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CELL-PERMEABLE PROTEIN THERAPY FOR COMPLEX I DYSFUNCTION

Salvatore Pepe¹, Robert M. Mentzer Jr.², and Roberta A. Gottlieb³

¹Heart Research, Murdoch Children's Research Institute, The Royal Children's Hospital, Melbourne, Australia, and Department of Paediatrics, University of Melbourne, The Royal Children's Hospital, Melbourne, Australia

²Department of Surgery, Wayne State University School of Medicine, Detroit, Michigan, USA

³Cedars-Sinai Heart Institute and the Barbra Streisand Women's Heart Center, Cedars-Sinai Medical Center, Los Angeles, California, USA

Abstract

Complex I deficiency is difficult to treat because of the size and complexity of the multi-subunit enzyme complex. Mutations or deletions in the mitochondrial genome are not amenable to gene therapy. However, animal studies have shown that yeast-derived internal NADH quinone oxidoreductase (Ndi1) can be delivered as a cell-permeable recombinant protein (Tat-Ndi1) that can functionally replace complex I damaged by ischemia/reperfusion. Current and future treatment of disorders affecting complex I are discussed, including the use of Tat-Ndi1.

The Importance of Complex I

The mammalian NADH:ubiquinone oxidoreductase (mitochondrial protein Complex I, EC1.6.5.3), a 900 kDa multiprotein complex of 45 subunits, facilitates the major entrance point of electrons into the mitochondrial respiratory chain[1-3]. Electrons arising from oxidation of NADH to NAD⁺ at Complex I are carried by the mobile electron carrier ubiquinone (Coenzyme Q₁₀) to Complex III then transferred via the Q redox cycle to cytochrome c and finally to Complex IV where O₂ is reduced to form 2H₂O. Coupled by redox reactions to this flow of electrons is the translocation of protons out of the matrix across the inner membrane at complexes I, III and IV, which generates a gradient that drives an influx of protons back to the matrix through Complex V (F₁F₀ ATP synthase), permitting phosphorylation of ADP to form ATP. Thus, considering the estimate that the average person turns over ~65kg ATP per day [4], a global dysfunction or deficiency of Complex I activity may have debilitating and potentially fatal consequences.

Genetic Disorders of the Respiratory Chain & Complex I

Respiratory chain disorders have been reported at an incidence of 1:5000 births with Complex I deficiency being the most common [5]. Complex I has 7 highly hydrophobic

subunits encoded by mitochondrial DNA (ND1 to ND6, ND4L; ND = NADH dehydrogenase), and 38 subunits encoded by nuclear DNA [2]. Mutations of these subunit-encoding genes have been identified as causes of Complex I dysfunction and are associated with a wide array of degenerative and fatal disorders that first present at birth or during infancy, including Leigh Syndrome (the most common), Lethal Infantile Mitochondrial Disease (LIMD), Leber Hereditary Optic Neuropathy (LHON), Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-Like Episodes (MELAS) [2].

Crucial to the proper functioning of Complex I are highly coordinated and regulated processes for correct synthesis and assembly of mitochondrial or imported nuclear subunits, with some 13 Complex I assembly factor proteins identified to date. Complex I-deficient patients have been identified with mutations in 9 different Complex I assembly factors, [6-10,5,11,12]. In addition, maintenance of Complex I assembly and stability requires apoptosis-inducing factor (AIF), a flavoprotein with pyridine nucleotide-disulfide oxidoreductase and DNA binding domains, which is normally located in the mitochondrial intermembrane space and associated with the inner membrane [13].

