nature portfolio

Peer Review File

Activation of Parkin by a Molecular Glue

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Sauve et. al. conduct structural and mechanistic experiments for a small molecule activator of parkin that was identified in a previous publication. The authors use a series of ubiquitination experiments to show how the molecule (BIO-2007817) affects autoubiquitination of parkin and binding of a secondary pUb molecule to the RING0 domain. They were unable to crystallize parkin with BIO-2007817 but did obtain a structure with a related compound – BIO-1975900, although this compound has an EC50 that is 20-fold lower. In organello experiments that test some substitutions in the Ubl domain (R42P, V56E) show that addition of 2007817 offers some recovery of mitofusin ubiquitination.

Overall, this is an interesting paper that focuses on the important problem of identifying small molecule activating molecules for parkin. As parkin and other E3 ligases have several steps needed for ubiquitination these proteins present challenging targets because there are multiple decision forks where intervention might be attempted. The current work attempts to use a small molecule that activates towards the end of parkin's activation pathway following translocation to mitochondria, activation of PINK1, phosphorylation of Ub (pUb) and phosphorylation of parkin. Whether this is the best strategy remains to be seen. In this work the authors present data that shows that addition of BIO-2007817 to parkin increases autoubiquitination following activation by pUb and present a crystal structure that shows the THPP building block of BIO-1975900 (along with a glycerol molecule) lands in a similar region as the "ACT" region observed in a crystal structure. Based on this data the authors propose that BIO-2007817 increases parkin's activity by mimicking the ACT region to displace the catalytic Rcat domain.

This work tackles an important problem. However, I feel it has several shortcomings that need to be addressed prior to further consideration and publication. A major concern is the use of, and interpretation of the crystal structure that utilizes BIO-1975900. While this molecule contains the THPP building block it lacks the most important portions of the molecule needed for increased activity and specificity. For example, the current (and previous publication) show that derivatization of the methyl-ketone terminus in BIO-1975900 OR having the R configuration (Fig. 1e) at the chiral center adjacent to the ketone group increase EC50 > 200-fold. Yet this portion of the compound is not present in BIO-1975900. The authors use NMR to show that W183 in the RING0 domain has a similar chemical shift change upon binding of BIO-1975900 or BIO-2007817. This is to be expected since W183 is close to the tetrahydroquinoline ring at the opposite end of the molecule that is common to all the small molecules tested. There is no information regarding interactions of the important methyl-pyrazole derivatization in BIO-2007817 present in the BIO-1975900 structure. The authors did do some docking to infer this (Supp. Fig. 2) but there is no validation for this. In the absence of a crystal structure to show these interactions or NMR data that monitor interactions near the methyl-pyrazole region of BIO-2007817 these experiments provide little information regarding the specificity and increased activity of BIO-2007817 that are the foundation of this manuscript. In addition, the authors do not provide activity assays (i.e. ubiquitination) that compare the activity of BIO-1975900 vs BIO-2007817.

The ubiquitination assays used to measure and compare activities (Fig. 1) appear to be well done but offer little information about substrate ubiquitination which ultimately is the functional target. In

addition, there are inconsistencies between the results obtained in Fig.1 and those described on p12 for in organello experiments (Fig. 4). Based on autoubiquitination experiments one would have expected far greater ubiquitination of Mfn2 for the S65A parkin species (Fig. 4) since this experiment is akin to pUb addition (only) used in Fig. 1. The experiments show addition of BIO-2008717 yields a significant change in autoubiquitination but substrate ubiquitination for S65A in Fig. 5b is minimal. Substrate ubiquitination should be completed in place of autoubiquitination for Fig. 1.

There are several other details that should be addressed.

The title does not accurately reflect the content of the manuscript. The compound tested does not help or increase pUbl/pUb binding.

-p4, top – the description of activation is written as a "matter of fact". My understanding is that there is no direct evidence to show the Rcat and REP domains are released upon phosphorylation of parkin. The idea arises from negative evidence in X-ray structures that lack these regions rather than observation that the Rcat has relocated elsewhere. I am sure the authors realize this, but the text should be more clearly stated.

-p6 (Fig. 1b) – the autoubiquitination assays with parkin and R0RBR use 200 uM BIO-2007817 which is about 100-fold excess compound compared to parkin. It would be appropriate to complete experiments at lower concentrations to help draw conclusions regarding competition or displacement with either the pUbl/pUb or ACT. These assays should utilize a substrate rather than parkin self-ubiquitination

-p6 – UbVS assays do not measure structural changes. They measure the accessibility and reactivity of a catalytic triad cysteine

-p6 (Fig. 1) – multiple studies have shown the pUbl domain from parkin, including those from the authors's groups, is a requirement for full activity yet no experiments are completed using parkin phosphorylation. The authors state the affinity of pUb/pUbl with the Rcat is ~400 nM – (in trans). The actual pUbl interaction is not in trans and its apparent affinity would be considerably tighter. How does this impact the relevance of drug binding to parkin?

