# Chronic Treatment of LRRK2 Inhibitors in Diet Produces an Effect in the Lung: Comparison of the Effect in Rats vs. Mice

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## 1 BACKGROUND

- Mutations in the Leucine-rich repeat kinase 2 (LRRK2), including G2019S, enhance kinase activity and leads to Parkinson's disease (PD), therefore, there is a strong therapeutic focus on the discovery of potent and safe LRRK2 kinase inhibitors capable of slowing disease progression.
- It has been reported that the chronic administration of LRRK2 inhibitors results in morphological changes of type II pneumocytes in the lung in rodents (1) and non human primates (NHP) (2), similar to that observed in LRRK2 KO rodent models (3,4).
- In this study, we have administered a selective LRRK2 inhibitor, PF-360, chronically in diet to C57Bl6 and G2019S mice as well as to CD rats and collected the lungs for qualitative histopathology assessment.

## 2 METHODS

**Animals**. *Mouse studies*: Total of 90 male C57Bl/6J mice (Charles River, Sulzfeld, Germany) and 105 male LRRK2-G2019S mice (C57Bl/6 background, n=75 10-12 weeks old; n=30 5-6 months, donated by Michael J Fox Foundation) were used in the studies. All mice received unilateral injection of AAV-A53T in the substantia nigra. The treatment with a diet containing PF-360 or vehicle started 7 days prior to injections. Five weeks after the infusions, the animals were subjected for end point tissue collection. Plasma bile acid levels of all study mice were analysed prior to the study to exclude mice with portal liver shunts. The mice were group housed up to 5 mice/cage.

*Rat study:* Total of 40 male CD rats (Charles River, Sulzfeld, Germany) weighting 350-400g were used for the study. The rats were treated either with vehicle diet or diet with PF-360 for 7 days. The rats were single housed for seven days, and body weight and the amount of food consumed was measured daily. After 7 days of treatment, the animals were subjected for end point tissue collection.

## 2 METHODS CONTINUED

**Quantification of PF-360 in brain and plasma.** An aliquot of each plasma sample was precipitated using acetonitrile. The resulting supernatant was diluted 5x and then injected onto the LC-MS/MS. Brain tissue samples were homogenized by sonication in PCA solution (6 µL per each mg tissue) and centrifuged. The resulting supernatant was used as brain extract sample and diluted 26x prior to injection onto the LC-MS/MS. Chromatographic separation was performed on a reversed phase analytical column (100 x 2.1 mm, 3.5 µm) held at a temperature of 35 °C. Components were separated using a gradient of acetonitrile-methanol (1:1) containing 0.1% formic acid in ultrapurified $H_2O$ containing 0.1% formic acid at a flow rate of 0.2 mL/min. The MS analyses were performed using an API 4000 MS/MS system, consisting of a API 4000 MS/MS a Turbo Ion Spray interface (both from Sciex, USA). The instrument was operated in multiple-reaction-monitoring (MRM) mode. The acquisitions were performed in positive ionization mode, with optimized settings for PF-360. Data were acquired and processed using the Analyst™ data system (v 1.6.2, Sciex, USA).

**Total and pSer935 LRRK2 protein lung assessment.** Total and phosphorylated LRRK2 were assessed using Time-Resolved Fluorescent Energy Transfer from lung homogenates. Equal amounts of protein were loaded in assay for normalization. Data was further normalized to the total LRRK2 protein levels of vehicle treated groups. Total and phosphorylated LRRK2 pSer935 levels were analyzed by Cytation3 according to the manufacturer's instructions (Cisbio, #6FNRKPEG and #FLRKPEG).

**Lung histopathology assessment.** Formalin fixed whole lungs (n=4-5/group) were processed to paraffin and 2 series of 5 µm thick transverse sections of the lung, including all lobes, were collected (4 sections at 100 µm interval), stained with H&E and assessed semi-quantitatively by pathologist blinded to the treatment groups. Alveolar epithelial cells were assessed and type II pneumocytes were identified by their location in the alveolar walls and the numbers of vacuolated and enlarged type II pneumocytes were counted. For qualitative assessment, tissues were evaluated by light microscopy. For quantitative assessment, slides were scanned using an Aperio slide scanner (Leica Biosystems) and ten X40 objective field photomicrographs of parenchyma were randomly selected from one transverse section from each slide (20 per animal). The numbers of vacuolated and enlarged type II pneumocytes were manually counted using Image J (NIH) imaging software. Means and standard deviation were calculated for each group.

## 3 RESULTS – CD RAT

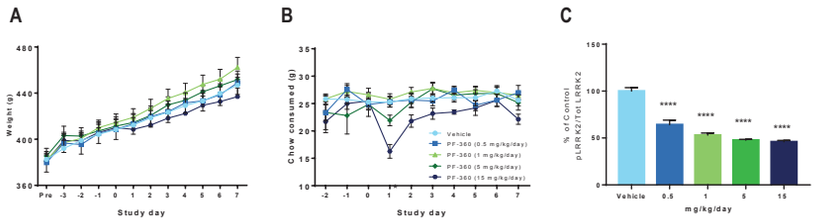


**Figure 1.** Body weight (A) and food consumption (B) in CD rats over 7 days of in-diet treatment with PF-360. Phosphorylated Ser935 LRRK2 levels in lungs (C) measured by HTRF assay. Data are presented as mean ± SEM, n = 8, , * p < 0.05, **** p < 0.001, Two-way repeated measures ANOVA, with Dunnett's multiple comparisons test.

