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Ex-vivo inhibition of LRRK2 phosphorylation by a new kinase inhibitor in peripheral blood cells of subjects with and without G2019S LRRK2 mutation.

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Introduction

In familial Parkinson's disease, the most common mutation of LRRK2 is G2019S. It leads to an increase in its kinase activity in transfected cells or transgenic knock-in mice [1]. In cell cultures, this gain of function is directly related to neurotoxicity[2].
LRRK2 is expressed mostly in brain, kidney and also peripheral blood mononuclear cells (PBMC) allowing to use them as a surrogate tissue to evaluate LRRK2 inhibitor activity in humans.
Serine 935 (S935) is a phosphorylation site known to be correlated to LRRK2 activity[3].
In order to further support clinical development and to monitor pharmacological activity of a new LRRK2 kinase inhibitor, ODS2005294*, we evaluated, using western blot and HTRF®-immunoassay, its effect on S935-LRRK2 phosphorylation. This assessment was performed after ex vivo incubation of fresh blood collected from PD patients with G2019S mutation, and their first degree asymptomatic relatives carrying or not carrying the same mutation.

***IPSEN-ONCODESIGN compound, *cf* Poster 763.012**

Methods

Patients and Relatives
All subjects included in this multicentric study were recruited in 5 hospitals via the French NS-Park-Network.

Blood exposure
Blood samples in EDTA within a maximum of 6 hours after sampling were exposed for 1 hour at 37°C to 30 µM of ODS2005294, corresponding to 180 nM of free compound taking into account plasma protein binding.

Genotyping of G2019S mutation by allelic Discrimination
DNA extraction from whole blood was based on the protocol of the ArchivePure™ DNA Blood Kit (30 ml) from 5 Prime using saline precipitation of proteins.
DNA samples were genotyped for the G2019S mutation using a TaqMan assay. Allelic discrimination was done automatically with Roche LightCycler LC480 sequence detection system.

Measure of pS935-LRRK2 inhibition
Analysis on PBMC isolated on Ficoll gradient was performed with 2 technologies:

- *Western Blot*

Western Blot analysis was used to detect total-LRRK2 and pS935-LRRK2 in the PBMCs. After separation on gel electrophoresis proteins were transferred (semi-dry) to a membrane (nitrocellulose). They were stained with antibodies specific for LRRK2 (MJFF2 C41-2, Abcam) and for pS935-LRRK2 (UDD2 10(12), Abcam).

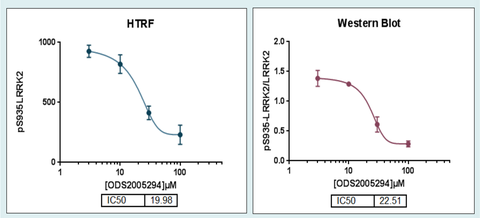
- *HTRF®-Immunoassay*

An immunoassay was developed in order to replace Western Blot in future clinical studies.
HTRF® sandwich assays use two antibodies coupled respectively to Lumi4®-Tb cryptate and to an HTRF® acceptor d2. The two conjugates bind to the antigen when present in the sample, thereby generating fluorescence resonance energy transfer (FRET).

Study design

➢ ***ODS2005294 ex vivo IC50 on S935 phosphorylation in human PBMC from volunteer donor is 20 µM (= 150 nM free compound). Western blot and HTRF® technologies demonstrated equivalent results.***

ODS2005294 Dose effect on pS935



30 µM (180 nM free compound) of ODS2005294 was selected as optimum concentration to be tested *ex vivo* on blood patients and their relatives.

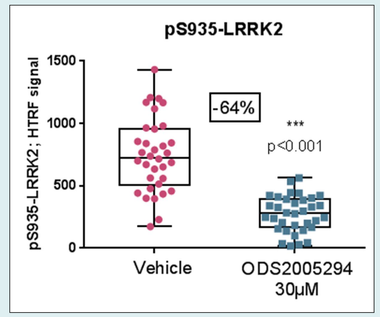
➢ ***Genotyping confirmed selected patients were G2019S carriers and among asymptomatic relatives comparable numbers of G2019S carriers and non carriers***