Fig. 1d should include a phosphorylated-parkin+pUb in the absence of compound as a control

Fig. 3 –the authors might want to be more cautious stating that that the two compounds bind at the same location – only a single signal is indicated and even then Fig. 3f shows differences between the CSP of the two compounds. This text should be modified to be less general. See comments above also.

p9 bottom – structure 5N2W has pUb bound so is not fully inhibited as stated. It would be more correct to refer to structures 5C1Z, 6HUE or 4ZYN and use these structures for reference even if the differences (ie. Fig. 3d) are small

p9 – the authors state that "BIO-2007817 competes with Rcat for binding to RING0 to promote its (Rcat) release …" This is not supported by data in this manuscript. The ubiquitination assays in Fig. 1 show that in the absence of pUb, BIO-207817 alone does not promote autoubiquitination. The structure shows pUb is bound to the RING0 domain. This indicates that the pUb binding is a

requirement for BIO-2007817 to further activate parkin as shown by a 5000-fold decreased affinity of BIO-207817. In addition the ITC experiments were completed in the absence of the Rcat domain so how can one state anything about competition of BIO-207817 with Rcat domain binding to the RING0 domain

Fig. 4 - is this predictive from docking?? Please clarify if data in the table are calculated from docking. If so, actual measurements should be completed (or included in the table) to confirm EC50 or binding affinities?

p12 (Fig. 5b) – it is stated that BIO-2007817 can “partially rescue” S65A and R0RBR and at “10 uM BIO-2008717 the ratio of mono-ubiquitinated to unmodified Mfn2 was roughly 50%”. It is the total ubiquitination that is important here and this is quite different between WT and S65A/R0RBR. Judging by the intensities of the unmodified Mfn2 the ubiquitination for S65A/R0RBR is much less than 50% using BIO-2008717 compared to WT. The authors should reword and re-quantify this.

Reviewer #2 (Remarks to the Author):

The manuscript 'ACTIVATION OF PARKIN BY A MOLECULAR GLUE' describes the functional and structural-biology characterization of a previously identified molecule that activates the PD-relevant E3-Ub ligase and might be useful for the treatment of certain familial, but potentially also idiopathic PD cases.
In a number of elegant biochemical and biophysical assays, authors show that the molecule activates Parkin only in the presence of pUB and that it indeed only binds with significant affinity to the complex of Parkin and pUB. Very importantly, the author solved the 2.5 A crystal structure of the Parkin R0RB and RING0 in complex with rUB and the Parkin activator. This structure explained the aforementioned biochemical and biophysical finding by showing that the Parkin activating molecule actually works as an orthosteric PPI stabilizer/molecular glue that binds in the interface of the RING0 domain and pUB.

This manuscript is another great example of the value of molecular glues as a promising therapeutic modality. Its activity to restore the activity of PD-related Parkin mutants and the potential use in idiopathic PD makes this molecule a great starting point for the development of an urgently new therapeutic molecule for this underserved patient population.

Minor remarks:

Page 15, first line: 'molecular glue' instead of 'molecule glue'
Page 15, first paragraph: 'In the inactive
conformation of parkin, the groove is filled by C-terminal residues of the Rcat domain. In the inactive conformation, the groove is occupied by elements of the Rcat domain..."
==> The first (or the second) sentence is redundant.

Reviewer #3 (Remarks to the Author):

Sauvé et al provide and structural and mechanistic study on the activation of parkin by a series of compounds that act as molecular glues between parkin and phospho-ubiquitin. The authors demonstrate that the compounds activate parkin by binding the RING0 domain in the place of the ACT element and recruit pUb. Through a crystal structure they show the equivalent positioning of an additional pUb where normally the parkin pUbl domain resides. They combine the crystal structure with a weaker compound with ligand docking studies with a tighter binder to do an SAR study. Their model satisfactorily explains why the molecular glues work only for the Ubl/PINK1 mutant cell lines.

Major points
1. Fig 1c: The RING1 mutant control at 0 min incubation time would be good to include.
2. The NMR spectra in Fig S1 show that BIO-2007817 adopts cis and trans isomeric states in water. Have the authors described these experiments in the methods section?
3. In the crystal structure results section, they mention that this isomerization could be a cause for the difficulty in crystallization with this compound. Are the authors proposing that both the isomers can bind parkin/pUb? Is that something they can investigate by docking?
4. Since the authors report a relatively small number of compounds, is there a reason they chose the standard precision mode over the extra precision mode?
5. Fig 4d: Docking scores vs pEC50 plot is missing one point for BIO-1983977. Also please report the R^2 value for trendline with this data point.
6. Could a similar plot be included for Docking score and -log(MM-GBSAdG) scores as well?

Minor points
1. The ubiquitination assay depicted in Fig 1b/c needs to be described better in the main text. Especially the use of pUbΔG76. While this is clearly described in the methods section, a line or two in the main text will help the reader understand the assay much quicker.

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> We thank the reviewer for recognizing the interest of identifying small molecules that activate parkin and the effectiveness of BIO-2007817 in parkin ubiquitination assays.

