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Minireview

Phenylalanine ammonia lyase (PAL): From discovery to enzyme substitution therapy for phenylketonuria

Harvey L. Levy[a,b,*], Christineh N. Sarkissian[c,1], Charles R. Scriver[d]

[a] Division of Genetics and Genomics, Boston Children's Hospital, 1 Autumn St., Boston, MA 02115, USA
[b] Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA
[c] Rubius Therapeutics, Inc., 325 Vassar St., Suite 1A, Cambridge, MA 02139, USA
[d] Department of Human Genetics, McGill University, Montreal, Quebec H3A 0C7, Canada

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ABSTRACT

Phenylketonuria (PKU) is a genetic inborn error in metabolism that impacts many people globally, with profound individual and societal consequences when left untreated. The journey of phenylalanine ammonia lyase (PAL) from plant enzyme to enzyme substitution therapy for PKU is a fascinating story that illustrates the importance of collaboration between basic scientists and industry in the drug development process. The story begins with the curiosity of plant physiologists about the origin of lignin, a polymer involved in maintaining the rigidity of plants. They learned that the critical element in this synthesis was an intermediary enzyme that deaminates phenylalanine to cinnamic acid and ammonia (later called phenylalanine ammonia lyase or PAL). Recognition of this ability to metabolize phenylalanine led to subsequent consideration of PAL as a treatment for PKU. This was initially attempted as enteral therapy with extracted enzyme, but that showed only minimal efficacy. Crucially, further development of PAL as a therapy for PKU required quantities of enzyme that could only be obtained after successfully cloning the gene, expressing the enzyme *in vitro* and modifying the protein *via* PEGylation to enable parenteral administration of this non-mammalian enzyme. Ultimately, PEGylated PAL was developed as an enzyme substitution therapy for PKU now approved under the name "Palynziq." The multidisciplinary academic-industrial partnership engaged throughout this process has been key to the successful pursuit of this therapeutic possibility and serves as a model for the development of future innovative therapies.

1. Introduction

Phenylketonuria (PKU) is the classical biochemical genetic disorder [1, 2]. It is characterized by a defect in phenylalanine hydroxylase (PAH), which normally catalyzes the conversion of phenylalanine (Phe) to tyrosine. The virtual lack of PAH activity in the presence of a normal diet results in markedly increased concentrations of Phe and Phe metabolites (Fig. 1). High Phe is neurotoxic, producing intellectual disability, often severe, and other neurologic features that can include autistic behavior, seizures, tremors, and ataxia. Phe-restricted dietary therapy lowers the blood Phe concentration to within a non-neurotoxic range and prevents these complications when initiated immediately after identification of the hyperphenylalaninemia (HPA) by newborn screening and subsequent confirmation of PKU. This process has dramatically altered the face of PKU from a profound neurologic disease to one in which affected individuals have normal intelligence and participate in all phases of life [3].

The preservation of optimal intellect and psychological health, however, requires strict adherence to a difficult Phe-restricted diet, perhaps for life [4, 5]. First, restriction of protein is required, often to as little as 10% of the normal requirement. This means that many of the usual foods in a traditional diet, such as meat, chicken, fish, eggs, cheese, and many others cannot be consumed, and common vegetables such as potatoes, corn, peas, and beans are only permitted in very small and measured quantities [6]. Although a number of low protein medical foods may substitute for some of the natural foods, their palatability is not optimal. Secondly, to prevent the nutritional deprivation that would result from this degree of protein restriction, a medical formula that

* Corresponding author at: Boston Children's Hospital, 1 Autumn St., Rm 526.1, Boston, MA 02115, USA.
E-mail addresses: Harvey.levy@childrens.harvard.edu (H.L. Levy), christineh.sarkissian@mail.mcgill.ca (C.N. Sarkissian), Charles.scriver@sympatico.ca (C.R. Scriver).
[1] Former Affiliation: Department of Human Genetics, McGill University, Montreal, Quebec H3A 0C7, Canada.

