

RuPHOTACs Provide Photocontrol Over Protein Degradation with Optimized Properties for Biological Applications

Corresponding Author: Professor Edith Glazer

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study offers a well-structured and innovative contribution to the field of targeted protein degradation. It describes the development and characterization of RuPHOTACs as a new class of light-activated PROTACs with enhanced potency upon irradiation. For this the authors synthesize a small library of PROTACs containing ligands for incorporation (and later light-induced release) into ruthenium complexes. The research combines chemical synthesis, cellular characterization, live-cell imaging, molecular modeling and a novel Dendra2-based approach to monitor protein degradation. As such, this manuscript is very well suited for Nature Communications. However, the study must be strengthened before publication by addressing several issues.

Major Issues:

What is the effect of the incorporation of different coordinating groups concerning off-targets/ neosubstrates that are recruited for degradation by the coordinating group? Given that many of the PROTACs here are more toxic than dBET1, a contribution of off-target activity on the toxicity should be considered. Similarly, effects of the Ru-complex as "POI-Ligand" for off-target proteins should be discussed. A great control would be the CRBN-Ru-complex bifunctional without JQ1 to see if it has a toxic effect.

Generally, an additional control experiment would be important to fully confirm the proposed mode of action. An effect of the BRD-targeted Ru-complex on stability of BRD2/3/4 (with and without irradiation) should be ruled out, for example by preparing the N-methylated glutarimide as a control compound.

Cellular uptake (e.g. P22, L410-412), I disagree with the statement that the in vivo activity demonstrates that the Ru(II) center does not prevent uptake. 8 is mostly inactive in the dark which could also result from blocked cellular uptake, and it is possible that only light-released 3 is taken up.

Generally, the data supporting cellular uptake of the Ru-complex (8) instead of released PROTAC (3) could be stronger. (If 3 is released mostly in the extracellular medium this might cause side effects by diffusion in a therapeutic setting.) Currently, the only strong indication of cellular uptake I can see is the difference in PI indexes for the different BRD proteins in light vs. dark. This is a general concern with many light-controlled systems (switching the bioavailability), but if the authors find more means to show cellular uptake (e.g. treatment to allow uptake and replacement of the cell culture medium before irradiation) that would further strengthen this work.

Fig. 2c shows a little of liberated 9 in the sample of 8 as well as other absorbing species. Is this instability of 9 or impurities? P12, L213-214: How do you explain the improvement in potency for BRD2+3 degradation of 8 over 3, when irradiated 8 should just give 3?

P12+Fig.3 (and P16+Fig.5d): The application of the PI (as the ratio between activity without and with irradiation) to Protein degradation levels is a bit problematic due to the hook-effect (which is also ignored by a DC50 value). Since no POI-degradation in the dark was observed for PHOTAC-I-3 by the Trauner group, the PI would be infinite and thus 8 would not have "the largest PI value for any photo-PROTACs reported". Maybe a different metric/statement would highlight the advantage of 8 over PHOTACs in terms of its light-controlled activity window more accurately.

Fig. 3b: Please also include data for 10000 (and 30000) nM after red-light irradiation to make it comparable to 3a and S23 to see if there is a hook-effect/where it is.

Fig. 3c: How was the PI for BRD2 calculated? From 3a+b it appears to be larger (~100).

The molecular docking does not seem relevant and could be moved to the supporting information, as it does not provide much value. While it is used to postulate that the Ru-complex brings "steric/hydrophilic interference" for the CRBN-BRD4 complex, this remains a hypothesis and looking at Hill slopes in Fig. 3d, the Hill slope is higher in the dark, which indicates that the Ru-complex could promote cooperativity (increasing the Hill coefficient) at this similar binding site.

P13, Fig. 3b: How do you explain that 8+Light does degrade PIM1, whereas 3, which should be formed after irradiation, does not according to Fig. S24? (I assume exposure/image settings in S24 are not ideal.)