This work tackles an important problem. However, I feel it has several shortcomings that need to be addressed prior to further consideration and publication. A major concern is the use of, and interpretation of the crystal structure that utilizes BIO-1975900. While this molecule contains the THPP building block it lacks the most important portions of the molecule needed for increased activity and specificity. For example, the current (and previous publication) show that derivatization of the methyl-ketone terminus in BIO-1975900 OR having the R configuration (Fig. 1e) at the chiral center adjacent to the ketone group increase EC50 > 200-fold. Yet this portion of the compound is not present in BIO-1975900. The authors use NMR to show that W183 in the RING0 domain has a similar chemical shift change upon binding of BIO-1975900 or BIO-2007817. This is to be expected since W183 is close to the tetrahydroquinoline ring at the opposite end of the molecule that is common to all the small molecules tested.

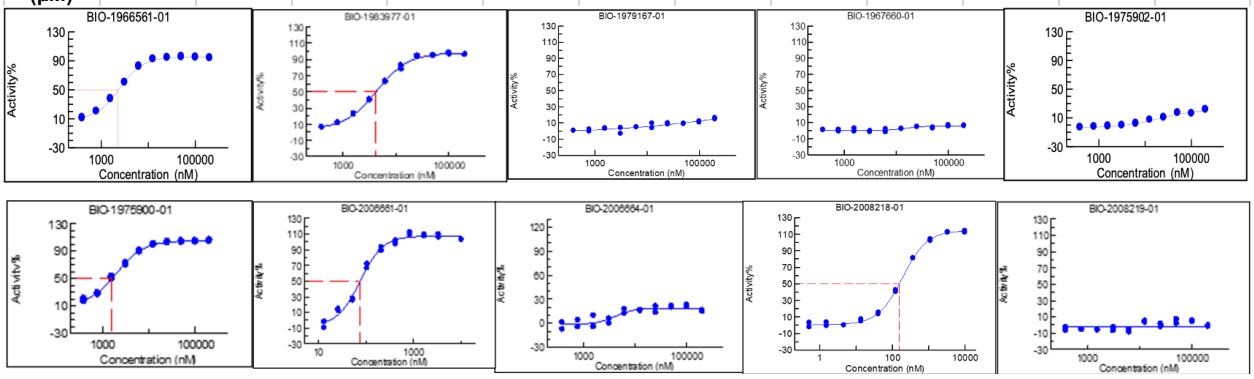
> We agree that, ideally, parkin would have been crystallized with the most active compound, BIO-2007817. Unfortunately, multiple attempts to crystallize or soak BIO-2007817 into crystals were unsuccessful. However, this does not invalidate or undermine the importance of the BIO-1975900 crystal structure.
>
> As the reviewer points out, multiple pieces of evidence confirm that the molecules bind to the same site. Firstly, NMR spectra confirm that the tetrahydroquinoline rings of both molecules are positioned close to W183. Secondly, mutagenesis of Phe146 in the BIO-1975900-binding site also decreases the affinity of BIO-2007817. Thirdly, the pUb dependence of BIO-2007817 binding is most simply explained by pUb contacts as observed in the BIO-1975900 structure. Lastly, BIO-1975900 and BIO-2007817 are ~80% identical with identical tetrahydroquinoline, acetophenone, and tetrahydropyrazolopyrazine groups. These observations validate the conclusion that both molecules bind at the interface between RING0 and pUb, which is a key conclusion of the paper.

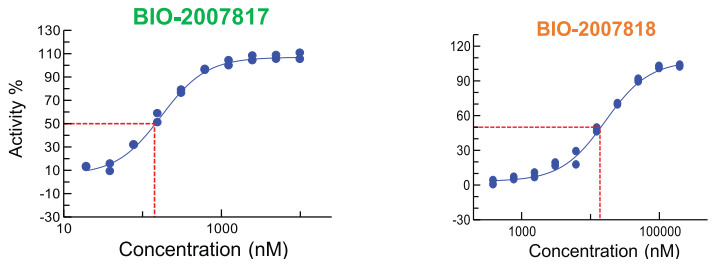
There is no information regarding interactions of the important methyl-pyrazole derivatization in BIO-2007817 present in the BIO-1975900 structure. The authors did do some docking to infer this (Supp. Fig. 2) but there is no validation for this. In the absence of a crystal structure to show these interactions or NMR data that monitor interactions near the methyl-pyrazole region of BIO-2007817 these experiments provide little information regarding the specificity and increased activity of BIO-2007817 that are the foundation of this manuscript. In addition, the authors do not provide activity assays (i.e. ubiquitination) that compare the activity of BIO-1975900 vs BIO-2007817.

> We apologize for not more clearly stating that the EC50 values in the figure are experimental measurements of autoubiquitination. The text has been revised to clarify this. The activity profiles for

the new compounds in Figure 5 are presented below and have been added as Supplementary Fig. 1. The activity profiles for BIO-2007817and BIO-2007818 were published previously (Shlevkov et al., 2022) and are shown for comparison.

