Cytotoxic effects of LRRK2 inhibitors in combined treatment with chemotherapeutic agents on a large panel of cancer cell lines

Franziska Fimm-Todt, Joachim Lauterwasser, Eva Egenter, Christian Weber, Daniel Feger, Katharina Schaich, Sarah Ulrich, Oliver Siedentopf, Frank Totzke, Michael Kubbutat and Jan Erik Ehlert

Reaction Biology Europe GmbH, Engesserstr. 4, 79108 Freiburg, Germany

Introduction

The leucine-rich repeat kinase 2 (LRRK2) is a multidomain-protein involved in the regulation of intracellular trafficking. Aberrant LRRK2 function causes neurotoxicity, dysfunctional autophagy as well as deregulated vesicle trafficking[1]. Several pathogenic LRRK2 mutations have been identified (e.g. G2019S) which ultimately cause aberrant LRRK2 hyperactivity and increased phosphorylation of downstream targets like Rab GTPases (e.g. Rab10) [2].

The association between aberrant LRRK2 activity and the onset of sporadic as well as familial Parkinson's disease (PD) is well described in the literature[3]. Thus, the inhibition of LRRK2 represents a promising target for the treatment of PD. However, several studies report about LRRK2-mediated deregulation of cancer-related pathways (e.g. ATM-p53-p21-pathway, JNK pathway)[4;5]. Pathogenic LRRK2 hyperactivity is suggested to increase the risk for the onset of different types of cancer (e.g. breast, thyroid, lung, leukemia)[6,7] or is described to protect from the development of non-skin-related cancer.

We hypothesized that LRRK2 inhibition may therefore influence the anti-cancer treatments in specific cancer cell types.

At Reaction Biology we established a cellular LRRK2 phosphorylation assay to study the effect of various LRRK2 inhibitors and compared the results to biochemical LRRK2 assays.

Additionally, we compared the direct and combinatorial effect of LRRK2 inhibitors in the presence or absence of the chemotherapeutic agent Doxorubicin on the proliferation of 140 (cancer) cell lines.

References

1. Marchand, A., et al (2020). Frontiers in neuroscience, 14, 527
2. Berwick, D. C., et al (2019). Molecular neurodegeneration, 14(1), 49.
3. Kluss, J. H., et al (2019). Biochemical Society transactions, 47(2), 651–661.
4. Chen, Z., et al (2017). Human molecular genetics, 26(22), 4494–4505
5. Jiang, Z. C., et al (2019). International journal of oncology, 55(1), 21–34.
6. Lebovitz, C., et al (2021). Scientific reports, 11(1), 2097.
7. Agalliu, I., et al (2019). Movement disorders : official journal of the Movement Disorder Society, 34(9), 1392–1398.

Contact Information

Franziska Fimm-Todt, PhD

Reaction Biology Europe GmbH
Engesserstr, 4
79108 Freiburg
Germany

+49-761-769996-1646
F.Fimm@reactionbiology.de
www.reactionbiology.com

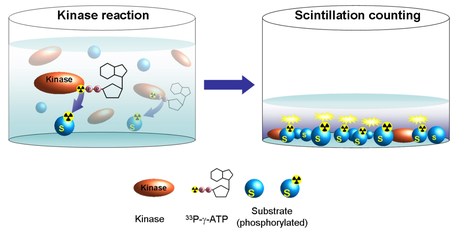
Validation of LRRK2 inhibitors

LRRK2 is a serine/threonine kinase which is able to catalyze autophosphorylation (e.g. S1292, S935) as well as the phosphorylation of selected substrates (e.g. Rab10, Rab29). Besides the kinase domain LRRK2 posseses a second catalytic GTPase domain and four additional domains (ARM, ANK, LRR and WD40 domains) which are supposed to facilitate different protein-protein interactions[2].

Interestingly, several pathogenic mutations have been shown to affect the kinase activity of LRRK2. The most common PD-related LRRK2 mutation G2019S is located in the kinase domain and elevates kinase activity. Several other disease-related LRRKs mutation sites have been identified[1].