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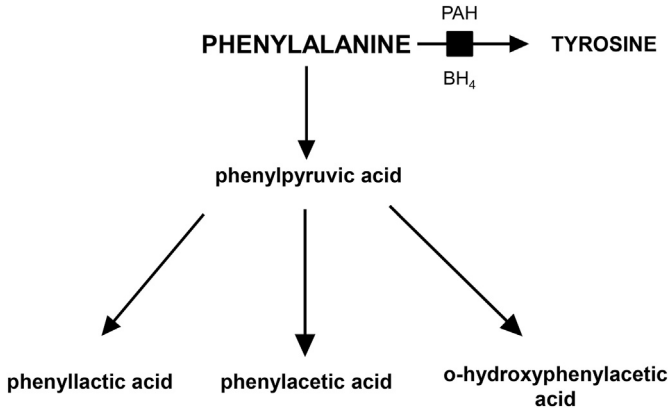

Fig. 1. Phenylalanine metabolism pathway. The phenylalanine metabolism pathway is mediated by phenylalanine hydroxylase (PAH) and the requisite cofactor tetrahydrobiopterin (BH_4). In PKU, PAH is defective, resulting in an increase in the phenylalanine concentration with reduction of tyrosine and presence of phenylalanine metabolites.

completely or largely excludes Phe but contains other amino acids and additional nutrients must be ingested several times a day. These formulas are often malodorous and very unpleasant to the taste [7, 8]. Third, the effect of these dietary restrictions may contribute to social maladjustment, which interferes with adherence to long-term treatment [9]. Consequently, many individuals with PKU do not consistently comply with these rigid dietary requirements, especially during adolescence and adulthood, resulting in blood Phe concentrations well above that required for optimal outcome [10, 11] and causing psychological and emotional difficulties that affect school or work performance, as well as family life [12].

With the multiple dietary-related treatment challenges faced by the patient, developing alternative non-dietary therapies for PKU has been a focus of the metabolic research community for a number of years. The first of these to be approved by the Food and Drug Administration was the drug sapropterin dihydrochloride,[2] a synthetic form of tetrahydrobiopterin (BH_4), the cofactor for PAH (Fig. 1). In some cases, megadoses of sapropterin can reduce blood Phe concentration and allow greater dietary tolerance for protein [13]. However, those with the most severe forms of PKU, who must maintain the most stringent dietary restrictions and are the most vulnerable to complications, rarely respond to this therapy [14] Moreover, even patients with less severe degrees of PKU do not always respond [15] and among those who do, the majority are only partially responsive and get limited relief from the dietary restrictions [16]. Accordingly, more universally beneficial treatments have been considered.

Somatic gene therapies for expression of normal PAH in liver or muscle are being extensively investigated, but these options remain at the experimental stage [17]. Enzyme replacement therapy (ERT), which has been effective for several of the lysosomal storage disorders [18], is not yet possible for PKU because of the instability of PAH as well as its other related complexities [19, 20]. However, enzyme substitution therapy (EST) with phenylalanine ammonia lyase (PAL), an enzyme that degrades Phe *via* a different pathway than PAH, holds promise as a non-dietary way to control the Phe level in PKU. PAL is a non-mammalian enzyme that converts Phe to *trans*-cinnamic acid and ammonia (Fig. 2). *Trans*-cinnamate has shown no embryotoxic effects in laboratory animals [21] and the amount that is generated daily with dietary Phe conversion by PAL (approximately 3 g) is predicted to be harmless to both those with PKU and normal individuals. It is excreted as hippurate in urine along with small amounts of cinnamate and benzoic acids [22, 23]. Finally, the ammonia formed is metabolically insignificant since it can be rapidly metabolized *via* the urea cycle [23, 24].

This paper explores the fascinating journey from the discovery of PAL as a plant enzyme to enzyme substitution therapy for PKU. It is a journey that vividly illustrates the importance of collaboration between basic scientists and industry in drug development.