BIO-#	2007817	2007818	1966561	1983977	1979167	1967660	1975900	1975902	2006661	2006664	2008218	2008219
EC50 (μM)	0.15	13.9	2.9	2.1	>50	>50	2.9	>50	0.1	>50	0.28	>50





The reviewer is correct that we have an incomplete picture of the importance of the compound-protein interactions in the methyl-pyrazole region. However, the docking studies and experimental EC50s (Figure 5), which correlate well together, bring substantial insights to the proposed binding modes. For instance, the docking scores and binding energies of the two enantiomers BIO-2007817 and BIO-2007818 are consistent with the higher affinity of the former. The binding of the tetrahydroquinoline group into the W183 pocket, which is common to BIO-2007817 and BIO-1975900, restrains the possible conformations of the molecules, and thus increase our confidence about the binding mode in the K48 pocket. Still, we acknowledge that additional studies are needed to fully explore the SAR of the methyl-pyrazole region before development of TTHP compounds as therapeutics.

The ubiquitination assays used to measure and compare activities (Fig. 1) appear to be well done but offer little information about substrate ubiquitination which ultimately is the functional target. In addition, there are inconsistencies between the results obtained in Fig.1 and those described on p12 for in organello experiments (Fig. 4). Based on autoubiquitination experiments one would have expected far greater ubiquitination of Mfn2 for the S65A parkin species (Fig. 4) since this experiment is akin to pUb addition (only) used in Fig. 1. The experiments show addition of BIO-2008717 yields a significant change in autoubiquitination but substrate ubiquitination for S65A in Fig. 5b is minimal. Substrate ubiquitination should be completed in place of autoubiquitination for Fig. 1.

The reviewer is correct - the relative importance of parkin substrates is poorly understood. Proteomics studies have shown parkin ubiquitinates a wide variety of mitochondrial outer membrane proteins, as well as itself (see e.g. Sarraf et al., Nature 2013, or Ordureau et al., Mol Cell 2018). Physiologically, parkin-mediated ubiquitination appears to be principally regulated by its localization and activation by pUb rather than by substrate selection (see Vranas et al., Open Biol 2022 for details), although we don't exclude that there are additional substrate anchoring steps (e.g. new paper on Miro1 selectivity by the Walden and Shaw labs, Koszela et al., BioRxiv 2024). We and others have shown that autoubiquitination activity correlates very well with substrate ubiquitination and mitophagy (e.g. Stevens et al., Life Sci Alliance 2023). As such, autoubiquitination is a convenient measure of ligase activity in the presence of small molecules or mutations. The goal of Figure 1 was not to assess target

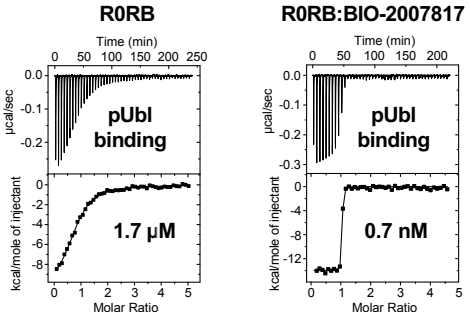
engagement, but rather to elucidate the mechanism of action of BIO-2007817. Our main finding here is that the Ubl is dispensable for activation by BIO-2007817, but pUb is not, which guided our ITC and structural studies. Thus, repeating the ubiquitination experiments in Figure 1 with a substrate is unnecessary since substrate (Mfn2) ubiquitination is assessed in Figure 6 (previously Figure 5). The key result is the differential effects of BIO-2008717 with different parkin mutants. These *in organello* and cellular experiments are more physiological and more relevant.

With respect to the expectation of greater ubiquitination of Mfn2 in Figure 6, we suspect that BIO-2007817 doesn't fully rescue the S65A and Ubl-deletions in the in organello experiments due to the requirement of two pUb molecules in close proximity – one to recruit parkin via RING1 and one to activate via RING0. Mitochondria for the in organello are isolated from HeLa cells which don't express Parkin, and thus have very low levels of endogenous pUb. In contrast, in the autoubiquitination assays, pUb is in solution and doesn't require parkin recruitment to the mitochondrial membrane. The experiments in Figure 1 only measure activation by pUb binding to RING0.

There are several other details that should be addressed.

The title does not accurately reflect the content of the manuscript. The compound tested does not help or increase pUbl/pUb binding.

We thank the reviewer for the suggestion. We previously showed pUbl binds to the parkin R0RB fragment with ~1 μM affinity (reproduced below). To test if BIO-2007817 truly acts as a molecular glue, we repeated the measurement in the presence of BIO-2007817. The new ITC (added as Fig 2g,h) shows that the pUbl affinity improves to ~1 nM. The >1000-fold increase in affinity is dramatic proof that the compound acts as a molecular glue.

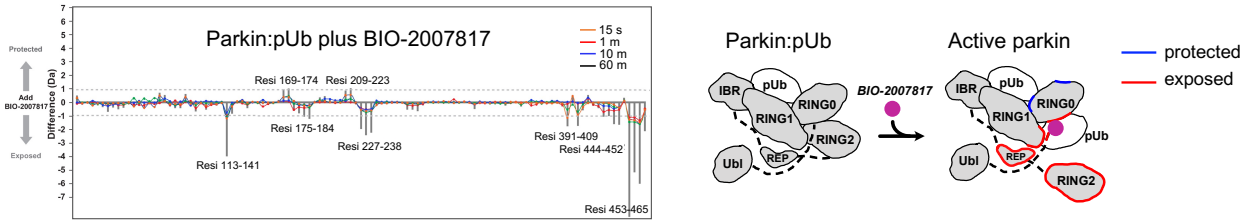


-p4, top – the description of activation is written as a "matter of fact". My understanding is that there is no direct evidence to show the Rcat and REP domains are released upon phosphorylation of parkin. The idea arises from negative evidence in X-ray structures that lack these regions rather than observation that the Rcat has relocated elsewhere. I am sure the authors realize this, but the text should be more clearly stated.

