Precision Medicine in Rare Diseases

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The own patient-derived cells can be used to perform personalized pharmacological screening in genetic rare diseases. For precision medicine to be successful at the therapeutic level, in addition of the information provide from genomics, pharmacogenomics, metabolomics and proteomics, our proposal argues that it is also necessary to know the cellular response, and therefore the behavior of particular mutations in vitro, to various therapeutic options. Precision medicine relies on the assumption that different mutations and marked inter-individual genetic variation can contribute significantly to drug response. The goal of personalized medicine is to maximize the probability of therapeutic efficacy for an individual patient.

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1. Precision Medicine in Rare Diseases

Despite the important emphasis placed on the investigation of rare diseases and the development of orphan drugs by national governments, the pharmaceutical industry and private foundations, there are no adequate treatments for approximately 95% of rare diseases. The advance of genomics and functional proteomics has placed medicine in recent years at the gates of a new revolution, precision medicine, characterized by the development of molecular, genetic and cellular therapies that materialize in specific treatments for particular patients [1]. The rationale for this approach is that different mutations and inter-individual genetic variability can influence significantly both the disease sensitivity and the response to particular pharmacological therapies. The goal of personalized medicine is to maximize the likelihood of therapeutic efficacy and minimize the risk of drug toxicity for an individual patient.

The last decade has witnessed a rapid acceleration in our understanding of the genetic basis of many diseases. With this greater understanding comes the possibility of redefining the disease at a higher resolution and, along with this, aiming for a more precise therapy.

The precision medicine strategy has been successfully applied in different areas of medical care such as cardiology, oncology and nutrition and has a hopeful future in rare diseases [2]

2. Braincure/Mitocure/Myocure Platforms

Our research group has developed three platforms for performing precision or personalized medicine in rare diseases: Braincure platform for rare neurodegenerative diseases with brain iron accumulation; Mitocure platform for mitochondrial diseases; and Myocure platform for congenital myopathies.

2.1. Braincure Platform

Neurodegeneration with brain iron accumulation (NBIA) is a group of rare neurodegenerative disorders of genetic origin, characterized by a dysfunction of the central nervous system and the accumulation of iron in certain areas of the brain that causes the progressive disability of patients [3]. Most NBIAs begin clinically in childhood and are inherited with an autosomal recessive pattern. Currently, there are no effective treatments for the great majority of these diseases.

The objective of this platform is to deepen the pathophysiology of the disease and find effective personalized treatments using fibroblasts and neuronal cells derived from NBIA patients. For this, we characterize the pathophysiological mechanisms and evaluate the effectiveness of a library of commercial pharmacological compounds in the recovery of pathological alterations in the patient-derived cells.

In a first stage, pharmacological screening is carried out in fibroblasts derived from NBIA patients. For high throughput screening, a library of 426 drugs approved by the U.S. FDA (United States Food Drug Administration) from Selleck Chemicals (Houston, TX, USA) including a selection of compounds frequently used in NBIA patients treatment (iron

chelators, antioxidants, pantothenate, creatine etc.) are routinely evaluated. As iron accumulation can be easily detected in fibroblasts derived from NBIA patients by Perls' Prussian blue reaction [4], drug hits are identified by a >75% reduction of intracellular iron accumulation. To confirm the defect rescue in NBIA mutant fibroblasts, in deep evaluation of iron metabolism alterations, expression levels of mutant enzymes, mitochondrial dysfunction, oxidative stress, lipid metabolism and spontaneous and induced apoptosis are examined in NBIA fibroblasts after treatment with selected favorable compounds. In parallel, INs are generated by direct reprograming from patient fibroblasts. The most favorable compounds in fibroblasts screening are selected for testing in mutant iNs.

2.1.1. Strategy for the Identification of Effective Treatments for Neurodegeneration Associated with Pantothenate Kinase (PKAN)

The most prevalent NBIA subtype is the neurodegeneration associated with pantothenate kinase (PKAN) due to mutations in the enzyme pantothenate kinase 2 (PANK2), which participates in the first reaction of the coenzyme A biosynthesis pathway. From the pathophysiological point of view, these mutations cause deficiency of coenzyme A, accumulation of iron and lipofuscin and a marked increase in oxidative stress. Mutations also cause low levels of expression of the mutant enzyme [5]. Our findings indicate that pantothenate treatment upregulates protein expression levels of PANK2 in fibroblasts obtained from patients carrying particular mutations. In addition, we have confirmed that pantothenate had also a favorable effect in iNs produced by direct reprogramming of patient skin cells. The restoration of PANK2 expression levels was accompanied by the correction of all the pathophysiological alterations such as coenzyme A deficiency, iron and lipofuscin accumulation, bioenergetics failure and increased oxidative stress [5]. These results suggest that pharmacological screening in cellular models can be a useful tool to identify PANK2 mutations with residual enzymatic activity that respond to pantothenate supplementation. Even more important, the existence of PANK2 residual expression that can be significantly corrected in cells obtained from patients points out to the possibility of an effective treatment with pantothenate at high doses. This hypothesis must be corroborated by comparing both the effect of pantothenate in vitro and in patients in controlled clinical trials. The knowledge of the multiple types of PANK2 gene mutations and their response to pantothenate supplementation gives new opportunities for the implementation of precision personalized therapies in PKAN.