2. The discovery of PAL

PAL as a plant enzyme is another in the long list of plant substances found to have important application in the treatment of human disease. It was discovered when scientists set out to understand the chemical basis of lignin, a primary structural component of plants. Lignin is the scaffolding around cellulose that allows plants to maintain rigidity (Fig. 3). As such, it is a critical constituent of all plants. As reviewed by the Canadian plant physiologists Brown and Neish [25], the idea that lignin is a polymer composed of phenylpropane (C_6, C_3) units was known for over a century, but the origin of its aromatic structure remained unknown until they identified shikimic acid (a C_6,C_1 monomer) in wheat and maple twigs as a likely precursor [26]. Suspecting that the pathway to lignin included phenylpyruvic acid (a C_6, C_3 monomer and metabolite of Phe), analogous to the *E.coli* pathway that led to the synthesis of Phe [27], they included Phe in their experiment and found that it was as efficient as shikimic acid in lignin synthesis. Subsequently, their group investigated cinnamic acid and L-phenylalanine, both C_6, C_3 monomers, and discovered that each was a good lignin precursor and that they might be interconvertible [26, 28, 29]. Soon afterward, they and others confirmed the role of both Phe and cinnamic acid in lignin synthesis [29] and established that Phe is converted to cinnamic acid in this synthetic pathway [30, 31], (Fig. 4).

During the early 1960s, the precise relationship between Phe and cinnamic acid was investigated by Koukol and Conn [32] who isolated a deaminase that converted L-phenylalanine to cinnamic acid and ammonia. Further study of this phenylalanine deaminase (the nomenclature later changed to phenylalanine ammonia lyase) showed that it was strongly inhibited by tyrosine [33] and was also found in yeast [34–36].

3. PAL for treatment of PKU – encapsulated enteral PAL

The discovery of PAL as an enzyme that could metabolize Phe eventually attracted the attention of those who were aware that the neurotoxicity in PKU was likely to be the result of increased Phe. In 1980, the first attempt was made to treat PKU with PAL by using enteral administration [24]. Although the pH range of the small intestine, typically 6.0–7.4, was lower than the optimal pH for PAL activity (pH > 8), ~50% of maximum activity was nonetheless anticipated with enteral delivery [37]. Hoskins and his group who performed this experiment also recognized the need to protect PAL from proteolytic degradation by digestive enzymes [38, 39]. Accordingly, they loaded the yeast-extracted PAL into semipermeable gelatin capsules and fed this product first to normal individuals with a co-dosed high protein load, and then to a patient with PKU. The mild Phe elevation induced by the protein load in the normal individuals was variably reduced, but the markedly increased blood Phe concentration in the patient with PKU was decreased 23%, from 30 mg/dL to 23 mg/dL. Subsequently, they showed that the cinnamic acid product of the PAL reaction was eliminated by excretion as hippuric acid [21, 23]. The *in vivo* evidence that PAL converted L-phenylalanine to *trans*-cinnamic acid, and the fact that the products (ammonia as well as trans-cinnamic acid) generated by this “therapeutic conversion” were metabolically manageable and harmless in an otherwise healthy patient, set the stage for the use of this enzyme as a substitute for PAH in the treatment of PKU.

As Hoskins was not a physician, we were intrigued as to how he would have: (i) known about PKU; (ii) considered PAL as a possible treatment; and, even more importantly, (iii) gained access and

[2] Kuvan (BioMarin Pharmaceutical, Inc., Novato, CA 94949).

