Biosensor-Enabled Optimization of SYNB1934, a Synthetic Biotic for Phenylketonuria Demonstrating Clinical Efficacy

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Catherine Monahan[1], Kristin Adolfsen[2], Isolde Callihan[2], Per Greisen[2], Vincent Isabella[1], David Lubkowicz[1], Jim Spoonamore[2], Lauren Fitch[2], Lindong Weng[2], Caitlin Allen[2], Adam Lawrence[2], Jay Konieczka[2], Caroline Kurtz[1], Marja Puurunen[1] and Ken Ndugga-Kabuye[1]

[1]Synlogic, Cambridge, MA [2]Zymergen, Medford, MA

Introduction

- Phenylketonuria (PKU) is a rare, human metabolic disease characterized by an inability to degrade phenylalanine (Phe), causing neurotoxicity.
- Approved treatments for PKU have limitations in efficacy and safety, leaving patients in need of new options.
- SYNB1618, a Synthetic Biotic designed to consume Phe in the GI tract using an *Escherichia coli* Nissle (EcN) chassis, was engineered to express two independent enzymes, PAL and LAAD, which metabolize Phe into the respective metabolites, *trans*-cinnamic acid (TCA) and phenylpyruvate (PP).
- In the Synpheny-1 phase 2 trial, SYNB1618 demonstrated >20% reduction in serum Phe levels in PKU patients.
- SYNB1934 is an optimized live bacterial therapeutic and was studied in Healthy Volunteers (HV) to compare its activity to SYNB1618.

TCA-responsive biosensor and pop-'n-sort screening methodology (Zymergen)

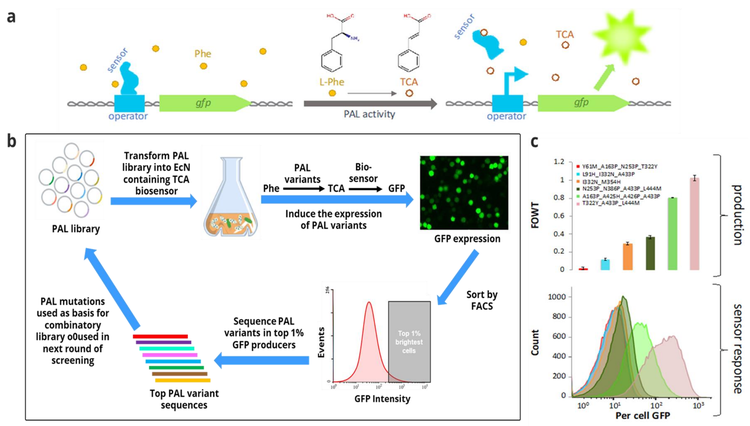

Fig. 1. (a) Specific TCA-responsive allosteric transcription factor biosensor represses expression of the fluorescent reporter gene *gfp* in the absence of TCA. When TCA is introduced, the repression is relieved and *gfp* is expressed. (**b**) Directed evolution method where a library of PAL variants were transformed into EcN with biosensor, encapsulated by microfluidics in water-in-oil droplets and sorted by FACS for highest GFP producers. Sequences of top 1% PAL variants used to design the next round of screening. This ultra-high-throughput screening approach allowed screening of >1M-member combinatorial libraries. **(c)** Ranking of PAL production corresponded to levels of GFP expression.

PAL evolution demonstrated improved TCA production

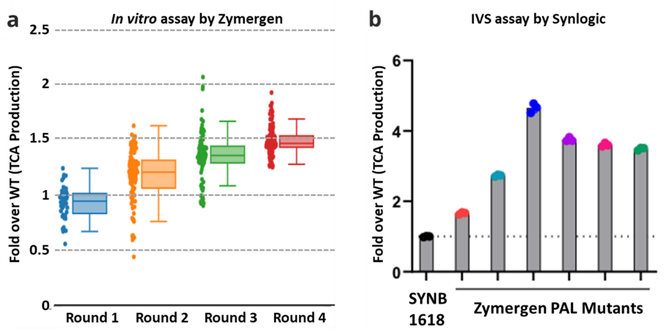

Fig. 2. (**a**) PAL variants with increased TCA production were enriched in four iterative rounds of evolution. (**b**) Fermenter produced cells were tested in the *in vitro* gastric simulation (IVS) model. TCA production was determined for cells incubated under microaerobic conditions in simulated gastric fluid containing Phe.

