RT001 in Progressive Supranuclear Palsy—Clinical and In-Vitro Observations.

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Abstract

Background: Progressive supranuclear palsy (PSP) is a progressive movement disorder associated with lipid peroxidation and intracerebral accumulation of tau. RT001 is a deuterium reinforced isotopologue of linoleic acid that prevents lipid peroxidation (LPO) through the kinetic isotope effect.

Methods: The effects of RT001 pre-treatment on various oxidative and bioenergetic parameters were evaluated in mesenchymal stem cells (MSC) derived from patients with PSP compared to controls. In parallel, 3 patients with PSP were treated with RT001 and followed clinically.

Results: MSCs derived from PSP patients had a significantly higher rate of LPO (161.8 ± 8.2% of control; p<0.001). A 72-hour incubation with RT001 restored the PSP MSCs to normal levels. Mitochondrial reactive oxygen species (ROS) overproduction in PSP-MSCs significantly decreased the level of GSH compared to control MSCs (to 56% and 47% of control; p<0.05). Incubation with RT001 significantly increased level of GSH in PSP MSCs. The level of mitochondrial DNA in the cells was significantly lower in PSP-MSCs (67.5%), compared to control MSCs. Changes in mitochondrial membrane potential, size, and shape were also observed.

Three subjects with possible or probable PSP were treated with RT001 for a mean duration of 26 months. The slope of the PSPRS changed from the historical decline of 0.91 points/month to a mean of decline of 0.16 points/month (+/- 0.23 SEM). The UPDRS slope changed from an expected increase of 0.95 points/month to an average increase in score of 0.28 points/month (+/- 0.41 SEM).

Conclusions: MSCs derived from patients with PSP have elevated basal levels of LPO, ROS, and mitochondrial dysfunction. These findings are reversed after incubation with RT001. In PSP patients, the progression of disease may be reduced by treatment with RT001.

Background

Progressive supranuclear palsy (PSP), or Steele-Richardson-Olszewski syndrome, is a sporadic, progressive neurodegenerative disease characterized by ocular motor dysfunction, postural instability, akinesia, and cognitive dysfunction. Freezing of gait, levodopa resistance, behavioral changes, and aphasia are often seen [1]. Symptoms typically begin after age 60 but can begin earlier. The exact cause of PSP is unknown. PSP is often misdiagnosed as Parkinson disease, Alzheimer disease, corticobasal syndrome and other neurodegenerative disorders [2-5]. At present, no effective therapies exist [6].

Neuropathological examination of the post-mortem PSP brain reveals intracerebral aggregation of the microtubule-associated protein tau in neurofibrillary tangles throughout the brain, most prominently in the brainstem, deep cerebellar nuclei and basal ganglia [7]. LPO byproducts such as toxic aldehydes are selectively associated with neurofibrillary tangles in the PSP patients [8]. A regionally specific increase in LPO has been observed [9], and other reports have demonstrated defects in oxidative phosphorylation in
muscle mitochondria from PSP patients [10]. Cerebrospinal fluid increases in superoxide dismutase and glutathione conjugated with 4-hydroxynonenal further support the association of LPO with PSP [11].

RT001 is a deuterated isotopologue of linoleic acid that makes membrane PUFAs resistant to LPO. A strong protective effect against LPO is seen when deuterated PUFAs replace non-deuterated PUFAs in liposomal lipid bilayers at levels exceeding 20% [12]. Treatment with RT001 has shown early signs of efficacy in patients with Friedreich's ataxia, a disorder of intracellular free-iron imbalance that initiates LPO, resulting in increased oxidative stress and mitochondrial dysfunction [13].

**Methods**

The current study evaluated the effect of RT001 on various oxidative and bioenergetic parameters in MSCs derived from patients with PSP. We also report on the results of expanded access use of RT001 in 3 patients with PSP, each of whom was treated for over 27 months.