We respectfully disagree. There is a great deal of evidence the Rcat and REP domains are released upon phosphorylation of parkin. Firstly, hydrogen-deuterium exchange experiments reported by three different groups show a dramatic increase in Rcat solvent exposure upon phosphorylation (Condos et al, EMBO J, 2018; Sauvé et al, NSMB 2018; Gladkova et al, Nature, 2018). Secondly, the Rcat catalytic cysteine, which is sequestered in the unphosphorylated structure, becomes reactive toward UbVS upon parkin activation. Thirdly, structures of activated parkin show the Rcat binding site is occupied by either pUbl (Sauvé et al, NSMB 2018; Gladkova et al, Nature, 2018) or pUb (Fakih et al., JBC, 2022). Finally, release of the Rcat domain is necessary for the transfer of the ubiquitin from the E2 enzyme to the Rcat. The E2 and parkin catalytic sites are separated by ~50 Å in inactive parkin so a large conformational change is required.

To confirm that activation by BIO-2007817 occurs through the mechanism of Rcat release, we carried out a complete series of HDX-MS experiments. These have been added as a new Figure 3. Addition of

BIO-2007817 to parkin in the presence of pUb led to a dramatic increase in solvent exchange for residues in the Rcat domain. The changes match the changes upon parkin phosphorylation and confirm that BIO-2007817 acts along the known mechanism of parkin activation – namely release of Rcat domain from RING0.



-p6 (Fig. 1b) – the autoubiquitination assays with parkin and R0RBR use 200 uM BIO-2007817 which is about 100-fold excess compound compared to parkin. It would be appropriate to complete experiments at lower concentrations to help draw conclusions regarding competition or displacement with either the pUbl/pUb or ACT. These assays should utilize a substrate rather than parkin self-ubiquitination

The reviewer raises an interesting point. A lower concentration could have been used in Figure 1b but it would less convincingly demonstrate the requirement for pUb. Even at a 100-fold excess, we observed no activation by BIO-2007817 without pUb. (As discussed above, we feel the experiments in Figure 6 address the substrate issue.)

-p6 – UbVS assays do not measure structural changes. They measure the accessibility and reactivity of a catalytic triad cysteine

We agree and have modified the sentence to read, "As previously reported[48], the activation was strictly dependent on the presence of pUb and could also be detected in ubiquitin-vinyl sulfone (UbVS) assays[51] that measure the accessibility of Rcat and are not dependent on the upstream components of the ubiquitination cascade (Fig. 1d)."

-p6 (Fig. 1) – multiple studies have shown the pUbl domain from parkin, including those from the authors's groups, is a requirement for full activity yet no experiments are completed using parkin phosphorylation. The authors state the affinity of pUb/pUbl with the Rcat is ~400 nM – (in trans). The actual pUbl interaction is not in trans and its apparent affinity would be considerably tighter. How does this impact the relevance of drug binding to parkin?

We agree that the BIO-2007817 binding affinity cannot compete with the affinity of Rcat. This is why the drug cannot bind in the absence of pUb. However, in Figure 1, we actually did carry out experiments with phosphorylated Parkin (Figure 1b, lanes 9-10). Once Parkin is phosphorylated, the pUbl domain indeed binds in "cis", and the BIO-2007817 compound does not further activate the protein. While the parkin pUbl domain is required for full activity, pUb can replace it to a limited extent. The potential therapeutic value of BIO-2007817 is that it enhances this secondary pathway we reported in Sauvé, EMBO J, 2022.

Fig. 1d should include a phosphorylated-parkin+pUb in the absence of compound as a control

The value of repeating the experiment with a phosphorylated-parkin + pUb control is unclear. Previous publications have shown that UbVS reacts with BIO-2007817-treated parkin (Shlevkov et al, iScience, 2022 and phosphorylated-parkin + pUb (e.g. Condos et al., EMBO J, 2018). The aim of Figure 1d is to demonstrate that BIO-2007817 induces a dose-dependent increase in UbVS reactivity, which is abrogated by the K211N mutation.

Fig. 3 –the authors might want to be more cautious stating that that the two compounds bind at the same location – only a single signal is indicated and even then Fig. 3f shows differences between the CSP of the two compounds. This text should be modified to be less general. See comments above also.