Key engineered genetic elements of SYNB1934 including key residues involved in increased PAL activity

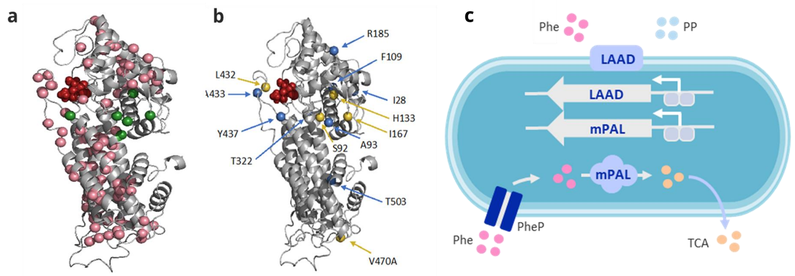

Fig. 3. A homology model of PAL was constructed, and active site residues were identified. **(a)** Active site residues (red), residues mutated in the initial library are highlighted (green), and additional mutations targeted during combinatorial mutagenesis (pink) (**b**) Active site residues (red), residues mutated in SYNB1934 (S92G, H133M, I167K, L432I, V470A) are highlighted in yellow, and residues mutated in other top hits (blue). **(c)** SYNB1934 contains chromosomally integrated genes encoding: PheP, a high affinity Phe transporter, four copies of mPAL, phenylalanine ammonia lyase from *Photorhabdus luminescens* with the engineered mutations (converts Phe to TCA), and LAAD, L-amino acid deaminase (converts Phe to phenylpyruvate, PP). Regulation of PheP and mPAL is carried out by IPTG inducible promoters and LAAD is L-arabinose inducible promoter. For biocontainment, the strain is a diaminopimelate (DAP) auxotroph.

SYNB1934 demonstrated higher activity *in vitro* and increased biomarkers *in vivo*

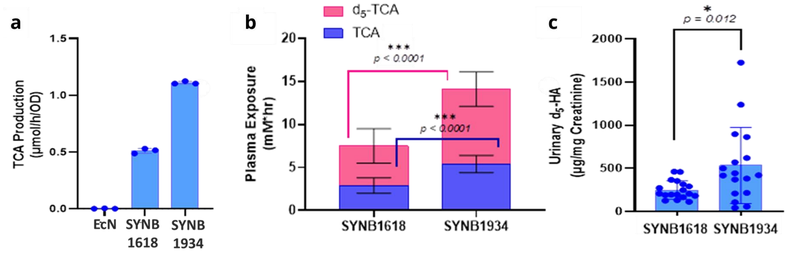

Fig. 4. (**a**) TCA production of fermenter produced cells grown under reduced oxygen conditions demonstrated that SYNB1934 is capable of consuming higher levels of Phe compared to SYNB1618 *in vitro* and the increase is due to expression of the evolved mPAL enzyme. Non-human primates (NHPs) were dosed orally with peptide + d_5-Phe bolus followed by dose of 1 x 10^{11} SYNB1618 or SYNB1934 cells. Plasma areas under the curve (AUCs) for strain-specific biomarkers TCA and d_5-TCA (**b**) and urinary d_5-HA normalized to creatinine (**c**) demonstrated a significant 2-fold increase in plasma TCA and d_5-TCA as well as urinary d_5-HA concentration with SYNB1934 treatment compared to SYNB1618.

Meal test to determine SYNB1934's ability to consume Phe in the gut

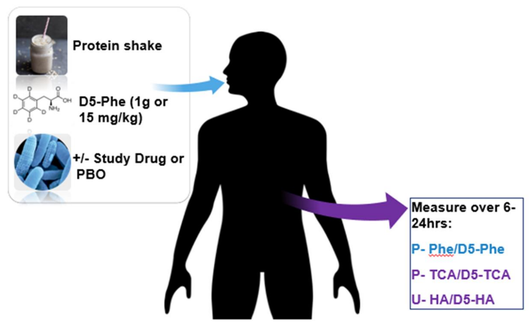

Fig. 5: Graphic representation of the D5-Phe meal test After an overnight fast, HV consumed: a protein shake (20g), D5-Phe (1g) diluted in water, and SYNB1934 within 15 minutes. Blood and urine samples were collected for up to 24 hrs. Plasma D5-Phe and its strain-specific metabolites plasma D5-TCA and urine D5-HA were measured.