**In vitro Methods**

MSC preparations from bone marrow (BM) were obtained from control subjects and from the PSP subjects following previously described protocols [14-18]. In short, MSCs were isolated from BM aspirates seeding 50,000 mononucleated cells/cm² in αMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), in T75 flasks. The cultures were incubated at 37 °C, 20% O2, 5% CO2. Medium changes were performed twice a week. Two weeks after initial seeding, primary MSC colonies were detached with a 10-minute incubation at 37 °C with TrypLE Select Enzyme (Thermo Fisher Scientific) and re-plated at 4000 cells/cm² in the same medium. MSC identity was previously assessed [18]. Subsequent passages were performed following the same steps. Passage 4-6 MSCs were used for all experiments. BM from PSP patients was collected in the context of a clinical protocol authorized by the local Ethics Committee of Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico (Italy), by the national competent authority for phase-I cell therapy at the National Health Institute (Istituto Superiore di Sanità) and approved by the Italian Medicines Agency (Agenzia Italiana del Farmaco, AIFA). The trial is registered at ClinicalTrials.gov (NCT01824121). All BM donors gave their written informed consent.

**Live cell imaging**

Lipid peroxidation was measured using confocal microscopy (Zeiss 710 LSM with an integrated META detection system). The rate of lipid peroxidation was measured using C11-BODIPY 581/591 (2 μM; Molecular probes) which was excited by the 488 and 543 nm laser line and fluorescence measured using a band-pass filter from 505 to 550nm and 560nm long-pass filter (40× oil-objective). Illumination intensity was kept to a minimum (0.1–0.2% of laser output) to prevent phototoxicity and the pinhole was set to give an optical slice of ~2 μm. Addition of a bright-field image allowed separation between neurons and glia that are visibly different and are situated on different focal planes.
For assessments of glutathione levels, the PSP-MSCs cultures were incubated with 50 μM monochlorobimane (MCB) (Molecular Probes, Invitrogen) for 40 minutes in HEPES buffered salt solution prior to imaging. Cells were then washed with HEPES buffered salt solution and images of the fluorescence of the MCB-GSH were acquired using a Zeiss 710 CLSM with excitation at 405 nm and emission at 435–485 nm. Mitochondrial ROS generation rate was assessed using MitoTracker® Red CM-H2XRos (Thermo Fisher Scientific) which accumulates into mitochondria upon oxidation. The fluorescence measurement was obtained by excitation with 561 nm laser and emission detected above 580 nm. Mitochondrial membrane potential (ΔΨm) was assessed using 25 nM tetramethylrhodamine methyl ester (TMRM, Thermo Fisher Scientific) at 560 nm excitation and fluorescence was measured above 580 nm. Z-stack images were collected and the fluorescence intensity of TMRM was analysed using Zen software (Zeiss).

RT001 effects on lipid peroxidation, mitochondrial function, glutathione, mitochondrial membrane potential, mitochondrial number, and mitochondrial structure were compared to the effects on MSCs derived from healthy control age-matched subjects. H2-LA and RT001 were added to cultures as described previously [17].

**Clinical Methods**

Patients with PSP were recruited to participate in this study at the California Movement Disorders Center, Los Gatos, CA. Ethics board approval was obtained, and all participants gave written informed consent. Participants met the Movement Disorders Society clinical diagnostic criteria for possible or probable PSP [4]. Participants underwent baseline assessment using the 28-item Progressive Supranuclear Palsy Rating Scale (PSPRS) [19] and the Unified Parkinson's Disease Rating Scale (UPDRS) [20,21]. They were then treated with RT001 (1.92 g BID; 5.76 g total daily dose) and observed for disease progression. Subject 2 increased the dose (2.88 g TID; 8.64 g total daily dose) after the first year of treatment. During the treatment period, scores in the 2 rating scales were determined every 3 months. Pharmacokinetic (PK) sampling was performed at month 3. These analytes included plasma and RBC membrane levels of D2-linoleic acid (D2-LA) and its centrally active metabolite D2-arachidonic acid (D2-AA).