> As enumerated above, we believe there are multiple reasons to believe that the two compounds bind to the same location.

p9 bottom – structure 5N2W has pUb bound so is not fully inhibited as stated. It would be more correct to refer to structures 5C1Z, 6HUE or 4ZYN and use these structures for reference even if the differences (ie. Fig. 3d) are small

> The reviewer is correct that the 5N2W structure has pUb bound and is not fully inhibited. We have removed the reference from the text but kept the 5N2W structure in the figure since, as the reviewer notes, the differences in the position of the Rcat domain are small and don't affect the interpretation of the figure.

p9 – the authors state that "BIO-2007817 competes with Rcat for binding to RING0 to promote its (Rcat) release …" This is not supported by data in this manuscript. The ubiquitination assays in Fig. 1 show that in the absence of pUb, BIO-207817 alone does not promote autoubiquitination. The structure shows pUb is bound to the RING0 domain. This indicates that the pUb binding is a requirement for BIO-2007817 to further activate parkin as shown by a 5000-fold decreased affinity of BIO-207817. In addition the ITC experiments were completed in the absence of the Rcat domain so how can one state anything about competition of BIO-207817 with Rcat domain binding to the RING0 domain

> We agree and have modified the sentence. Our rationale for stating that the THPP compounds compete with the Rcat domain is based on the crystal structures, which show that W462 and F463 in Rcat occupy the same sites as the two ends of the THPP molecule (compare Fig. 4c and 4d). These interactions are mutually exclusive and cannot happen at the same time. This is further confirmed by our new HDX-MS data, which show that addition of BIO-2007817 leads to the release of the Rcat domain.

Fig. 4 - is this predictive from docking?? Please clarify if data in the table are calculated from docking. If so, actual measurements should be completed (or included in the table) to confirm EC50 or binding affinities?

> We apologize for the lack of clarity in the original manuscript. The EC50 values are experimental autoubiquitination measurements. The response curves have been added as Supplementary Fig 1.

p12 (Fig. 5b) – it is stated that BIO-2007817 can "partially rescue" S65A and R0RBR and at "10 uM BIO-2008717 the ratio of mono-ubiquitinated to unmodified Mfn2 was roughly 50%". It is the total ubiquitination that is important here and this is quite different between WT and S65A/R0RBR. Judging by the intensities of the unmodified Mfn2 the ubiquitination for S65A/R0RBR is much less than 50% using BIO-2008717 compared to WT. The authors should reword and re-quantify this.

> The reviewer is correct. We should have written at "10 uM BIO-2008717 the ratio of ubiquitinated to unmodified Mfn2 was roughly 50%". To quantify, we had indeed performed densitometry for the total ubiquitination and divided by the sum of unmodified and total ubiquitination bands. It is this ratio which is 50% for S65A and R0RBR. The "partially rescued" statement is still correct.
>
> We thank the reviewer for identifying the error.

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The manuscript 'ACTIVATION OF PARKIN BY A MOLECULAR GLUE' describes the functional and structural-biology characterization of a previously identified molecule that activates the PD-relevant E3-Ub ligase and might be useful for the treatment of certain familial, but potentially also idiopathic PD cases.

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> We thank the reviewer for the encouraging remarks and are also thrilled to report this new mechanism of activation for Parkin.

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> The errors have been corrected.

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Major points
1. Fig 1c: The RING1 mutant control at 0 min incubation time would be good to include.

> We agree it would have been better to include it. The control was omitted in order to accommodate the other lanes on the same gel.
>
> Given the similarity of the +BIO-2007817 condition at 60 min for the three parkin variants (H302A/A320R, WT, K211N), we are very confident that the missing 0 min sample (H302A/A320R) would be identical to the 0 min samples shown (WT, K211N).

2. The NMR spectra in Fig S1 show that BIO-2007817 adopts cis and trans isomeric states in water. Have the authors described these experiments in the methods section?

> A description of the NMR experiments used has been added to the Methods section.

3. In the crystal structure results section, they mention that this isomerization could be a cause for the difficulty in crystallization with this compound. Are the authors proposing that both the isomers can bind parkin/pUb? Is that something they can investigate by docking?

> The suggestion that the isomerization could prevent crystallization was speculative. Given the exchange between the two forms is fast relative to the rate of protein crystallization (and their near

equal amounts in solution), it is unlikely that the presence of two isomers would significantly hinder crystallization.

Based on docking, there does appear to be specificity in the binding site. Efforts to dock the BIO-2007817 compound with the amide bond in *cis* vs. *trans* demonstrated a much lower energy conformation for the *cis* conformation vs. *trans* (-7.3 kcal/mol vs. -1.8 kcal/mol). This is why we docked the *cis* amide pose in Fig. 5a.

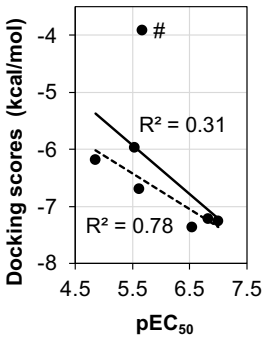
4. Since the authors report a relatively small number of compounds, is there a reason they chose the standard precision mode over the extra precision mode?

We thank the reviewer for the interesting question. Internally, over a series of different projects, we have seen that glide SP docking with enhanced sampling and binding pocket restraints does a very good job at finding accurate docked poses. During ligand conformer generation, we repeated the enhanced conformational sampling four times changing the value in the advanced setting of the docking panel. We also checked the expanded sampling option during selection of initial poses so that more poses pass through the initial Glide screens.