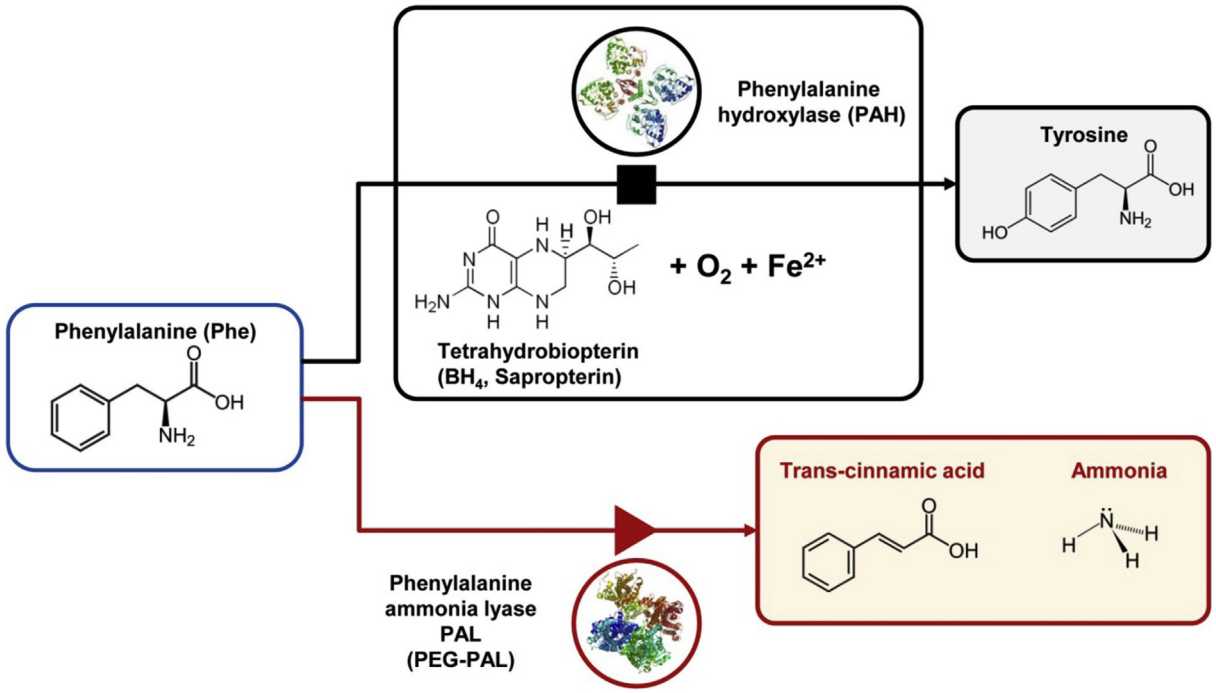

Fig. 2. Phenylalanine ammonia lyase (PAL) is an alternative enzyme that can substitute for PAH by reducing the phenylalanine concentration in PKU. As a lyase (or deaminase) PAL removes the amine (NH_2) and a proton (H^{+}) from phenylalanine to form ammonia (NH_3) leaving a deaminated and desaturated phenylalanine (*trans*-cinnamic acid). The *trans*-cinnamic acid is converted to benzoic acid which is conjugated with glycine in the liver and excreted as hippuric acid (benzoylglycine) while the ammonia is metabolized *via* the urea cycle and largely excreted as urea. PAL is a non-mammalian protein, so has been PEGylated (PAL-PEG) to reduce its immunogenicity.