**Table 1**. PF-360 total plasma concentration in male CD rat after 7 days of in diet dosing. Data presented as mean+ SEM.

| | Plasma (nM) | Plasma (ng/mL) | Brain extract (nM) | Brain extract (ng/g) |
|---|---|---|---|---|
| Vehicle | < LLOQ | < LLOQ | < LLOQ | < LLOQ |
| 0.5 mg/kg/day | 36.2 ± 5.6 | 11.2 ± 1.7 | 3.9 ± 0.5 | 7.3 ± 0.9 |
| 1 mg/kg/day | 173.0 ± 14.5 | 53.3 ± 4.5 | 16.1 ± 1.0 | 29.7 ± 1.9 |
| 5 mg/kg/day | 348.3 ± 42.3 | 107.4 ± 13.0 | 37.4 ± 5.8 | 69.3 ± 10.0 |
| 15 mg/kg/day | 679.1 ± 137.4 | 209.4 ± 42.4 | 64.8 ± 14.2 | 119.8 ± 26.3 |

**Table2.** Lung Type II Cells Vacuolation / Enlargement Severity Grade in CD rats weighing 350-400 grams, n=4/group.

| | Vehicle | 0.5 mg/kg/day | 1 mg/kg/day | 5 mg/kg/day | 15 mg/kg/day |
|---|---|---|---|---|---|
| Not detected | 3 | 4 | 3 | 2 | 3 |
| Minimal | 1 | - | 1 | 2 | 1 |
| Mean No. Of vacuolated Type II Pneumocytes | - | - | - | - | - |

## 4 RESULTS – C57BL/6J and G2019S MOUSE

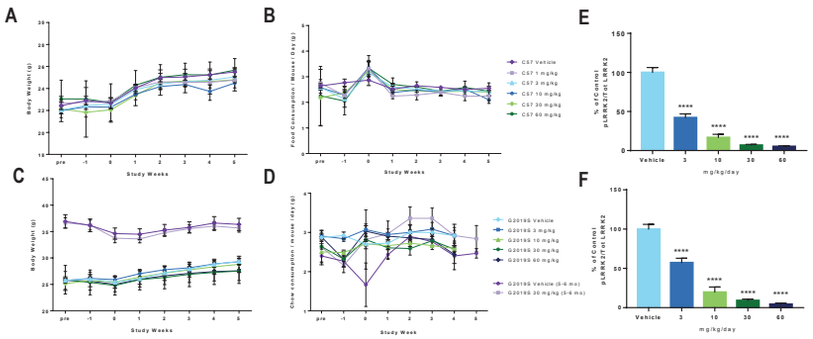


**Figure 2.** Body weight and food consumption in C57Bl6J (A, B) and G2019s (C,D) mice over 42 days of in-diet treatment with PF-360. Phosphorylated Ser935 LRRK2 levels in lungs of C57Bl6J (E) and G2019s (F) mice measured by HTRF assay. Data are presented as mean ± SEM, n=15 (A, C), n=8-10 (E-F), * p < 0.05, **** p < 0.001, Two-way repeated measures ANOVA, with Dunnett's multiple comparisons test.

| | Plasma (nM) | Plasma (ng/mL) | Brain extract (nM) | Brain extract (ng/g) |
|---|---|---|---|---|
| Vehicle | < LLOQ | < LLOQ | < LLOQ | < LLOQ |
| 1 mg/kg/day | 6.0 ± 1.1 | 1.9 ± 0.3 | < LLOQ | < LLOQ |
| 3 mg/kg/day | 18.4 ± 1.8 | 5.7 ± 0.5 | 2.9 ± 0.3 | 4.7 ± 0.6 |
| 10 mg/kg/day | 220.9 ± 74.5 | 68.1 ± 23.0 | 27.6 ± 9.7 | 51.1 ± 18.0 |
| 30 mg/kg/day | 388.1 ± 79.7 | 119.7 ± 24.6 | 51.0 ± 8.4 | 94.4 ± 15.6 |
| 60 mg/kg/day | 954.9 ± 170.8 | 294.4 ± 52.7 | 108.9 ± 19.7 | 165.9 ± 28.2 |

**Table 3.** PF-360 total plasma concentration in 8-10 weeks old C57Bl/6J mice after 42 days of in diet dosing. Data presented as mean + SEM.