In addition to docking, we performed MM-GBSA calculations to rescore docked poses. MM-GBSA score is more rigorous than glide docking scores (SP or XP) as it involves solvation/desolvation penalty and ligand strain, both of which are lacking in the docking scoring function. Therefore, we performed glide SP docking with enhanced sampling to get accurate poses and then re-scored using MM-GBSA as opposed to using the XP score. Overall, this workflow has proven as a good way for us to dock and filter compounds *in silico*. We have added details of enhanced sampling docking method to the manuscript.

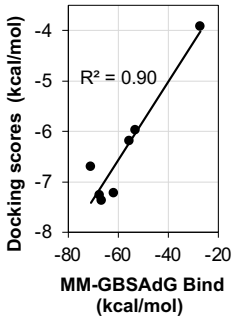
5. Fig 4d: Docking scores vs pEC50 plot is missing one point for BIO-1983977. Also please report the R^2 value for trendline with this data point.

We have added the data point for the non-THPP compound (marked #) and included the R2 value for the trendlines with and without it.



6. Could a similar plot be included for Docking score and -log(MM-GBSAdG) scores as well?

As requested, we have added the plot of the docking score versus MM-GBSAdG to Fig 5d (previously Fig 4d).



Minor points

1. The ubiquitination assay depicted in Fig 1b/c needs to be described better in the main text. Especially the use of pUbΔG76. While this is clearly described in the methods section, a line or two in the main text will help the reader understand the assay much quicker.

We have added a sentence to the main text to explain the use of the ubiquitin glycine truncation.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In response to other earlier comments, the authors have done good job at clarifying most of these. The HDX experiments are a nice addition to complement the rest of the paper. Several earlier points have been modified that improve the accuracy of the paper and remove textual overstatements.

Regarding comments about the differences for the BIO1975900 and BIO2007819 compounds I do not feel these were adequately addressed and the authors missed my earlier point. It is true that the template portions of BIO1975900 and BIO2007819 are identical, and the authors show by crystallography/docking that these bind to the same site in parkin. This is very clear and not being questioned. All the tested compounds have this backbone yet there are enormous differences in EC50 so it is the additional functional groups that are most important. What the authors have not addressed is that the methoxy-methylpyrazine group, present in BIO2007819 is absent in BIO1975900 and in the crystal structure. Further, chirality of the methoxy group – also missing in the x-ray structure - is important. The EC50 data show that these portions of the molecule are the major contributor to increased autoubiquitination activity based on data presented in Fig. 5. The docking experiments provide little structural insight into the improved EC50 of BIO2007819 and how the methoxy and pyrazine groups might direct this. This is clearly where future modifications to improve these compounds will occur. The authors should acknowledge this shortcoming and discuss this.

I have a few minor corrections the authors may want to consider.

(1) The earlier comments and suggested experiments regarding substrate ubiquitination has been partially addressed. In their absence, the authors should acknowledge that levels of substrate ubiquitination and profiles may differ from autoubiquitination. This has been demonstrated in the literature. Thus, it is possible that affects of a compound on autoubiquitination may not represent those for a substrate.

(2) Abstract (line 6) - enhance parkin activity should read enhance parkin autoubiquitination. Also, allosteric should be removed.

(3) Abstract (line 8) – Autoubiquitination assays ….

(4) Abstract – “The compound …. enhances the affinity of pUb for RING0 by more than 1000-fold.” This is inaccurate as it refers to the subject of the previous sentence (BIO-1975900) and there is no data in the paper that supports this. This data is for BIO-2007817 and refers to pUbl binding (Figure 2g/h) not pUb binding.

(5) P4 – regarding the inability of R42P and V56E to be phosphorylated by PINK1 – some literature shows these can be phosphorylated by PINK1. Do the authors mean inability of R42/V56E to be recruited to mitochondria or have defects in mitophagy?

(6) P6 … promotes detachment? Do the authors mean this? Also p8 – release of the Ubl domain. HDX shows there is increased exposure, nothing else. Crystal structures show the Ubl is still “attached” to rest of the protein and is bound to the RING1 domain.

Reviewer #2 (Remarks to the Author):

This revised version of the manuscript satisfies all points raised from the first manuscript. I believe it is now fit for publication.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed my comments and included the requested figures.

Minor point: pg 9 has a typo in the word crystallization in "As cvrystallization trials"

REVIEWER COMMENTS – Round 2

Reviewer #1 (Remarks to the Author):

In response to other earlier comments, the authors have done good job at clarifying most of these. The HDX experiments are a nice addition to complement the rest of the paper. Several earlier points have been modified that improve the accuracy of the paper and remove textual overstatements.

> We thank the reviewer for the comments and suggestions for improving the manuscript.