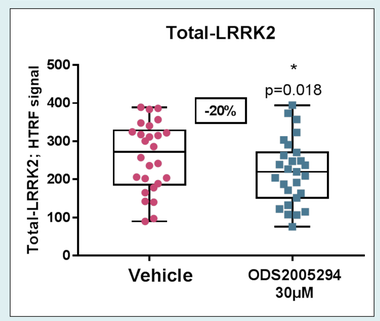
	PD Patients	Asymptomatic relatives	
	G2019S	G2019S	No mutation on LRRK2
Number of subjects	11	13*	10
Age (year)	43 to 76	20 to 61	24 to 69
Sex M / F	9 / 2	6 / 7	3 / 7

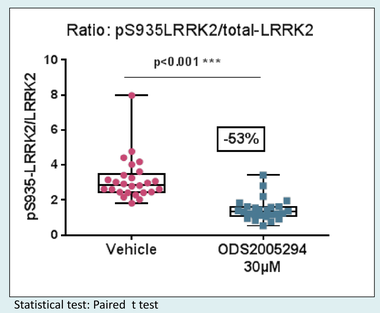
* only one relative was homozygote for G2019S mutation.

Results

➢ ***After ex vivo exposition to ODS2005294 at 30 µM (180 nM free compound):***
- ***pS935-LRRK2 level in PBMCs was highly and significantly decreased for every patient or relative.***
- ***total-LRRK2 was weakly decreased but not for every subject.***





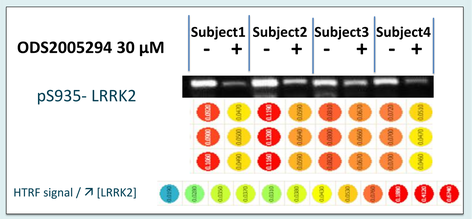


Statistical test: Paired t test

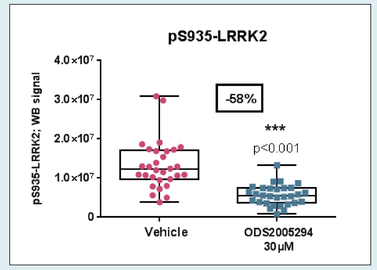
pS935-LRRK2 or its ratio on total LRRK2 in PBMC are reduced to the same extent after exposition to ODS2005294.

➢ ***Both Western blot and HTRF techniques lead to the same conclusion***

Example of Western Blot versus HTRF® signal



Global Inhibition measured with Western Blot



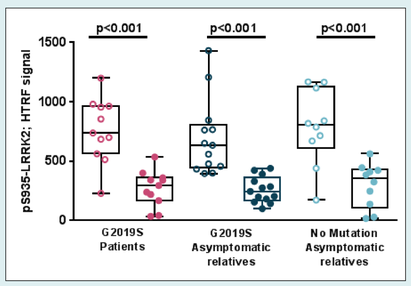
Statistical test: Paired t test

Global % of inhibition obtained with western blot is in the same range as HTRF®.

➢ ***ODS2005294 inhibited phosphorylation on S935 with the same potency independently of mutation or disease status.***

Comparison of the 3 different groups

○ vehicle, ● ODS2005294



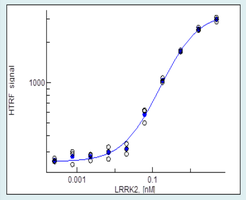
Statistical test: Paired t test

Ranges of pS935-LRRK2 and its inhibition with ODS2005294 , were equivalent in the 3 groups

➢ ***HTRF®-immunoassay : characteristics & robustness***

- Format : 384 wells
- Sample volume/well: 4µL
- Replicates easy to perform
- Homogeneous test
- Results in 5h
- Dynamic range 0.01 to 3 nM

Standard curve



	LRRK2	pS935-LRRK2
IC50 (nM)	0.44	1.9
Z	0.90	0.90
CV	12%	4.3%

LRRK2-HTRF® assay more convenient in clinical study than Western Blot

Conclusions

Altogether, we showed that ODS2005294 inhibited the phosphorylation on S935-LRRK2 in PMBC from G2019S PD patients, as well as from their asymptomatic relatives with or without G2019S mutation. We were able to monitor this effect by an HTRF® immunoassay suitable for future use in clinical studies.

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