Supporting Information:

Synthetic intermediates are characterized with different level of detail: Sometimes ¹H, ¹³C, MS, sometimes only ¹H and sometimes no analytical data. In the synthesis of 2 there was certainly enough material present to support ¹³Cs. Further, yields are missing in some cases S8, S9. NMRs spectra as supp. figures for intermediates should also be provided. Number of Protons assigned in NMR data sometimes exceeds the number of protons in the molecules (for example compound 5, 50 assigned, 49 in molecule) or is missing protons (for example S4: Only 23H of 24H), in other cases signals appear to be missing (e.g. ¹³C of S2, S4).

Please also provide original uncropped western blot images as an extra supporting file (I assume they are not included in the reviewer data).

In summary, this is an important scientific project and I support publication once the issues are addressed. There are no doubts about the validity of the results, but some claims should receive stronger support by data. I would also like to encourage all authors to perform a thorough proofreading of the SI to ensure this section matches the high quality of the main manuscript.

Minor issues:

P3 L55-57: (2) is not true. For the PHOTACs by Carreira and Crews, the stable isomer is actually the active form (which is a strong drawback by itself) but the isomers are also highly stable (half-lives of days). If a half-life of ~9h for the Trauner PHOTACs is a "low thermal stability" is debatable. Maybe it would be best to make this point about the difficult incorporation about photoswitches into the PROTAC structure that can yield dark-active PROTACs.

P4 L81-82: Since the production of potentially toxic byproducts is mentioned as drawback, are the Ru-complexes here truly non-toxic, the corresponding controls are not included in the data.

P6, L109 : Should be S20, 21 instead of S19.

P10 Fig 2a: The net +2 charge could be indicated in the structure, as it is a key feature of 8 over 7.

P10 Fig. 2e: One more data point for the cytotoxicity would be so interesting to see if 8 becomes more toxic in the dark than after irradiation or if it behaves the same, but I understand that solubility might be at the limit.

Fig. 3a: Concentrations do not display properly for me (windows).

P14: The discussion of the Hill-coefficient and the urge to use it more in the development of optical tools is very important, but maybe it could be condensed a bit, since the majority of cases reports worse compounds with low PI, but there are also better compounds and the PI>100 is only for protein degradation, not toxicity.

Figure 3a,b : Ideally both would be run on the same immunoblot. E.g. BRD2 seems to have different image settings here which makes it more difficult to compare both.

Fig. 5c: If you were to run this experiment again, also include a dBET or JQ1+Light control. (However, I see limited value in the treatment of xenograft cells in a transparent animal, so this is explicitly not a request to rerun any of those animal experiments).

P21, L390-393: A therapeutic window with a PI>100 was not completely reached with the Cytotox-PI of 87, so I would not state it as a prominent goal here which could make it read like this great study failed its goal.

P23, L435-437: HaloTag-fusions could provide the same level of information, by labelling with a HT-dye (e.g. red) and then washing out unbound dye (and possibly washing in a second HT-dye (green) to label newly synthesized protein). I am not sure if it has been used in this context though.

P28, Line 556: Please state final DMSO concentrations.

Supporting Information:

Page S6: Number of ¹³C signals exceeds number of carbons in 6 by far, please correct or explain this observation.

Synthetic procedures are written somewhat inconsistent, sometimes amounts are stated as either mg or mmol, sometimes both is given (which I would prefer).

Chemical formulas for HRMS ions should be corrected (chemical formula for [M] is written, but then stated as [M+H]⁺ with the mass for [M+H]⁺)

Page S13 Figure S23: BRD2 lane of 3, 3 uM should be rerun.

Figure S19, the main text states "no degradation observed over 72 hours" but a small broad peak can be seen to form at ~7.2 min after 48 h in Fig. S19.

Figure S21, cytotox concentration should probably be in uM not nM (else Table 1 is incorrect), Western blot is missing indications of the concentration.