Although genetic Complex I disorders can affect single organs (e.g. LHON), effects are generally widespread, with organs having a high demand for ATP being particularly susceptible (e.g. brain, skeletal muscle, heart). Presenting phenotypes include neonatal lactic acidosis, myopathy, or encephalopathy. In severe conditions of Leigh Syndrome, central nervous system neurodegeneration involves marked symmetrical lesions of necrosis and capillary proliferation, with severe symptoms of muscular hypotonia, ataxia, dystonia, optic atrophy, ophthalmoparesis, and marked lactic acidosis. However, between 17% and 40% of patients with respiratory chain disorders are found to have a cardiomyopathy [6,14-17], a condition which rapidly diminishes quality of life and contributes further to early death. Of these most involve Complex I predominantly or as combined Complex I,III,IV defects. They predominantly present with hypertrophic cardiomyopathy (left or bi-ventricular hypertrophy and myofibrillar disarray), dilated cardiomyopathy, or combined ventricular hypertrophy with systolic dysfunction and left ventricular non-compaction and often also feature atrio-ventricular block and cardiac arrhythmias. Complex I patients may exhibit fat deposition in tissues, accumulation of hydroxy-acylcarnitine but depletion of plasma carnitine, and increased renal excretion of dicarboxylic fatty acids, with potentially severe disorder due to the combined and marked interference of carbohydrate and fatty acid metabolism. Transmission electron microscopy imaging studies report enlarged dysmorphic cardiac mitochondria with centralized densely packed cristae. These cardiac mitochondria also appear disordered in their localization between disorientated myofibrils and displaced sarcomeres, and there are heterogeneous regions of mitochondrial proliferation and fragmentation [6,15,17,18]. Underlying the mitochondrial proliferation are adaptive changes that involve the induction of genes promoting mitochondrial biogenesis such as PGC1 α , NRF1, NRF2, Tfam [18], and mitochondrial and nuclear respiratory genes and other genes involved in metabolic pathways [19,20], and have been demonstrated in a variety of organs from patients with mitochondrial disease. However, although ATP synthetic capacity may partly rise, continued proliferation of dysfunctional mitochondria and interference with sarcomeric function ultimately cause adverse cardiac remodeling.

Clinical Manifestations and Management

Complex I deficiency patients with cardiac manifestation present much earlier with more severe and rapid disease progression. However, therapy to date for these patients has targeted symptoms and palliative management rather than direct correction of the specific underlying defect [6,14-17]. Patients presenting early with neurological conditions, but no clinical cardiac symptoms, are often not directly assessed for cardiac conditions, and are only detected sub-clinically via ECG assessment or inadvertently at autopsy. It has thus become evident that less overt myocardial disorder often underlies many Complex I disorders. More recently, clinical algorithms for cardiac screening and management in many mitochondrial disorders, particularly arrhythmogenic conditions have been advocated [14]. However patient management predominantly targets symptoms rather than defects *per se*.

Metabolic therapies have long been attempted to override or bypass deficiencies, however these have been greatly limited by the heterogeneous nature of mitochondrial disorders or poor target specificity and molecular efficacy [21,22]. A common presentation is lactic acidosis in plasma due to decreased pyruvate dehydrogenase activity and increased pyruvate conversion to lactate by lactate dehydrogenase. Dichloroacetate (DCA), has long been shown to lower lactate via activation of pyruvate dehydrogenase and inhibition of its inhibitory kinase (PDK), with clinical benefit in the treatment of children with PDH and other mitochondrial deficiencies [23-26]. Although generally tolerated, peripheral neuropathy has arisen in some patients, and the long-term benefits of DCA have been difficult to establish because of the marked severity of the disease at the start of treatment [26].

Coenzyme Q₁₀ (ubiquinone, CoQ₁₀) is a critical electron acceptor promoting transfer in the respiratory chain and as an important redox agent limiting free radical formation. Although diminished CoQ₁₀ levels have been reported in human heart failure and supplementation has been advocated, efficacy has been controversial, in part due to formulation and dosage [27]. The recently presented data from the long running Q-SYMBIO trial suggests early promise in limiting all-cause mortality and cardiac-dependent events and hospitalization [28]. Benefits have been reported for pediatric patients with CoQ₁₀ deficiency including those with dilated cardiomyopathy [29,30]. Molecules specifically targeted to the mitochondria that have REDOX action, while demonstrating benefit in abrogating excessive reactive oxygen species in cell culture models, may translate to clinical implementation [31]. Recently EPI-743 a synthetic molecule with attributes in common to idebenone and CoQ₁₀ has been fast tracked by the FDA to phase III trials in Friedreich's Ataxia patients and this may eventually hold promise in other mitochondrial disease settings (Leighs & Lebers) [32,33], particularly when its specific redox mechanism of action is clarified.

