior to 350 µM for all CBS-deficient mice. This first study shows that all the CBS-deficient mice that have been injected with **Erymethionase**, with pyridoxine administered by intraperitoneal or gastric routes, had their total plasma homocysteine level decreased as described in the table below:

	Total plasma Homocysteine depletion (%)			
	Group 1	**Group 2**	**Group 3**	**Group 4**
Day 1 vs Day -8	47%	40%	17%	57%
Day 6 vs Day -8	28%	34%	17%	40%

Erymethionase reduces by at least 40% the total plasma homocysteine level in all CBS-deficient mice. As indicated with the Anova one-way analysis, total plasma Hcy levels of group 3 (mock-loaded RBC) are not statistically different at Day 1 and Day 6 versus Day -8 (baseline). However, the effect of pyridoxine alone is to be studied in the next study to discard any effect of mock-loaded RBC.
Betaine supplementation in pyridoxine-containing drinking water (group 4) induces a higher total plasma homocysteine depletion than the depletions observed in groups 1 and 2. However, the effect of betaine alone need to be assessed on the total plasma homocysteine level of CBS-deficient mice in further studies.
Plasma methionine level has been assayed for all the CBS-deficient mice of this study and no significant plasma level difference has been observed between all the groups.

6 - Conclusion

Erymethionase, entrapment of Methionine-γ-Lyase inside Red Blood Cells, greatly improves the pharmacological properties of the enzyme. In healthy mice, half-life of the entrapped enzyme compared to the free form or the PEGylated form of MGL is highly increased in blood circulation.
When injected to CBS-deficient mice, **Erymethionase** has demonstrated a real efficacy on the very high plasma homocysteine concentrations displayed by these mice. Indeed, 24h after administration, plasma homocysteine concentration was reduced by more than 40% when **Erymethionase** was administered either with pyridoxine given by intraperitoneal or by gastric route. Six (6) days after **Erymethionase** administration plasma level of tHcy were still significantly lower compared to baseline.
Based on these results, further investigations are undergoing to determine the potential of **Erymethionase** as a therapeutic option for homocystinuria.

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Disclosure

E. Dufour, A. Meyzaud, F. Horand and A. Scheer are employees at Erytech pharma.