| | Plasma (nM) | Plasma (ng/mL) | Brain extract (nM) | Brain extract (ng/g) |
|---|---|---|---|---|
| *G2019S 11-12 wk* | | | | |
| Vehicle | < LLOQ | < LLOQ | < LLOQ | < LLOQ |
| 3 mg/kg/day | 17.9 ± 3.0 | 5.5 ± 0.9 | 4.5 ± 0.8 | 8.3 ± 1.5 |
| 10 mg/kg/day | 195.4 ± 38.9 | 60.3 ± 12.0 | 41.0 ± 7.8 | 75.9 ± 14.4 |
| 30 mg/kg/day | 604.5 ± 101.6 | 186.4 ± 31.3 | 123.4 ± 18.5 | 228.3 ± 34.3 |
| 60 mg/kg/day | 1808.2 ± 235.2 | 557.6 ± 72.5 | 351.6 ± 40.1 | 650.5 ± 74.5 |
| *G2019S 5-6 mo* | | | | |
| 30 mg/kg/day | 423.79 ± 65.0 | 130.7 ± 20.1 | 54.2 ± 9.64 | 100.3 ± 17.8 |

**Table 4**. PF-360 total plasma concentration in 11-12 weeks old G2019S and 5-6 months old G2019S mice after 42 days of in diet dosing. Data presented as mean + SEM.

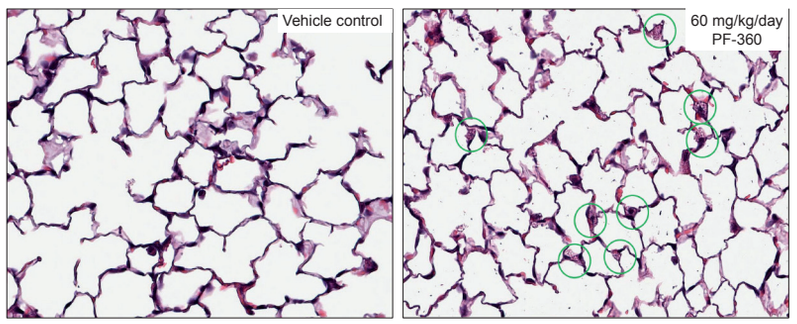


**Figure 3.** Example images demonstrating enlarged vacuolated Type II pneumocytes (green circles), x 400 magnification, H&E stain.

**Table 5.** Lung Type II Cells Vacuolation / Enlargement Severity Grade in 8-10 wk old C57BL/6J at (n=5/group).

| | Vehicle | 1 mg/kg/day | 3 mg/kg/day | 10 mg/kg/day | 30 mg/kg/day | 60 mg/kg/day |
|---|---|---|---|---|---|---|
| Not detected | 5 | 3 | 3 | 0 | 1 | 0 |
| Minimal | 0 | 0 | 2 | 2 | 0 | 2 |
| Mild | 0 | 0 | 0 | 0 | 0 | 2 |
| Moderate | 0 | 0 | 0 | 0 | 2 | 1 |
| Mean No. Of vacuolated Type II Pneumocytes (pneumocytes/50 fildes x40) | 0.7 | 1.5 | 1.0 | 3.4 | 5.1 | 3.9 |

**Table 6**. Lung type II cells vacuolation / enlargement severity grade in 11-12 wk old and 5-6 mo old G2019s mice (n=5/group).

| | Vehicle | 1 mg/kg/day | 3 mg/kg/day | 10 mg/kg/day | 30 mg/kg/day | 60 mg/kg/day |
|---|---|---|---|---|---|---|
| *G2019S 11-12 wk* | | | | | | |
| Not detected | 0 | N/A | 0 | 0 | 0 | 0 |
| Minimal | 3 | N/A | 1 | 5 | 1 | 1 |
| Mild | 1 | N/A | 3 | 0 | 2 | 2 |
| Moderate | 1 | N/A | 1 | 0 | 2 | 2 |
| Mean No. Of vacuolated Type II Pneumocytes (pneumocytes/50 fildes x40) | 1.5 | N/A | 1.5 | 1.3 | 2.4 | 2.7 |
| *G2019S 5-6 mo* | | | | | | |
| Not detected | 3 | N/A | N/A | N/A | 2 | N/A |
| Minimal | 2 | N/A | N/A | N/A | 3 | N/A |
| Mild | 0 | N/A | N/A | N/A | 0 | N/A |
| Moderate | 0 | N/A | N/A | N/A | 0 | N/A |
| Mean No. Of vacuolated Type II Pneumocytes (pneumocytes/50 fildes x40) | 1.8 | N/A | N/A | N/A | 1.6 | N/A |

## 5 CONCLUSIONS

- In histological examination and quantitative analysis of rat lung, low numbers of large and vacuolated alveolar epithelial cells, consistent with Type II pneumocytes, were observed both in vehicle treated controls and animals receiving PF-360. However, no differences were seen between vehicle and PF-360 treated groups. This further confirms previously reported findings that LRRK2 inhibitors do not develop the lung phenotype in rats.[3]
- In C57Bl6J mice, morphologic changes in the lung, consistent with increased numbers of enlarged type II pneumocytes, were observed at doses higher than 10 mg/kg/day. This observation is in line with previously reported data with PF-360.[5]
- In G2019S mice, morphologic changes in the lung, consistent with increased numbers of enlarged type II pneumocytes, were observed at doses higher than 30 mg/kg/day. To our knowledge, this is the first time the lung phenotype has been detected with PF-360 in G2019s mice. The finding is similar to what has been shown with MLi-2 in this strain.[5]

## 6 REFERENCES

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