Other approaches include exercise as well as adjunct combinations small molecules with DCA activation, or sirtuin (SIRT) pathway activated mitochondrial biogenesis [34-41]. Other drugs that activate mitochondrial biogenesis, such the bezafibrate PPAR agonists may also hold promise. NAD⁺, a redox coenzyme of the mitochondrial electron transport chain, which is also a rate-limiting co-substrate for the SIRT enzymes has been shown to enhance fat oxidation in as an adjunct metabolic therapy agent [35]. Increased mitochondrial

biogenesis and oxygen consumption has been demonstrated in patients with mitochondrial disease undergoing moderate exercise therapy [34,36,38,39]. Resveratrol, a polyphenol acting via SIRT1 and SIRT3, has also been used in combination with exercise to synergize mitochondrial biogenesis and respiratory activities, greater than the individual treatments alone [41]. A more recent experimental approach has been the use of rapamycin (first trialed in human cancer studies). The target of rapamycin (mTOR) signaling pathway has been shown to activate cell survival pathways, including prolongation of life span in animal models. Most notably, rapamycin treatment has recently been reported to limit disease progression in a Complex I-deficient mouse model of Leigh syndrome with *Ndufs4* deletion [42].

Complex I Is a Critical Target of Cardiac Reperfusion Injury

A major feature of cardiac ischemia-reperfusion injury is the marked decrease in Complex I respiration and increased formation of reactive oxygen species (ROS) [43-45], which contribute to post-oxidative modifications and dysfunction to Complex I proteins, mitochondrial DNA, plus other mitochondrial proteins and lipids [13]. Sustained ROS-induced ROS release triggers prolonged opening of the mitochondrial permeability transition pore, membrane depolarization, loss of ATP synthesis, mitochondrial swelling with outer membrane permeabilization and cell death via apoptosis and necrosis [43,46-48,13]. Impaired Complex I-oxidation of NADH forms superoxide at the fully reduced flavin mononucleotide group of Complex I on the matrix side of the inner membrane. The 75, 51, 49 and 24 kDa subunits within Complex I contain Fe-S clusters which make them highly vulnerable to superoxide and consequent oxidative modification of protein structure [44]. Subsequent oxidation of cardiolipin and other proximal inner membrane phospholipids promote cytochrome c release and apoptosis, including the translocation of AIF to the nucleus where it interacts with cyclophilin A to become an active DNase [13]. Although Complex I is a major target of ischemia-reperfusion injury, other respiratory chain and Krebs Cycle proteins, lipid peroxidation, redox reactions and ROS management are also markedly impacted [44,49]. However, depending on the severity and timing, systems not subjected to overt genetic deficiencies are able to access intrinsic adaptive mechanisms that permit survival. In contrast, in Complex I deficiency due to gene mutation etiology, increased ROS activity is not overtly evident in patients with (*i.e.*, *Ndufs6* mutations) [50], although unmanaged ROS may feature subsequent to progression of severe cardiomyopathy. Notably, in cultured skin cells from patients with *Ndufs7* or *Ndufs8* mutations, augmented ROS disrupts intracellular calcium signaling and homeostasis [51]. As often patients with mitochondrial respiratory complex disorders are also subject to congenital organ malformations, surgical or other palliative interventions which superimpose acute oxidative stress can further exacerbate Complex I dysfunction.

Current Non-Drug Approaches to Management and Treatment of Complex I Deficiency

Mitochondrial transplantation

As mitochondria had their start as endosymbionts, it is not so farfetched to imagine that they might be introduced into recipient cells for therapeutic effect. This possibility was pioneered by McCully *et al.* using skeletal muscle mitochondria that were injected into the wall of the left ventricle of an ischemic heart, just prior to reperfusion [52]. These mitochondria were internalized by cardiomyocytes 2-8hr after injection, and resulted in improved oxygen consumption, high-energy phosphate synthesis, and reduced infarct scars when measured 4 weeks later [53]. However, the efficiency of transfer was fairly low, and it is unknown whether the autologously transplanted mitochondria persisted for that long. Another group used a protein transduction peptide Pep-1 to deliver mitochondria into fibroblasts of patients with MERRF syndrome (myoclonic epilepsy with ragged red fibers). They showed that the delivered mitochondria restored mitochondrial function and enhanced mitochondrial biogenesis; the mitochondrial genome persisted for at least 21d in cell culture [54]. Thus mitochondrial transplantation may offer yet another approach for prolonged treatment, or possibly even cure, of mitochondrial diseases.