In addition, these personalized detection strategies in PKAN can facilitate the identification of new pharmacological chaperones (PC) able to restore the expression levels and activity of the dysfunctional enzyme. Preliminary results from our group have detected several commercial drugs, which are able to correct PANK2 expression levels as well as most of the physiopathological alterations in fibroblasts derived from PKAN patients.

A large number of mutations related to human diseases cause the destabilization of specific proteins. Interestingly, molecules that function as PC can rescue the activity of unstable proteins [6][7][8]. However, in a specific disorder, PC therapy will be adequate depending on its genotype [9]. Corroborating this hypothesis, our findings have shown that many mutations in PANK2, but not all, can respond positively to pantothenate supplementation [5][10]. Therefore, a strategy to identify more PCs capable of correcting PANK2 expression and activity in cells such as fibroblasts or/and induced neurons can lead to potential treatments in particular patients. Following this strategy, several drugs have been already repositioned as a PC for rare diseases treatment [11]: doxorubicin, an antitumor anthracycline, for cystic fibrosis [12]; diltiazem, an antihypertensive, for Gaucher's disease [13]; ambroxol, a mucolytic agent, for Fabry and Gaucher disease [14]; acetylcysteine, another mucolytic agent, for Pompe disease [15]; pyrimethamine, an antiparasitic drug, for GM2 gangliosidosis [16]; carbamazepine, a dibenzazepine, for hyperinsulinemic hypoglycemia [17]; and salicylate, a known anti-inflammatory, for Pendred syndrome [18]. Recently, a PC allosteric activator of PANK (PZ-2891) has been identified [19]. Interestingly, PZ-2891 crosses the blood-brain barrier and improves the phenotype in a mouse model with cerebral coenzyme A deficiency.

2.1.2. Precision Medicine in PKAN

The implementation of precision personalized medicine for the identification of potential treatment of neurodegenerative disorders such as PKAN seems to be very promising in contrast to the traditional "single drug for all patients" strategy [20]. In fact, neurodegenerative disorders can have variable clinical characteristics even in patients with the same mutation; therefore, it is very unlikely that all the patients will respond to a single drug. In this context, a precision medicine approach using fibroblasts and iNs derived from patients with PKAN may represent an attractive opportunity to find effective treatments.

Braincure platform performs precision medicine in the most prevalent NBIA disorders: PKAN, pantothenate kinase-associated neurodegeneration, caused by mutations in the PANK2 gene; PLAN, PLA2G6-associated neurodegeneration, due to mutations in the PLA2G6 gene; BPAN, Beta-propeller protein-associated neurodegeneration, caused by mutations in the WDR45 gene; MPAN, Mitochondrial-membrane Protein-associated Neurodegeneration, due to mutations in the

C19orf12 gene; and FHAN, Fatty acid hydroxylase-associated neurodegeneration, caused by mutations in the FAH2 gene). We are currently performing personalized medicine in more than 50 patients from both Spain and abroad (Brazil, Colombia, Mexico, USA, France, United Kingdom, Holland, Hungary and Poland).

2.2. Mitocure Platform

Mitochondrial diseases include a group of chronic and progressive muscular and neurodegenerative disorders caused by a great variety of mutations in nuclear (nDNA) or mitochondrial DNA (mtDNA), most of which have no effective treatment [21]. These diseases have a great heterogeneity and fundamentally affect the energy production capacity of the cells.

Current pharmacological therapies are based primarily on: (1) Eliminate toxic metabolites; (2) Try to circumvent the blockages of the respiratory chain; (3) Administer metabolites and cofactors to improve the synthesis of ATP; and (4) Prevent oxidative stress [22].