Reviewer #2

(Remarks to the Author)

The authors seek to develop a novel RuPHOTACs platform to achieve light controlled degradation of target protein such as Brd4 as a novel anti-cancer approach. The authors gathered experimental evidence via various chemical, biochemical, and in vivo animal modeling approaches to demonstrate the potency of their novel platform for targeted degradation of target protein upon light induction as potential new tool to combat human diseases.

The manuscript is clearly written. However, the following minor concerns should be addressed.

1). Figure 3b, it will be nice for the authors to show if degradation of BRDs can be blocked by addition of free pomalidomide or free JQ1?

2). Figure 3a-b, it will be nice to demonstrate if these effects can be observed in different cell lines and whether different Brd

isoform degradation kinetics differ in different cell lines

3). Figure 3c, it will be nice for the authors to monitor changes in Brd isoform mRNAs.

4). Figure 3, it will be nice to show the degradation of BRDs can be blocked by MLN4924 or MG132.

5). Figure 5: it will be nice for the authors to show if BRD4 protein abundances were reduced effectively in vivo.

Reviewer #3

(Remarks to the Author)

The manuscript "RuPHOTACc Provide Photocontrol..." by Heidary, Glazer, and co-workers reports design and application of Ru-based PROTAC analogues - the design of a small molecule that attracts an ubiquitin ligase to the protein of interests, and results in its ubiquitinylation and degradation, has been enhanced with light-induced deprotection which can be achieved in situ with red light. A set of various molecular architectures has been tested with a Bet ligand linked to thalidomide (E3-ligand), and the ruthenium complex attached via diverse ligands. The authors tested their design using cell lines and in vivo with zebrafish embryos. They also designed a clever optical assay (the D2 assay) compatible with high-throughput screening, which shows the protein degradation activity in real time.

The manuscript is coherent, brings significant progress to the field, it uses a broad spectrum of analyses to support the claims, and introduces an original technology with sufficiently high phototherapeutic index, that it can be realistic to expect practical applications. The methodology and data analysis are sound and coherent. Enough details are provided to warrant reproducibility.

The manuscript should be published with a minor revision:

- on the lines 188-195, authors discuss the advantages of ruthenium-caging vs. classical photocages which produce nitrosoarenes as side products which potentially contaminate the treated organism. Could the authors add a discussion here, what happens in vivo to ruthenium residues that are released upon their red-light-driven uncaging process? Are ruthenium salts toxic at such concentrations? Could they accumulate in the organism?

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have reworked their manuscript on light-activated degraders termed RuPHOTACs, addressed all open questions and performed several additional control experiments. The extra work put into this manuscript, including new control compounds and experiments to confirm the intended mode of action, provides a very robust framework for the conclusions. I also appreciate the rigorous improvement of the supporting information.

As such, the revised version of this manuscript greatly elevates the overall quality and reliability of the study. I congratulate the authors on their work and I strongly support the timely publication in Nature Communications.

Minor issues:

P21, line 404: Should say Figure 5d instead of 4d.

P27: At your own discretion, you might consider adding an outlook section (1-3 sentences) at the very end of the Discussion as an opportunity to highlight the impact of this work. Discussing broader implications/potential utility/future avenues would be a valuable addition and a satisfying conclusion for the reader.

(Concerning bullet point 8. in your rebuttal letter, I would propose that dark-cytotoxicity can also arise from BRD4 inhibition and not degradation as a kind of on-target, "off-mechanism" effect but as you mentioned we can both only speculate on this in the absence of clear experiments. And your cytotoxicity-PI makes your system undoubtedly better suited for applications in higher biological systems.)

Reviewer #2

(Remarks to the Author)

The authors have addressed most of the raised concerns.

Reviewer #3

(Remarks to the Author)

The quality of the initial manuscript has been considerably improved by addressing issues raised by all the reviewers. My comments have been fully addressed. Therefore I can recommend publication of the manuscript in its current shape.