Gene therapy

Gene replacement for complex I disorders has been stymied by the fact that mutations or deletions in the mitochondrial genome affect gene products that have multiple membrane-spanning domains and would be difficult to import if translated from cytosolic mRNA. In the few cases where the defect in complex I is due to a nuclear gene mutation, then gene therapy might be feasible, but would still face numerous technical challenges of achieving long-term gene expression in the most severely-affected tissues. Despite these pessimistic concerns, Guy and colleagues developed a novel AAV vector in which the adeno-associated virus capsid protein VP2 was fused with a mitochondrial targeting sequence to carry the ND4 gene into the mitochondrial matrix, where it would be transcribed and translated by the host mitochondrial machinery. Preclinical work with this vector introduced by vitreal injection has shown promise in a mouse model of Leber hereditary optic neuropathy (LHON) [55]; introduction of the vector into ex vivo human eyes resulted in efficient expression of ND4, and no adverse reactions were encountered in primates receiving the AAV vector [56]. Regionally targeted gene therapy also has potential for rescuing the dopaminergic neurons in Parkinson Disease (PD).

Ndi1 as replacement for complex I

With respect to gene therapy, the introduction of yeast Ndi1 as a potential replacement for complex I has been a novel advance. The yeast internal NADH quinone oxidoreductase (Ndi1) was first proposed for complex I replacement by Yagi and colleagues [57]. Studies in rodents have confirmed its feasibility [58,59] and it offers considerable promise for long-term therapy for PD. Lentiviral delivery of Ndi1 into cancer cells has been shown to suppress tumor progression in a mouse xenograft model [60]. Functionally, although Ndi1 lacks the proton-pumping capability of mammalian complex I, it effectively transfers

electrons from NADH to ubiquinone in mammalian mitochondria, although it slightly decreases the efficiency of ATP production [57,61]. A second concern has been that the persistent expression of a yeast-derived protein would lead to a deleterious immune response. However, lentiviral expression of Ndi1 in skeletal muscle of rats over a span of 7 months failed to elicit antibody production or local inflammation [62,63]. This was hypothesized to be due to the fact that the foreign protein, which is localized to the mitochondrial matrix, would be hidden from immune detection. The protein would be degraded by mitochondrial proteases or in the lysosome when the whole mitochondrion is degraded by mitophagy; therefore little antigenic material would escape from intact cells. There is more work required to rule out the possibility that the foreign protein would be released from apoptotic or necrotic cells and could trigger an immune response. However, at first blush, gene therapy with Ndi1 holds promise as a means to replace or bypass complex I, regardless of the underlying genetic or acquired defect.

Protein transduction therapy for mitochondrial defects

The recognition that short peptide sequences can be used to deliver recombinant proteins to cells [64] has led to a number of novel therapeutic approaches, a few of which have been explored for treatment of mitochondrial disorders. Our group utilized the protein transduction domain of HIV TAT fused to Apoptosis Repressor with CARD domain (TAT-ARC) [65] and a peptide corresponding to the BH4 domain of Bcl-xL (TAT-BH4)[66] to prevent ischemia-reperfusion injury in isolated perfused rat hearts. These agents targeted the mitochondrial outer membrane, but Payne's group focused on delivery of TAT fusion proteins to the mitochondrial matrix [67]. TAT-TFAM was used to upregulate mitochondrial biogenesis and enhance bioenergetics in mouse skeletal muscle [68]. Correction of COX deficiency was achieved in cell culture using TAT-SCO2 recombinant fusion protein [69], and TAT-frataxin was shown to improve cardiac function and lifespan in a mouse model of Friedreich's ataxia [70]. Our group showed that TAT-Ndi1 was able to attenuate myocardial ischemia/reperfusion injury by functioning in place of damaged complex I in isolated perfused rat hearts [71], and more recently we have demonstrated efficacy *in vivo* in a rat model of ischemia/reperfusion [72]. While TAT-Ndi1 show efficacy for acute replacement of complex I function, with persistence of the protein for up to 48hr, it remains to be shown whether it will be useful in chronic settings; a study is underway in a mouse model of complex I deficiency developed by Thorburn's group [73]. The assembly factor NDUFAF4, delivered as a recombinant TAT-fusion protein has been shown to restore complex I activity in cells from patients with isolated complex I deficiency [74].