Given the diversity of mutations and the different therapeutic options, our proposal argues that a personalized therapeutic approach is required in mitochondrial diseases. In the Mitocure platform, we evaluate the therapeutic effectiveness of currently available treatments in fibroblasts derived from mitochondrial patients and in neuronal cells generated by direct reprogramming. To achieve this objective, we study the effects of these treatments on the pathophysiological alterations present in mutant cells. As a screening strategy, we examined cell proliferation and/or cell death in galactose culture medium (which forces energy to be obtained by the mitochondria) [23][24], using an automated platform for live-cell imaging (Celldiscoverer 7, Zeiss). Combined analyses of phase-contrast and fluorescence images allow assessment of treatment effects on cell proliferation as well as the extent and kinetics of cell death. A pharmacology library of compounds frequently used in the treatment of mitochondrial patients is evaluated: antioxidants, AMP-activated protein kinase (AMPK) activators, autophagy/mitophagy modulators, mitochondrial dynamics modulators; inflammasome inhibitors; mitochondrial unfolded protein response (UPR^{mt}) activators; and combinations of several treatments.

Next, positive compounds are confirmed by assessing their effect on mitochondrial respiratory chain activity, expression levels of mitochondrial proteins, mitochondrial membrane potential, oxidative stress, and the activation of mitophagy and/or apoptosis in fibroblast and iNs. The relevance of cellular models derived from patients with inherited mitochondrial disorders for pathomechanistic studies and evaluation of therapies have been previously explained by the group of Ann Saada [25][26]. Currently, Mitocure platform is performing precision measurement in more than 20 mutations that directly affect mitochondrial oxidative phosphorylation.

2.3. Myocure Platform

Congenital myopathies are a group of genetic muscle diseases that are classified based on the histopathological characteristics observed in muscle biopsy [27]. They are subdivided by the predominant structural pathological change on muscle biopsy, resulting in five subgroups [28]: (1) core myopathies; (2) nemaline myopathies; (3) centronuclear myopathies; (4) congenital fiber-type disproportion myopathy; and (5) myosin storage myopathy. To date, only supportive treatments are available.

Nemaline myopathy (NM), which is part of this group of diseases, was described for the first time as a non-progressive congenital musculoskeletal disorder, characterized by the presence of inclusions in muscle fibers called “nemalinic rods” [29]. This pathology contains a wide genetic heterogeneity that produces a similar phenotype [30]. Its incidence is 1 in 50,000 live births; although in our country the incidence of this pathology is unknown, since in clinical practice it is a rare disease [31]. Mutations have been found in more than 10 different genes that cause the disease; seven of which code for sarcomeric thin filament components (NEB, ACTA1, TPM2, TPM3, TNNT1, CFL2, LMOD3) and 3 genes (KBTBD13, KLHL40 and KLHL41), belonging to the BTB-BACK-kelch family of proteins (BBK) involved in the ubiquitin-proteasome pathway [32].

The objectives of the Myocure platform are: (1) To establish cellular models to understand the pathophysiological mechanisms of NM. (2) To identify potential therapies by developing a pharmacological screening methodology in cell models of NM.

As a screening strategy, we assess the correct formation of actin filaments by fluorescence microscopy techniques using the CellDiscoverer7 Image Analysis platform (Zeiss). Positive compounds are those that are capable of restoring the correct formation of actin filaments. Next, favorable compounds are confirmed by verifying the improvement of all pathophysiological alterations. A collection of compounds are routinely tested; phalloidin (stabilizer of actin filaments); several combinations of amino acids (tyrosine, carnitine, taurine, or creatine); salbutamol (selective ß2-adrenergic agonist

of bronchial smooth muscle); Ras homolog family member A (Rho) stimulators (forskolin); Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitors (Y-27632 2HCl); myostatin inhibitors (anti-myostatin antibodies, ACE-031); follistatin activators (natural myostatin inhibitor); UPR stimulators: nicotinamide.

Currently, the Myocure platform is performing precision medicine in 8 patients with nemaline myopathy. In a second phase, if the results are positive in the pharmacological screening in fibroblasts, we will confirm the positive findings in skeletal muscle cells generated by direct reprogramming of patients' fibroblasts [33].

This mutation-specific therapeutic approach can potentially be applied for other hereditary muscular diseases such as Duchenne muscular dystrophies (DMD) and facioscapulohumeral muscular dystrophy. In the particular case of DMD, as molecular treatments aimed at dystrophin restoration are increasingly available as commercialized drugs or within clinical trials, genetic diagnosis has become an indispensable tool in order to determine eligibility for specific treatments [34]. Thus, DMD patients harboring deletions in exon 44, 45, 51 or 53 may be eligible for inclusion in one of several ongoing clinical trials of exon skipping. Patients harboring nonsense mutations that cause the synthesis of the dystrophin protein to stop prematurely may be eligible for treatment with ataluren, which promotes ribosomal read-through of premature stop codons [34]. It is reasonable to propose that particular mutations should be also evaluated at the cellular level and confirm in vitro the effectiveness of potential treatments to minimize the frequency of non-responder patients.

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