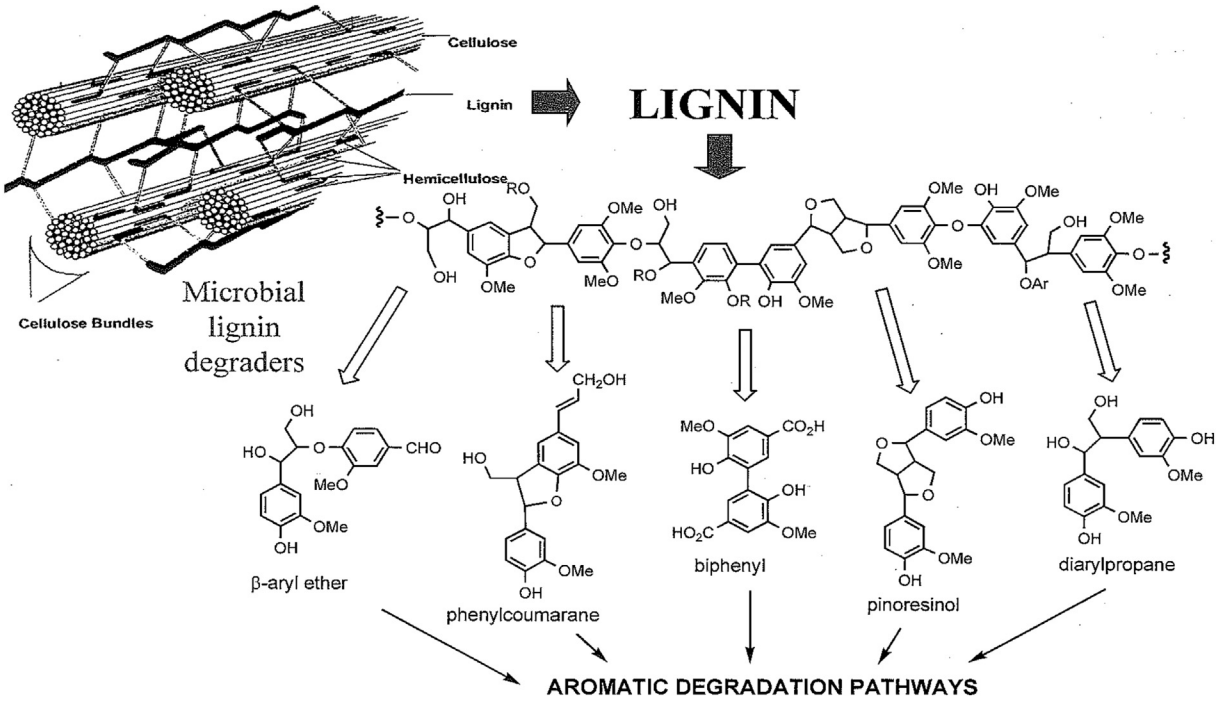

Fig. 3. Lignin forms scaffolding around the cellulose bundles of plants, serving to maintain the rigidity of plants (trees, branches, stems, and leaves). It is a polymer, ultimately composed of phenylpropane (C_6C_3) monomers.

permission to administer PAL to a patient. Fortunately, we were able to locate Dr. Hoskins, now retired, through the Medical Research Council (MRC) Toxicology Unit in Carshalton, England where this work was undertaken; he graciously told us the story.

Seven years prior to his 1980 publication (24), Hoskins had joined a unit of the MRC as an organic chemist with a particular interest in analytical techniques. His assigned task was to examine the possibility of metabolic differences between the two phases of bipolar disorder. The laboratory he joined, however, shared space with the regional newborn PKU screening program and Dr. Rodney Pollitt, an international authority on neonatal screening, was his supervisor. Dr. Pollitt stimulated an interest in determining unidentified differences in amino

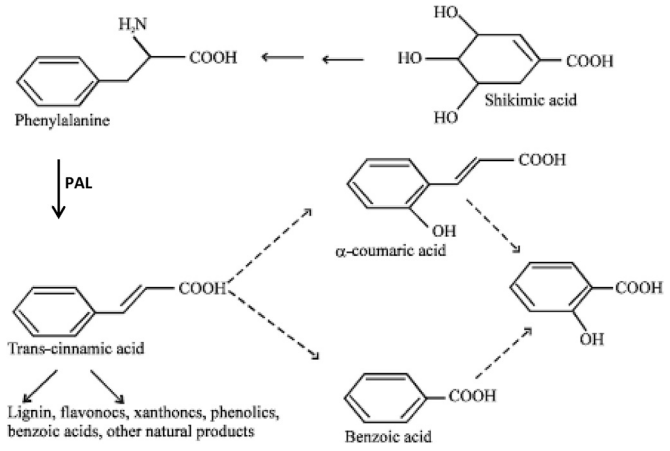

Fig. 4. Pathway of shikimic acid through phenylalanine which is converted by PAL to trans-cinnamic acid. The cinnamic acid is reduced to its alcoholic derivatives which serve as the monomers for the lignin phenylpropanoid polymer.