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REVIEWER REPORTS AND RESPONSES:

We are deeply grateful to the reviewers for their time, careful evaluation, and insightful feedback, which have greatly contributed to strengthening the quality of our manuscript. We were gladdened to see that all three reviewers were strongly supportive of publication in Nature Communications.

We have carefully considered each point raised and have performed multiple experiments to address questions and concerns, gaining new data to support each conclusion. We have revised the manuscript accordingly. Please find our detailed responses to the comments below, and the revised manuscript and SI uploaded to the portal.

Reviewer 1 Comments to the Author

This study offers a well-structured and innovative contribution to the field of targeted protein degradation. It describes the development and characterization of RuPHOTACs as a new class of light-activated PROTACs with enhanced potency upon irradiation. For this the authors synthesize a small library of PROTACs containing ligands for incorporation (and later light-induced release) into ruthenium complexes. The research combines chemical synthesis, cellular characterization, live-cell imaging, molecular modeling and a novel Dendra2-based approach to monitor protein degradation. As such, this manuscript is very well suited for Nature Communications.

We sincerely thank the reviewer for the positive feedback and the recommendation for publication. We are pleased to address all questions and suggestions to enhance the manuscript.

However, the study must be strengthened before publication by addressing several issues.

Major Issues:

1. What is the effect of the incorporation of different coordinating groups concerning off-targets/neosubstrates that are recruited for degradation by the coordinating group? Given that many of the PROTACs here are more toxic than dBET1, a contribution of off-target activity on the toxicity should be considered.

To address this question, we have performed additional experiments. Notably, we find the cytotoxicity of the new PROTACs correlates well with their degradation potency for BRD proteins, consistent with on-target activity. The variable cytotoxicity in the different cell lines that we present also is consistent with literature supporting the dependence of the Ramos cell line on BRD protein activity, with each PROTAC exhibiting less potent cytotoxicity in the SF8628 cell line (see Supplementary Table 2). This again correlated with DC₅₀ values for the PROTAC targets (see new Supplementary Figures 39 and 40). All these results for the new PROTACs reported are consistent with the behavior of dBET1, the data for which is shown in the same tables. This supports an interpretation that there is no new off-target toxicity for the new PROTACs, at least relative to dBET1. Potential off-target toxicity for the PROTACs was also considered and assessed in vivo in zebrafish, and this data is now shown in Supplementary Table 4. The in vivo studies showed no significant toxicity, supporting the biocompatibility (and absence of general off-target toxicity) of the new PROTACs. We further discuss this question below and in the context of a comment from Reviewer 3.

2. Similarly, effects of the Ru-complex as “POI-Ligand” for off-target proteins should be discussed. A great control would be the CRBN-Ru-complex bifunctional without JQ1 to see if it has a toxic effect.

To address this question, we synthesized several Ru(II) complexes to test as negative controls: the Ru(II) photocaging scaffold released from RuPHOTAC 8 (compound 9) and the CRBN-Ru-complex suggested by the reviewer as a Ru(II) complex conjugated with thalidomide (compound S20). In addition, we synthesized and tested a Ru(II) complex conjugated with the JQ1 group but no thalidomide (compound S19). All compounds were evaluated in Ramos cells and exhibited no

cytotoxicity at concentrations up to 10–30 μM (note the light induced cytotoxicity of the Ru-PHOTAC **8** is 0.33 μM). This new data is shown in Supplementary Figure 29. The lack of cytotoxicity is gratifying and supports the interpretation that the Ru-complex and conjugates with either POI ligand or thalidomide do not have off-target toxic effects at concentrations relevant to this study.

3. Generally, an additional control experiment would be important to fully confirm the proposed mode of action. An effect of the BRD-targeted Ru-complex on stability of BRD2/3/4 (with and without irradiation) should be ruled out, for example by preparing the N-methylated glutarimide as a control compound.