**Results**

**In-vitro results**

Using the LPO-specific probe BODIPY C11, PSP MSCs had a significantly higher rate of LPO compared to controls. After a 72-hour incubation of the cell lines with RT001, PSP MSCs returned to normal levels, while PSP MSCs incubated with non-deuterated linoleic acid ester (H2-LA) remained elevated (Figure 1, Panel A). The time course of lipid peroxidation for the various MSCs and treatments is displayed in Figure 1, Panel B.
Glutathione levels were measured using MCB. The MCB intensity was reduced in PSP MSCs compared to HC. After incubation with RT001, glutathione levels were restored to HC levels, while glutathione levels remained low in PSP MSCs after incubation with H2-LA (Figure 1, Panel C). Representative images of these cells are shown in Figure 1, Panel D.

**Panel A)** Bar chart quantification of the efficacy of RT001 on the rate of lipid peroxidation using C11-Bodipy (PSP alone vs. PSP + RT001, p < 0.0001). **Panel B)** Representative time course traces of lipid peroxidation in MSCs derived from HC (light green), and PSP (red), PSP treated with RT001 (orange), and PSP treated with H2-LA (dark green), respectively. **Panel C)** Measurements of monochlorobimane (MCB) fluorescence intensity as an indicator of glutathione (GSH) levels (PSP alone vs. PSP + RT001, p < 0.0001). **Panel D)** Representative images showing MCB (GSH) fluorescence intensity for HC, PSP, and PSP + RT001. MCB intensity is reduced in PSP compared to HC, but are restored after incubation with RT001. The coarse dash lines approximate the cell borders of an individual MSC (fine dash line). Data are represented as mean ± SEM. Total number of cells per well n = 10 – 50 from 3 – 6 culturing wells. All experiments were repeated 2 – 3 times (N, independent culturing conditions). *p<0.05, **p<0.001, ***p<0.0001.

The fluorescence intensity of TMRM was increased in the PSP MSCs relative to HC MSCs, indicating in increase in the $\Delta \psi_m$. This increase remained elevated when the cells were incubated with H2-LA, but $\Delta \psi_m$ normalized after RT001 (Figure 2, Panels A and B). The changes seen in the $\Delta \psi_m$ were also seen in the fluorescence of MitoTrackerCM-H2Xros. Fluorescence intensity of MitoTrackerCM-H2Xros for the PSP MSCs was increased more than 2.5 times HC at baseline, indicating an increase in mitochondrial ROS. Incubation with H2-LA reduced mitochondrial ROS generation slightly, but RT001 reduced mitochondrial ROS back to near normal levels (Figure 2, Panels C and D). Fluorescence intensity with Pico Green exhibited an inverse correlation with the other studies. PicoGreen fluorescence was decreased at baseline for the PSP MSCs relative to HC, indicating a reduced amount of mitochondrial DNA. After incubation with H2-LA, fluorescence increased slightly, but was far more pronounced for the RT001 incubated cells. In addition to increased mitochondrial DNA amount, the structure and number of mitochondria were also increased by RT001 treatment (Figure 2, Panels E and F).