acid metabolism among those with PKU and instructed Hoskins to look for any such differences in urine specimens from these individuals. Hoskins showed that unlike normal individuals, those with PKU did not produce meta-hydroxyacetic acid and, therefore, concluded that the production of this metabolite required PAH activity [40]. Shortly thereafter, funding for this research was discontinued and Hoskins moved to the MRC Toxicology Unit in Surrey, a civilian offshoot of the Porton Down World War II chemical warfare research facility. Adjacent to the Toxicology Unit were the Porton Down Microbiological Laboratories, which had been commercialized as the Public Health Laboratory Services (PHLS) and housed the regional newborn screening program in that section of greater London. When Hoskins arrived, he was encouraged to continue his work on PKU until a new area of work in toxicology could be found. Meanwhile, he learned that the director of the PHLS, Dr. Henry Wade, had an enzyme called phenylalanine ammonia lyase (PAL), which Wade thought could be of interest in PKU treatment. Wade knew that PAL metabolized Phe and that PKU required Phe reduction. Accordingly, Hoskins began to explore the use of PAL as a potential treatment for PKU (mentioned in the original article was earlier work at Glaxo, now part of GlaxoSmithKline pharmaceutical company, that showed the capability of PAL to deplete Phe derived from ingested food protein in dogs).

To begin his work, Hoskins had to learn how to formulate and prepare the enterically coated capsules used at Glaxo in the dog experiment. However, before using encapsulated PAL on a patient with PKU, he was required to investigate the safety and impact of the formulation on normal individuals. For this, he conducted his protein-feeding experiment (a large steak!) with encapsulated PAL administration on 15 normal human volunteers [24]. Finding no adverse events, he was then allowed to perform the experiment on a patient with PKU. At Dr. Wade's suggestion, Hoskins contacted Dr. Jan Stern, director of metabolism at St. Mary's Hospital for Children, which happened to be adjacent to the Toxicology Unit where he worked. Through Dr. Elaine Wright, senior consultant psychiatrist, Dr. Stern arranged access to the PKU patient whom Hoskins studied in his classic experiment to treat PKU with PAL. Several of those individuals noted above are co-authors of the original paper [24].

4. Parenteral PAL for PKU – immobilized enzyme in extracorporeal reactor

In the late 1970's, investigators at Yale University and Roswell Park Memorial Institute in Buffalo, New York [41, 42] had begun considering parenteral PAL as a direct and perhaps effective approach to reducing circulating Phe in PKU. They knew that since PAL was a non-mammalian protein, it would be highly immunogenic and rapidly cleared from the blood with repeated administrations [43]. To circumvent this problem, they devised an innovative method of isolating PAL in the porous shell of a multitubular enzyme reactor. This extracorporeal enzyme-containing device allowed PAL access to the free Phe in the blood, but prevented it from entering into systemic circulation [41, 42, 44, 45]. Following their initial experiments, Ambrus and collaborators applied the extracorporeal enzyme reactor to a patient with PKU, and after three rounds of hemodialysis observed a Phe decrease of 30%, from the initial level of 1820 μM (30 mg/dL) to 1270 μM (21 mg/dL) [46]. The limited reduction was partially explained by the marked loss in enzyme activity in the reactor; PAL activity in this state was only 1–3% of that measured with free enzyme [44]. However, beyond the limited efficacy was the fact that this approach was far too invasive to even be considered for PKU treatment. Larue et al. in France [47] also developed an extracorporeal enzyme reactor for similar use, but it was never applied to a patient with PKU.

5. Recombinant PAL toward the generation of pegylated-PAL – An academic-industry partnership

In 1994, Christineh Sarkissian joined the laboratory of Charles Scriver at McGill University (Canada), marking a critical event in the development of PAL as a treatment for PKU. From 1994 to 2001 Sarkissian and Scriver, together with scientists from IBEX Technologies in Montreal, cloned and expressed the PAL gene providing an economically feasible supply of the PAL enzyme. They then provided proofs of the pharmacological and physiological principles for both enterally and parenterally administered recombinant PAL formulations in the treatment of PKU [48].

5.1. Before recombinant PAL

Before Sarkissian's arrival, however, a group led by T.M.S. Chang (a McGill University researcher and physician) had also examined enteral PAL for PKU treatment. Chang had pioneered a semi-permeable microencapsulation technique to protect enzymes from proteolysis while allowing access of micromolecules such as amino acids for metabolic conversion [49]. He and his collaborators thought that their encapsulation of PAL might work as enteral therapy despite evidence that PAL in their capsules was only 20% as active as the free enzyme [50]. Their protocol was a more sophisticated reevaluation of the encapsulated PAL method that Hoskins et al. [24] had originally examined as enteral therapy for PKU.