Overview of Tat Protein Therapy

The ability of the HIV TAT protein to cross the cell membrane was discovered in 1988 [75,76], and the domain responsible for this was identified in 1994 [77]; domains with similar functionality were recognized [78,79]. The use of the protein transduction domain for delivery of recombinant proteins was pioneered by Dowdy's group [64] and was shown to be feasible in mice in 1999 [80]. Since then, this approach has been expanded to deliver not only peptides and proteins but also nucleotides such as morpholino oligos, and other macromolecules. Targeting of recombinant cell-permeable proteins to mitochondria has

been accomplished by several groups [74,70-72]. A 28 aa cell-penetrating peptide from azurin, a bacterial protein, is in Phase II clinical trial as an antiangiogenic cancer drug [81], and a cell-permeable peptide inhibiting protein kinase C delta was tested in a clinical trial for patients with acute myocardial infarction [82]. Morpholinos are in clinical trial for treatment of Duchenne muscular dystrophy and may be more effectively delivered if conjugated to cell-penetrating peptides (CPPs).

The approach holds great promise because of the ability to optimize tissue and organelle specificity. The ability to deliver proteins, oligonucleotides, or particles adds to its versatility. As the delivered materials are taken up rapidly by cells and degraded over time, there is less biologic risk than virus-mediated gene therapy. This may be a disadvantage if a cell-penetrating protein is being used chronically to treat a genetic disease, as repeated delivery is necessary, and an immune response may eventually arise. However, because the proteins are taken up rapidly by cells, and are degraded via intracellular mechanisms, it is possible that they will be less likely to precipitate an immune response than recombinant extracellular proteins such as clotting factors or cytokines.

Challenges

A number of technical challenges for TAT-fusion protein therapy exist. Production of recombinant proteins in bacteria often entails re-engineering the cDNA sequence to eliminate rare codons which may result in premature termination of protein translation. Efficient expression of full-length protein in bacteria may result in formation of inclusion bodies, or insoluble protein aggregates. This is aggravated by the fact that the amphipathic alpha helix of the protein transduction domain tends to form aggregates. Thus protein production in therapeutically feasible quantities can be challenging. Once purified, the protein can aggregate and precipitate, creating additional challenges for storage and stability. In preclinical work, the denatured protein may be stored in urea and desalted immediately before use; however, this is impractical in the clinical setting, so developing a suitable biocompatible buffer is important, and further re-engineering of the protein may be necessary.

Determining the appropriate dosage and characterizing the pharmacokinetics and pharmacodynamics is also challenging. The volume of distribution comprises both intracellular and extracellular space but is non-equilibrium, because TAT-fusion proteins that enter the cell may not exit as readily, resulting in a build-up over time. This also means that the extracellular protein concentration may not be a helpful parameter in dose-response studies. TAT-fusion proteins can cross the blood-brain barrier, but perhaps to a lesser extent, depending on specific characteristics of the protein. In a study of enzyme replacement therapy for lipoamide dehydrogenase (E3) in mice, the TAT-E3 was cleared from plasma quite rapidly (50% within 30min), and resulted in an increase in a 50-70% increase in enzyme activity in organs in the same time, with maximal increases seen at 4hr [83]. By 24hr, enzyme activity was down to ~10%, but detectably activity persisted to 48hr. Interestingly, replacement enzyme activity increased to the greatest degree in heart and brain (90-100% increase), which may reflect the abundance of mitochondria in these organs.

Immunogenicity is a theoretical impediment to protein therapy with cell-permeable recombinant proteins. If synthesized in bacteria, removal of any residual lipopolysaccharide is important as this might enhance an immune response. Based on the rapid egress of TAT-E3 from plasma and its accumulation in mitochondria-rich organs, it seems likely that mitochondria-targeted TAT-fusion proteins will not remain in circulation long enough to trigger an immune response. However, the intracellular degradation pathway may give rise to antigenic peptides. In the case of TAT-Ndi1, which is a yeast gene, the protein and its peptides may be recognized as foreign. However, in mice that expressed Ndi1 delivered by lentivirus for several months, no immune response was detected. Further work will be required to determine whether an immune response to mitochondria-targeted TAT-fusion proteins will be problematic.