Regarding comments about the differences for the BIO1975900 and BIO2007819 compounds I do not feel these were adequately addressed and the authors missed my earlier point. It is true that the template portions of BIO1975900 and BIO2007819 are identical, and the authors show by crystallography/docking that these bind to the same site in parkin. This is very clear and not being questioned. All the tested compounds have this backbone yet there are enormous differences in EC50 so it is the additional functional groups that are most important. What the authors have not addressed is that the methoxy-methylpyrazine group, present in BIO2007819 is absent in BIO1975900 and in the crystal structure. Further, chirality of the methoxy group – also missing in the x-ray structure - is important. The EC50 data show that these portions of the molecule are the major contributor to increased autoubiquitination activity based on data presented in Fig. 5. The docking experiments provide little structural insight into the improved EC50 of BIO2007819 and how the methoxy and pyrazine groups might direct this. This is clearly where future modifications to improve these compounds will occur. The authors should acknowledge this shortcoming and discuss this.

> We agree that there are significant differences in the EC50s of the compounds that are not explained by the molecular docking. We have added an acknowledgement of the importance of the methoxy-methylpyrazine group in the discussion of optimization strategies for future development.
>
> "Notably, the chiral methoxy-methylpyrazine group, which makes important contributions to the binding affinity, is not present in the crystal structure." (page 16)

I have a few minor corrections the authors may want to consider.

(1) The earlier comments and suggested experiments regarding substrate ubiquitination has been partially addressed. In their absence, the authors should acknowledge that levels of substrate ubiquitination and profiles may differ from autoubiquitination. This has been demonstrated in the literature. Thus, it is possible that affects of a compound on autoubiquitination may not represent those for a substrate.

> The reviewer is correct that autoubiquitination and substrate ubiquitination rates can differ. We have added the following sentence to the section on the *in organello* assay to clarify the importance of looking at ubiquitination of physiological substrates.

"As substrate ubiquitination and autoubiquitination rates can differ, it was important to test the activity of the compounds on a physiologically relevant substrate." (page 12)

(2) Abstract (line 6) - enhance parkin activity should read enhance parkin autoubiquitination. Also, allosteric should be removed.

We thank the reviewer for the suggestion and correction. We have removed the word "allosteric"; however, we feel it remains more appropriate to refer to parkin "activity" than "autoubiquitination". In addition to enhancing autoubiquitination, the compound increases parkin reactivity toward UbVS, increases ubiquitination of mitofusin-2 in *in organello* assays, and increases mitophagy in cultured cells expressing parkin Ubl mutants.

(3) Abstract (line 8) – Autoubiquitination assays ....

See above.

(4) Abstract – "The compound .... enhances the affinity of pUb for RING0 by more than 1000-fold." This is inaccurate as it refers to the subject of the previous sentence (BIO-1975900) and there is no data in the paper that supports this. This data is for BIO-2007817 and refers to pUbl binding (Figure 2g/h) not pUb binding.

We thank the reviewer for pointing out the error. We have deleted the sentence.

(5) P4 – regarding the inability of R42P and V56E to be phosphorylated by PINK1 – some literature shows these can be phosphorylated by PINK1. Do the authors mean inability of R42/V56E to be recruited to mitochondria or have defects in mitophagy?

We thank the reviewer for raising this point. We have removed the statement that the R42P and V56E mutations are unable to be phosphorylated. They likely have multiple effects. The sentence now reads:

"Mutations that affect the PINK1-mediated activation such as Ubl mutations R42P and V56E, or K211N in RING0 which impairs pUbl-binding, could all be rescued by synthetic activating mutations that disrupt auto-inhibitory interactions." (page 4)

(6) P6 ... promotes detachment? Do the authors mean this? Also p8 – release of the Ubl domain. HDX shows there is increased exposure, nothing else. Crystal structures show the Ubl is still "attached" to rest of the protein and is bound to the RING1 domain.

We thank the reviewer for the opportunity to discuss this important point. There are many experimental results that show that pUb binding promotes detachment of the Ubl domain. Firstly, as the reviewer notes, HDX experiments show increased solvent exposure of the Ubl domain upon pUb binding to RING1 (Figure 3a & published

literature). Secondly, NMR experiments show decreased binding of the free Ubl binding (in trans) to R0RBR parkin in the presence of pUb (Sauvé et al., EMBO J, 2015). Conversely, ITC experiments show that deletion of the Ubl domain increases the affinity of pUb binding 20-fold. The conformational change responsible for this allosteric coupling was first imaged by Wauer et al., Nature, 2015 who stated, "PhosphoUb binding leads to straightening of a helix in the RING1 domain, and the resulting conformational changes release the Ubl domain from the PARKIN core." Finally, multiple groups have observed that pUb binding to RING1 facilitates Ubl phosphorylation. As the phosphorylation site is inaccessible when the Ubl domain is bound to RING1, parkin can only be phosphorylated when the Ubl domain is detached. Proteins are dynamic and rapidly sample multiple conformations. pUb binding to RING1 doesn't prevent the Ubl from binding to RING1 but rather it shifts the equilibrium of the interaction.

Reviewer #2 (Remarks to the Author):

This revised version of the manuscript satisfies all points raised from the first manuscript. I believe it is now fit for publication.

We thank the reviewer for their interest and support.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed my comments and included the requested figures.

Minor point: pg 9 has a typo in the word crystallization in "As cvrystallization trials"

Corrected. We thank the reviewer for finding the typo.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have clarified several sections of the manuscript and improved the accuracy of several statements.