Chang and his team began their work in 1985. Using commercially obtained PAL from natural sources, they microencapsulated the enzyme and studied its effect on Phe levels in rats rendered hyperphenylalaninemic by Phe loading and the administration of a PAH inhibitor, *p*-chlorophenylalanine. They reported the depletion of plasma Phe up to 75% [51, 52], as well as substantial reduction in intestinal Phe [53]. However, a later study using the ENU2 mouse, a true genetic and biochemical model for PKU, failed to show a significant reduction in the plasma Phe concentration [54].

5.2. Recombinant PAL

When Sarkissian joined the Scriver laboratory at McGill, IBEX Technologies, under the leadership of Dr. Robert Heft, had become interested in engineering PAL for PKU treatment. The goal was to generate a variant candidate molecule that retained its integrity and function while safely and efficaciously reversing hyperphenylalaninemia. IBEX and the Scriver lab were joined under a novel government, MRC-funded, academic-industrial collaborative effort headed by the Network of Centres of Excellence - Canadian Genetic Diseases Network. Both IBEX Technologies and the Scriver lab became critical players, scientifically and fiscally, and their combined efforts resulted in a

collaboration that proved to be very fruitful.

The task assigned to Sarkissian along with the team at IBEX was to investigate the potential application of PAL as a therapy for PKU. The first step in the process was to obtain large amounts of expressed enzyme, which they did by employing the cloning and expression methods described by Gilbert et al. [55] and Orum and Rasmussen [56]. The next step was to demonstrate the *in vivo* effectiveness of the recombinant PAL. Using the $Pah^{enu2/enu2}$ mouse model for PKU and a new heteroallelic $Pah^{enu1/enu2}$ mouse model [57], they showed that intraperitoneal injections of recombinant PAL lowered blood Phe to therapeutically significant levels even in the most severely affected mice. In addition, both enteral enzyme administration expressed in a non-pathogenic *E. coli* organism using the cell itself to provide a protective environment for PAL and the free enzyme co-administered with aprotinin (a protease inhibitor), significantly reduced the blood Phe concentration in these mice [48]. However, the required long contact time with Phe in the intestine and the low activity of the oral formulations meant that very large amounts of the enzyme would be necessary for enteral treatment. It was apparent that oral PAL needed further development and the technology to make it therapeutically viable was not yet at hand.

In 2001 BioMarin Pharmaceutical,[3] under the leadership of Dr. Emil Kakkis, acquired the PAL project from IBEX. The McGill team now began to work with this young California company interested in developing a parenteral PAL formulation. They initially showed that injected PAL resulted in substantial lowering of plasma Phe in the murine model, as well as a significant reduction in brain Phe [58], but the effect was not sustained when repeated injections induced an immune response and resultant inactivation of the enzyme, as had been reported in their earlier work [48].

5.3. PEGylated and mutant PAL forms

Seeking ways to counteract this immunity led further to the enlistment of Dr. Raymond Stevens and his team at The Scripps Research Institute in La Jolla, California to the project. Stevens and colleagues had determined the atomic structure of PAH in 1997 [59], and had subsequently begun to apply that structural knowledge to produce protected forms of PAH for ERT in PKU [20]. While the concomitant requirement of BH_4 for PAH efficacy and inherent instability of PAH made this a complicated venture with uncertain viability as a therapeutic, their experience with introducing chemical modifications on PAH would soon be key to the development of modified PAL. Utilizing knowledge of the crystal structures of PAH [59] and PAL [60], the Scripps team were able to apply structure-based molecular engineering to attach polyethylene glycol (PEG) molecules to stabilize and protect the PAL protein. Further, they applied their understanding of protein structure and activity to create and test many mutations, and mutation combinations, for their ability to improve the protein's characteristics – all in search of an appropriate formulation for a potentially injectable enzyme *substitution* treatment for PKU [61, 62].