**Panel A)** Histogram demonstrating the mitochondrial membrane potential ($\Delta \psi_m$) measured using the fluorescence intensity of TMRM (tetramethylrhodamine). $\Delta \psi_m$ was increased in PSP MSCs compared to HC. $\Delta \psi_m$ was reduced after incubation with RT001, but not after incubation with H2-LA (p=0.0009). **Panel B)** Representative images depicting the mitochondrial shape, distribution, and fluorescence intensity at baseline for HC and PSP MSCs (fine dash line represents the approximate cell border of a MSC). **Panel C)** Quantitative histogram of MitoTrackerCM-H2Xros fluorescence intensity shows baseline elevations in ROS in the PSP MSCs are reduced to near normal levels after incubation with RT001 (p<0.0001), but not after incubation with H2-LA (p=0.0801). **Panel D)** MitoTrackerCM-H2Xros fluorescence over time for HC, baseline PSP, PSP + RT001, and PSP +H2-LA. Baseline fluorescence elevations for the PSP MSCs over HC MSCs were restored to near normal after RT001, but not after H2-LA incubation.
images of the mitochondrial DNA content of PSP MSCs at baseline (top panel) and after incubation with RT001 (bottom panel): note the difference in the extranuclear distribution of the PicoGreen fluorescence. A fine dash line represents the approximate cell border of a MSC. Quantification bar chart of the PicoGreen Intensity as a measure of mitochondrial DNA content. Baseline (orange columns) reductions in mitochondrial DNA were seen in the PSP MSCs (middle and right histograms). Incubation with H2-LA (middle histogram) resulted in a small increase in mitochondrial DNA (middle histogram, green column; $p=0.0689$), while RT001 restored PSP MSCs to normal levels (right histogram, green column; $p=0.0010$). Data are represented as mean ± SEM. Total number of cells per well $n = 10 – 50$ from 3 – 6 culturing wells. All experiments were repeated 2 – 3 times (N, independent culturing conditions). *$p<0.05$, **$p<0.001$, ***$p<0.0001$.

Clinical results

Baseline demographic information for the 3 subjects is displayed in Table 1.

**Table 1. Baseline characteristics for the 3 subjects at the onset of treatment**

| Characteristic                  | Subject Number |
|--------------------------------|----------------|
|                                | 1  | 2 | 3  |
| Age (years)                    | 66 | 73| 74 |
| Sex                            | Male| Male| Female |
| Pre-treatment symptom duration (years) | 6  | 3 | 2  |
| PSP diagnosis                  | Probable| Possible| Probable |
| Baseline PSPRS                 | 17 | 12| 13 |
| Baseline UPDRS                 | 44 | 36| 21 |

The linear regression slopes of the PSPRS and UPDRS scores for the 3 subjects were plotted against those obtained from disease progression predicted by previous longitudinal studies of untreated PSP patients. Figure 3 shows the slope of the PSPRS changed from the historical decline of 0.91 points/month to a mean of decline of 0.16 points/month ($+/- 0.23$ SEM). The UPDRS slope changed from an expected increase of 0.95 points/month to an average increase in score of 0.28 points/month ($+/- 0.41$ SEM).

Pharmacokinetics

Mean plasma and RBC membrane levels of drug were 21% and 19% of total linoleic acid. Levels of di-deuterated arachidonic acid in both plasma and RBC also increased, indicating normal enzymatic processing of the stabilized LA into stabilized AA.

Safety
Overall, RT001 was well tolerated. One serious adverse event occurred (cerebrovascular event) that was not drug-related.

**Discussion**

RT001 reduced LPO, mitochondrial ROS production, and improved other measures of mitochondrial health in MSCs derived from patients with PSP. Oral RT001 was well tolerated in 3 subjects with PSP over a minimum treatment period of 24 months, and this treatment was associated with a stabilization in the rate of decline in functional rating scales over time.

The pivotal role for increased ROS and LPO in the pathophysiology of PSP has been identified previously [18]. Because of its down-regulation of LPO, RT001 affords a novel, yet specific approach to preventing these harmful effects on lipids in mitochondrial and other membranes in PSP. Improvement in these *in vitro* parameters were seen with RT001 pre-treatment, leading to improvement in mitochondrial number, function, and structure. Treatment of PSP with RT001 may be a reasonable therapy to interrupt the causal pathway leading to mitochondrial dysfunction, tau accumulation and cell death.

In PSP, misfolded and aggregated tau incorporates into plasma and mitochondrial membranes, causing depolarization and flux through various ion channels. The resulting cellular and mitochondrial calcium overload activates cytosolic and mitochondrial ROS production, calcium-induced Caspase-3 activation, and cell death cascades. [24, 25]. Incorporation of RT001 into cell membranes should reduce LPO and PUFA degradation compounds such as toxic isoprostanes and bifunctional aldehydes and should ultimately reduce the neurodegenerative consequences of tau accumulation.