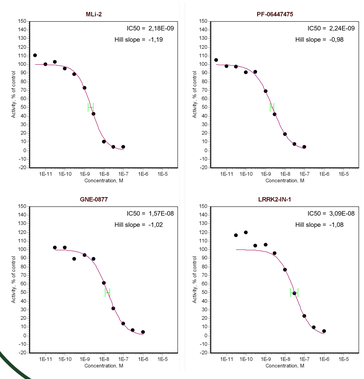
In this study, the effect of different LRRK2 inhibitors on the kinase activity of LRRK2 was analyzed. To this end the LRRK2 inhibitors were subjected to the radiometric ^{33}PanQinase™ assay. Furthermore, selected LRRK2 inhibitors were subjected to a cellular LRRK2 phosphorylation assay to assess the effects on Ser925 phosphorylation.

Biochemical ^{33}PanQinase™ assay



Principle of the radiometric ^{33}PanQinase™ assay

A radiometric, Flashplate® based assay setup was used to determine the phosphorylation of LRRK2 substrates by different LRRK2 variants.
Kinase and substrate were incubated in presence of ATP containing ^{33}P-γ-ATP as tracer. After the kinase reaction was stopped, the proteins were immobilised on the Flashplate reaction vessel surface and the incorporated radioactivity measured by scintillation counting.



Results of the biochemical kinase assay for selected LRRK2 inhibitors

The LRRK2 inhibitors MLi-2, PF-06447475, GNE-0877 and LRRK2-IN-1 were subjected to the radiometric ^{33}PanQinase™ assay.

The following assay conditions were used:

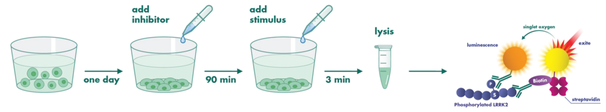
Kinase Name	*Kinase Concentration (ng/50µl)*	*Kinase Concentration (nM) **	*ATP Concentration (µM)*	*Substrate Name*	*Substrate Concentration (µg/50µl)*
LRRK2	50	4,9	0,3	GSK3(14-27)	4,0

Cellular LRRK2 phosphorylation assay

Principle of the the cellular LRRK2 assay using the AlphaLISA Technology

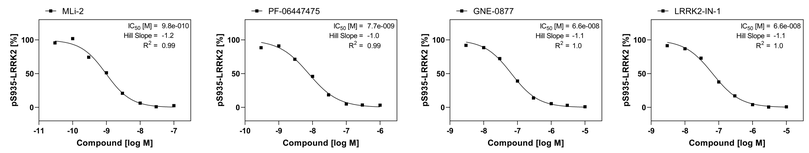
In the cellular LRRK2 phosphorylation assay the human non-small cell lung cancer cell line A549 is used. This cell line endogenously expresses high levels of LRRK2, resulting in constitutive autophosphorylation at Ser935, which is impaired in response to LRRK2 inhibition.

A549 cells were plated in DMEM supplemented with 10% FCS in multiwell cell culture plates. The next day, cells were treated with compounds (2,5 h at 37°C) in complete culture medium. Cells treated with 1,0E-06 M MLi-2 were used as low control. After cell lysis, quantification of pS935-LRRK2 was assessed in 96-well plates using the AlphaLISA (**a**mplified **l**uminescent **p**roximity **h**omogeneous **a**ssay) Technology. In this homogeneous assay system, an LRRK2 kinase-specific and an anti-phospho-Ser935 antibody immobilized to specific Acceptor and Donor beads were used.



Results of the cellular LRRK2 assay for selected LRRK2 inhibitors

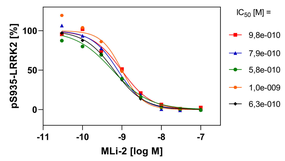
The LRRK2 inhibitors MLi-2, PF-06447475, GNE-0877 and LRRK2-IN-1 were subjected to the cellular LRRK2 phosphorylation assay using A549 cells. LRRK2 inhibition results in reduced autophosphorylation at LRRK2 Ser935 which can be analyzed using the AlphaLISA technology.