Tat-Ndi1 Preclinical Advances

With evidence that ischemia/reperfusion (I/R) in the heart is associated with damage to mitochondria and complex I [84], a number of studies have been conducted to determine whether Ndi1 delivered by protein transduction might protect the heart against I/R injury. The rationale is based on the concept that Ndi1 could attenuate reactive oxygen species (ROS) production from complex I damaged by myocardial I/R. This innovative strategy in the heart was first tested [71] in HL-1 cardiomyocytes and neonatal rat ventricular cardiomyocytes (NRVM). After preliminary studies in which Ndi1 was delivered to cells by transient transfection, cardiomyocytes were transduced with recombinant Ndi1 expressed as a fusion protein with the 11 amino acid protein transduction domain of HIV TAT, as well as a hemagglutinin epitope tag for immunodetection. It was found that 100% of the cardiomyocytes took up TAT-Ndi1, which localized to mitochondria.

To assess its cardioprotective properties, transduced HL-1 cells and NRVMs were subjected to 2hr simulated ischemia and 24hr reperfusion. Cell death was reduced almost four-fold in the presence of Tat-Ndi1. Moreover, the protection was associated with a 51% reduction in ROS production and preservation of ATP. With respect to mitochondrial damage, transduction resulted in substantial retention of cytochrome *c* in mitochondria compared to untreated cardiomyocytes, indicating preservation of mitochondrial integrity. Electron microscopy of adult rat cardiomyocytes transduced with TAT-Ndi1 and subjected to sI/R revealed that 89% of the mitochondria had well-defined invaginations of cristae in contrast to the untreated cells in which only 48% of the mitochondria retained detectable cristae.

When introduced into Langendorff-perfused rat hearts, the TAT-Ndi1 protein was localized to mitochondria. Infusing the protein into isolated perfused rat hearts subjected to 30min ischemia and 2hr reperfusion resulted in suppression of ROS production, maintenance of ATP levels, and attenuation of mitochondrial permeability transition pore opening. The TAT-Ndi1 infusion prior to ischemia was associated with a 62% reduction in infarct size. Of major import was the finding that TAT-Ndi1 infused at the onset of reperfusion was equally cardioprotective. Collectively, these findings suggest that in cells and the Langendorff perfused heart that cytoprotection and cardioprotection can be achieved by preventing complex I dysfunction by xenotransplantation with Ndi1.

To determine whether Ndi1 could be used to protect the heart in vivo, TAT-Ndi1 or placebo was administered to female Sprague-Dawley rats intraperitoneally [72]. Two hours later the rats were subjected to 45min regional myocardial ischemia and 2hr reperfusion, after which the hearts were harvested for mitochondrial isolation and infarct size determination. Western blot detection of the hemagglutinin tag and the presence of rotenone-insensitive NADH oxidase activity indicated that the enzymatically active protein was present in the mitochondria of the Tat-Ndi1 transduced hearts. Infarct size was significantly smaller in rats that received TAT-Ndi1 compared to placebo, i.e., 34% versus 61%. These findings suggest that it is possible to achieve myocardial protection by administering a cell-permeable functional enzyme that compensates for complex I dysfunction and stabilizes mitochondria.

Taken together these experimental findings suggest it may be possible to treat complex I dysfunction associated not only with heritable mitochondrial diseases but also acquired diseases such as mitochondrial damage secondary to acute myocardial infarction. Should these preclinical observations be confirmed in large-animal studies, the stage could be set for the study of this metabolic strategy to overcome complex I deficiencies in a clinical setting.

Conclusions

A variety of approaches to managing Complex I disorders have been evaluated in animal models and a few have progressed to clinical trials; however, protein therapy with Tat-Ndi1 is still at an early preclinical stage of development. Its feasibility for acute correction of complex I dysfunction has been demonstrated, and it is being evaluated for potential benefit in a mouse model of complex I deficiency with chronic administration. If chronic therapy is shown to be feasible, then Tat-Ndi1 offers hope as a therapeutic approach to a variety of human diseases including Parkinson disease and congenital complex I deficiencies as well as ischemia/reperfusion injury.

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