To address this question, we synthesized and characterized the effect of control Ru-JQ1 complex **S19** on BRD protein levels in cells, as well as PIM1 and c-MYC. This compound, lacking the thalidomide group, exhibited no cytotoxic effect at a concentration of 10 μM . Immunoblot dose responses for **S19** confirmed that this control compound does not alter protein levels (see Supplementary Figure 30). Activity was not observed in either the dark or the light. To further demonstrate that the RuPHOTAC acts via a PROTAC mechanism, not by some other indirect or less precise mechanism, we evaluated singlet oxygen ($^1\text{O}_2$) production. The intact compound **8** and the released Ru(II) cage **9** do not produce $^1\text{O}_2$; this data is now shown in Supplementary Figure 31. We discuss the implications in the body of the manuscript in section “Development of RuPHOTACs” and in the “Conclusions”.

Text changes relevant to points 2 and 3 are copied below for ease of review:

*“The possibility was raised that the RuPHOTAC did not function as a photocaged PROTAC, but rather bound to the BRD targets and then caused protein degradation due to the presence of the Ru(II) center. To address this, a control compound **S19** was synthesized that contains the JQ1 but not the thalidomide portion. As anticipated, this compound did not induce cytotoxicity in Ramos cells in the dark or following irradiation (Supplementary Figure 29). Western Blot analysis (Supplementary Figure 30) also demonstrated a lack of induced protein degradation, consistent with the requirement for the full PROTAC structure for activity. Moreover, compound **8** and photoproduct **9** did not produce detectable $^1\text{O}_2$ (Supplementary Figure 31). This is consistent with the expectation that the biological activity of **8** does not stem from destabilizing the BRD target or from direct photodamage through a mechanism like chromophore assisted light inactivation (CALI) where the chromophore generated- $^1\text{O}_2$ photo-inactivates proteins.³³”*

4. Cellular uptake (e.g. P22, L410-412), I disagree with the statement that the in vivo activity demonstrates that the Ru(II) center does not prevent uptake. **8** is mostly inactive in the dark which could also result from blocked cellular uptake, and it is possible that only light-released **3** is taken up. Generally, the data supporting cellular uptake of the Ru-complex (**8**) instead of released PROTAC (**3**) could be stronger. (If **3** is released mostly in the extracellular medium this might cause side effects by diffusion in a therapeutic setting.) Currently, the only strong indication of cellular uptake I can see is the difference in PI indexes for the different BRD proteins in light vs. dark. This is a general concern with many light-controlled systems (switching the bioavailability), but if the authors find more means to show cellular uptake (e.g. treatment to allow uptake and replacement of the cell culture medium before irradiation) that would further strengthen this work.

In order to address this question, we performed additional experiments to quantify the cellular uptake of compound **8** and control compounds **9** and **S19**. We used ICP-MS, and this new data is provided in Supplementary Figure 28 and Supplementary Table 1. The values obtained for Ru indicated ~2–12 ng/ 10^6 cells, which is consistent with other literature reports of Ru complexes, which we cite to provide context. These new results are discussed in the body of the paper in the section “Development of Ru-PHOTACs”, “In vivo validation of RuPHOTACs”, and “Discussion”. We found a good correlation between hydrophobicity and cellular uptake, which could even be observed upon visual inspection of the cell pallets, which were pink. We also were able to measure the Ru content

in the zebrafish, and found good uptake. This is shown in Supplementary Table 3. An additional interesting finding was that there was a lower level of Ru(II) in vivo after photoactivation, which correlates with the LogP of the released cage. This implies that the inert and hydrophilic Ru cage partitions out of the zebrafish and into the media, which is an asset for a photocaging strategy.