SYNB1934 demonstrated dose-dependent Phe consumption in HV

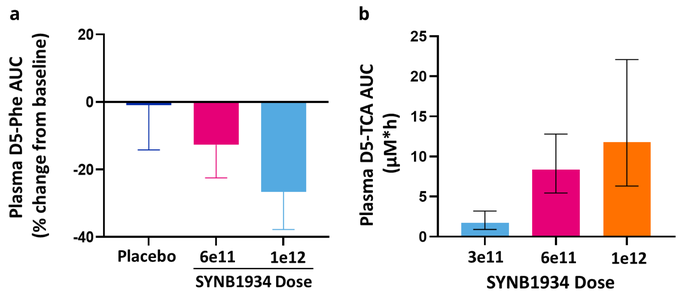

Fig 6. An ongoing phase 1, dose-escalation, placebo- and active-controlled crossover study to assess the safety, tolerability, and pharmacodynamics of SYNB1934 in HV (NCT04984525, n=12) was conducted. Three cohorts of HV each received a different dose of SYNB1934: 3e11, 6e11, and 1e12 cells (or placebo). Subjects were dosed, after meals, 3 times per day on Day 1 and once at breakfast on Day 2. A D5-Phe Meal Test was performed on Day –1 (baseline) and on the last day of dosing. A safety follow-up visit occurred 28 days after the last dose (**a**) SYNB1934 demonstrated dose dependent reduction in plasma D5-Phe AUC in HV, shown as change from baseline (**b**) SYNB1934 demonstrated dose dependent production of strain biomarker D5-TCA AUC in HV.

SYNB1934 demonstrated 2-fold increase in biomarkers compared to SYNB1618 in HV

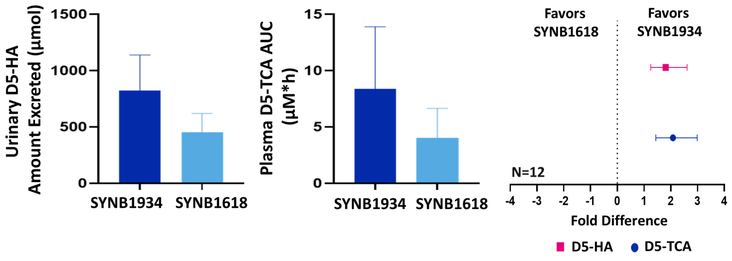

Fig 7. For the crossover study in the phase 1 trial, cohort 2 HVs entered a second treatment period with 6e11cell dosing of SYNB1618 or SYNB1934 (N=6) or placebo (N=2) after a ≥7-day washout period (treatment sequence was randomized). A D5-Phe Meal Test was performed on Day –1 (baseline) and on the last day of dosing. Strain-specific biomarkers: urinary D5-HA (**a**) and plasma D5-TCA (**b**) produced by SYNB1934 both demonstrated a significant 2-fold increase compared to SYNB1618 in HV. (**c**) A comparison of the ratio of biomarker production, D5-HA and D5-TCA, produced by SYNB1618 and SYNB1934 demonstrated 2X activity of SYNB1934 relative to SYNB1618 in HV.

Conclusions

- A specific TCA-responsive allosteric transcription factor biosensor and a large library of PAL variants were developed for initial HTS in whole cell format using microfluidics and FACS.
- Using the directed evolution platform to screen >1M-member combinatorial library, PAL variants were identified with increased activity.
- SYNB1934 was developed by integrating four copies of top PAL in *E. coli* Nissle containing PheP and LAAD.
- In vitro, SYNB1934 demonstrated 2-fold higher TCA production compared to SYNB1618. In non-human primates, SYNB1934 produced increased levels of strain biomarkers compared to SYNB1618.
- In a phase 1 clinical trial, SYNB1934 demonstrated dose-dependent Phe lowering in serum and dose-dependent production of a strain specific biomarker, TCA.
- In head-to-head testing with SYNB1618 in a phase 1 trial, SYNB1934 demonstrated 2-fold higher TCA and HA production compared to SYNB1618 in HV. SYNB1934 is currently being evaluated in PKU patients in the Phase-2 Synpheny trial.