In addition to tau, other intrinsically disordered proteins like alpha-synuclein and beta-amyloid are characterized by the formation of aggregates that have similar membrane perturbation capacity. Restoration of membrane oxidative status with RT001 prevents the acute aggregate-membrane interaction, calcium dysregulation, and cell death in human IPS-derived neurons with triplication of alpha-synuclein [26]. Thus, RT001 has the potential to be effective in other types of neurodegenerative diseases in which protein misfolding and lipid peroxidation are pathophysiologic [27].

The interpretations of the clinical results reported here are subject to the inherent limitations of an open-label study without concurrent placebo controls. However, previous studies have suggested the absence of a significant placebo effect in PSP clinical trials [28]. Further exploration of the effects of RT001 in PSP is warranted in a randomized, placebo-controlled trial of appropriate size and duration.

**Conclusions**

In summary, *in vitro* studies of MSCs derived from PSP patients demonstrate increased rates of LPO and mitochondrial dysfunction that can be reversed with RT001 pre-treatment. Expanded access treatment of PSP patients with RT001 slows the rate of PSP progression. RT001 represents a potential therapy for PSP patients that should be studied in randomized, controlled clinical trials.
Abbreviations

ΔΨm Mitochondrial membrane potential
AA Arachidonic acid
BM Bone marrow
D2-AA di-deuterated arachidonic acid
D2-LA di-deuterated linoleic acid
H2-LA Non-deuterated linoleic acid
LPO Lipid peroxidation
MCB monochlorobimane
MSC Mesenchymal stem cell
PK Pharmacokinetic
PSP Progressive supranuclear palsy
PSPRS Progressive Supranuclear Palsy Rating Scale
ROS Reactive oxygen species
RT001 di-deuterated linoleic acid ester
SEM Standard error of the mean
TMRM tetramethylrhodamine methyl ester
UPDRS Unified Parkinson’s Disease Rating Scale

Declarations

Ethics Approval and consent to participate

In vitro and clinical protocols received IRB approval and informed consent was obtained from participants. The in vitro studies received Ethics Committee approval from Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico (Italy), by the national competent authority for phase-I cell therapy at the National Health Institute (Istituto Superiore di Sanità) and approved by the Italian Medicines Agency (Agenzia Italiana del Farmaco, AIFA). The trial is registered at ClinicalTrials.gov (NCT01824121). The
expanded access clinical protocols received IRB approval from Western IRB through the Parkinson's Institute and Clinical Center in Sunnyvale, CA.

Consent for publication

All subjects provided informed consent for publication.

Availability of data and materials

In vitro and clinical data generated or analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

Competing Interests

Authors PA, KA, and MB have no competing interests to declare.

Author OT is employed by Retrotope.

Authors MM, PA, PM, FH, and MS are stockholders and employees of Retrotope.

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Retrotope provided RT001 for in vitro and clinical use. Retrotope provided funding for the in vitro research materials.

Author contributions

| Author | Contribution |
|--------|--------------|
| PA     | Designed, conducted and analyzed in vitro experiments. Wrote in vitro sections of the manuscript. Constructed in vitro figures. |
| KA     | PI for clinical protocols. Assisted in manuscript writing |
| MM     | Medical monitor for study. Wrote initial manuscript draft, constructed figures, coordinated manuscript submission. |
| MB     | Provided source for MSCs, reviewed and edited manuscript |
| PA     | Analyzed data, edited manuscript |
| OT     | Assembled and analyzed clinical data, constructed clinical figure |
| PM     | Conceived clinical studies, analyzed in vitro and clinical data, edited manuscript |
| FH     | Designed clinical protocols, managed safety data, edited manuscript |
| MS     | Invented RT001, conceived of in vitro and clinical studies, edited the manuscript |

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