Ultimately, in order to optimize PAL for the best therapeutic outcome, multiple enzyme species, including PALs from *Rhodosporidium toruloides, Anabaena variabilis*, *Nostoc punctiforme*, and *Petroselinum crispum* were studied [63–65]. Pharmacological *in vivo* examinations of PEGylated derivatives of PAL (PEG-PAL) from each wild-type species as well as structurally engineered mutants were then conducted by the team at McGill. These formulations generally provided short- and long-term reversal of hyperphenylalaninemia in both blood and brain tissue of the PKU mice in a dose-dependent manner, with diminished manifestations associated with hyperphenylalaninemia. Each formulation, however, had its own defined and valuable characteristics. After multiple rounds of *in vitro* and *in vivo* study, the PEGylated double mutant (C503S, C565S) form of PAL from the filamentous cyanobacteria, *Anabaena variabilis* (*Av*), was finally chosen to enter human trials [65]. While this mutant did not have the highest specific activity, it did have the most favorable biochemical and structural specifications (*i.e.* enhanced thermal stability and resistance to proteolytic cleavage) and promise to reverse hyperphenylalaninemia in patients [64]. Notably, a subsequent preclinical study in the PAH^{enu2} mouse model for PKU suggested that treatment with PEGylated PAL might partially restore brain tyrosine hydroxylase, lending hope that treatment with this enzyme might have a positive effect on the neuropathology of PKU [66].

6. Clinical trials with PEGylated PAL

Since PEG-PAL might reduce or even normalize the elevated Phe level without diet in patients with any degree of PKU, its potential as treatment was extremely attractive. Consequently, after successful preclinical efforts on PEG-PAL were completed in 2007, BioMarin applied to the FDA to begin human trials and received approval. Clinical trials with PEG-PAL (originally renamed pegvaliase following completion of the Phase I trial) have been ongoing since 2008. Results from these trials have consistently reported a substantial reduction of plasma Phe in the majority of participants following a period of titrated application of the enzyme. Subcutaneous administration of a single dose of PEG-PAL was apparently safe and well-tolerated in adult patients with PKU who participated in the Phase I trial [67]. Phase II trials further reported substantial and persistent long-term decrease in mean plasma Phe, with the treatment well-tolerated by most subjects, showing only grade 1 or 2 adverse events. All subjects developed a sustained antibody response against PAL and most subjects developed a transient antibody response against PEG [68]. The Phase III trials added to these findings by measuring improvements in attention scores compared to pre-treatment baseline for subjects who continued long-term treatment and follow-up. Subjects with the greatest blood plasma Phe reduction from pre-treatment baseline had the greatest change in attention deficit hyperactivity disorder rating scale (ADHD-RS) inattention scores [69]. This information very recently appeared in two publications in this journal [70,71].

On August 29th, 2017, the FDA accepted BioMarin's Pegvaliase Biologics License Application (BLA) and granted Priority Review Designation [72] and on May 24th, 2018 the FDA approved Palynziq™ (pegcaliase-pqp3) in the United States as enzyme therapy for PKU [73]. On March 28th, 2018 the European Medicines Agency (EMA) accepted BioMarin's submission of a Marketing Authorization Application (MAA) for Pegvaliase [74].

7. Conclusion: importance of academic-industrial partnership in drug discovery

From the discovery of PAL, to Hoskins pioneering work, to the McGill and IBEX collaboration, and to the collaborations of BioMarin with Scripps and McGill, PEG-PAL has now been approved in the United States under the name Palynziq™ as an enzyme substitution therapy (EST) for PKU. The long and complex development of PAL as a promising enzyme therapy that could benefit thousands of PKU patients would not have been possible without this close and collegial relationship between academic and industrial scientists with their varied interdisciplinary approaches and the fiscal support of industry. Each element was key to the successful outcome, and this multidisciplinary, academic-industrial approach, which has quickly become a standard model for more recent drug development efforts, is also lending itself to second generation drug development activities in varied platforms such as cell and gene therapies.

Conflict of interest and financial disclosure

HLL is an investigator in clinical trials of BioMarin Pharmaceutical,

[3] BioMarin Pharmaceutical, Inc., Novato, CA 94949.

Inc. and serves on their advisory boards. RCS was a consultant and on the Scientific Advisory Board to BioMarin Pharmaceuticals from 2002 to 2012.

Competing interests

None of the authors have competing interests.

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