A comparison of the IC50 values is for all four LRRK2 inhibitors analyzed in the biochemical and cellular assays:

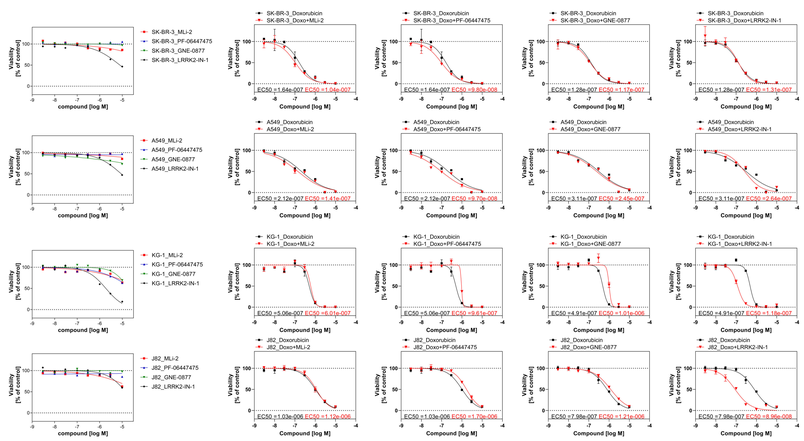
	Biochemical ^{33}PanQinase™ assay	Cellular LRRK2 pSer935 assay
MLi-2	3,01E-09	9,80E-10
PF-06447475	2,66E-09	7,70E-09
GNE-0877	1,32E-08	6,60E-08
LRRK2-IN-1	3,21E-08	6,60E-08

AlphaLISA Technology is well-validated and provides an excellent detection window. A simple add-mix-read protocol with no wash steps guarantees high reproducibility as shown for a reference inhibitor MLi-2:



Reference ID	IC50 (mean) [M]	SD IC50
MLi-2	8,0E-10	1,9E-10

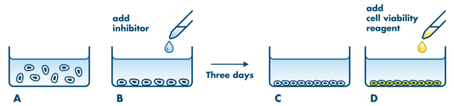
Potential combinatorial effects of LRRK2 inhibitors and Doxorubicin on cell proliferation



Principle of the the cell proliferation assay using CellTiter-Glo Reagent

Cells were seeded in 384-well plates. The next day, compounds were added using a nanodrop-dispenser (Tecan D300E). After incubation for 3 days, the CellTiter-Glo dye was added and the number of viable cells was determined by luminescence readout.

Promega's CellTiter-Glo dye is used to determine the number of viable cells by generation of a luminescent signal proportional to the amount of ATP present based on an ATP-dependent luciferase reaction.



Analysis of cytotoxic effects on a panel of 140 human (cancer) cell lines

The cytotoxic effects of the LRRK2 inhibitors MLi-2, PF-06447475, GNE-0877 and LRRK2-IN-1 were analyzed in 140 cell lines. To this end, cells were treated with the LRRK2 inhibitor alone or in combination with the chemotherapeutic agent Doxorubicin.

Exemplary plots for SK-BR-3 (breast cancer), A549 (lung cancer), KG1 (blood cancer) and J82 (bladder cancer) are depicted on the left.

In summary, treatment with the four LRRK2 inhibitors alone did not show significant cytotoxicity on almost all tested cell lines. LRRK2-IN-1 showed the strongest cytotoxic effects (especially in blood (cancer) cell lines) which are probably not strictly LRRK2-dependent.

Furthermore, the combinatorial treatment with LRRK2 inhibitors and Doxorubicin did not show significant LRRK2-dependend synergistic or antagonistic effects. We thereby could not confirm results of a study showing a synergistic effect of LRRK2 inhibition and Doxorubicin treatment in mouse cell lines[4] in our panel of 140 human cell lines.

Conclusion

LRRK2 inhibition seems to be a promising target for PD treatment, but it is highly disputed if inhibition of LRRK2 may also increase the risk to develop certain types of cancer.

In this study we could show that:

- **a cellular LRRK2-pSer935 phosphorylation assay in A549 cells can be used to analyze the effect of LRRK2 inhibitors**
- **the potent inhibition of selected LRRK2 inhibitors could be validated in the cellular as well as biochemical assay**
- **LRRK2 inhibitors cannot significantly inhibit cancer cell growth tested in a proliferation assay with using a panel of 140 cell lines**
- **the combinatorial treatment of 140 human cell lines with LRRK2 inhibitors and Doxorubicin show only minor synergistic or antagonistic effects on cancer cell growth**