The added discussion is copied below for ease of review:

Development of RuPHOTACs:

*Considering the size of the RuPHOTAC and questions about cellular uptake, especially given that impaired uptake is commonly found with PROTACs, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was performed to quantify the Ru(II) content of the cells treated with **8** and control compounds. As shown in Supplementary Figure 28, the pink color of the RuPHOTAC could be readily discerned in the cell pellets both in the dark and following irradiation. Quantification demonstrated a correlation between LogP values for the Ru(II) compounds, with the more lipophilic compounds demonstrating better uptake. Cells treated with **8** (10 μ M concentration for 17 hours) were found to have ~2.6–5.5 ng Ru/ 10^6 cells. These values are in agreement with other studies where the metal center is acting as the active agent³¹ or as a caging protecting group.³² The value for Ru(II) uptake were within error for the cells irradiated vs. kept in the dark, which suggests that the complex enters the cells equally well with and without irradiation.*

In vivo validation of RuPHOTACs:

*The effects of **8** in the dark on zebrafish mortality and physiology matched the No Compound control, and the effects of **8** following irradiation was similar to what was observed with irradiation alone, as shown in Supplementary Figure 41. The RuPHOTAC was detected in vivo by ICP-MS (Supplementary Table S3), and the Ru(II) content for **8** in the dark is ca. 6-fold higher than the value when activated by light. This suggests that the photo-released **9** partitions out of the zebrafish and into the media, which is consistent with the higher hydrophilicity of **9** (Supplementary Figure 28). This could be an attractive feature, where the biologically innocuous Ru(II) complex released upon uncaging does not accumulate in the organism. These studies highlight the biocompatibility of the Ru(II) caging group, with low toxicity for both intact **8** and the released cage **9**. Notably, new PROTACs **5** and **6** also demonstrated no toxicity in zebrafish (Supplementary Table 4). This is in contrast to other PROTACs like dBET1, suggesting that toxicity in vivo is not directly correlated to on-target PROTAC activity inducing degradation of BRD proteins, as **5** and **6** exhibited the most potent values for protein degradation. It also showcases an apparent absence of off-target toxic effects in vivo for these new systems.*

Discussion:

How the Ru(II) center achieves this outcome is the subject of ongoing mechanistic studies, but we have found a correlation between the LogP of the RuPHOTAC (and control compounds) and their uptake in cells and in zebrafish, as quantified by ICP-MS. The levels of Ru(II) from the RuPHOTAC were notably similar to much smaller compounds reported in the literature, where the metal center is either used as a cage or as the active agent.³¹⁻³²

5. Fig. 2c shows a little of liberated **9** in the sample of **8** as well as other absorbing species. Is this instability of **9** or impurities?

Due to concerns about trace impurities in the chromatograms obtained using an old column, all compounds shown in Fig. 2c. were retested on a new Luna 5 μ m C18(2) 100 Å column. The revised results are shown in Figure 2c and demonstrate no impurities in sample **8**.

6. P12, L213-214: How do you explain the improvement in potency for BRD2+3 degradation of **8** over **3**, when irradiated **8** should just give **3**?

We thank the reviewer for this insightful question, which led us to realize we had a typo in our manuscript. The comparison that was being made was to dBET1, not to compound **3**. This is now

corrected in the manuscript. The finding is that there is excellent agreement in the cytotoxicity values and DC₅₀ values for **3** and **8** upon light activation.

7. P12+Fig.3 (and P16+Fig.5d): The application of the PI (as the ratio between activity without and with irradiation) to Protein degradation levels is a bit problematic due to the hook-effect (which is also ignored by a DC₅₀ value).

We agree that the reviewer has a valid philosophical point, as the hook effect reverses PROTAC efficacy upon reaching a specific concentration. However, we did not observe a hook effect for **8** for protein degradation in the dark or upon irradiation, so the concern raised regarding hook effects and DC₅₀ values does not apply to the RuPHOTAC. (The only hook effects found were for the organic PROTACs.)

There is an important utility for DC₅₀ values (which are common in the PROTAC field) and ratios like PI (common in photocaging) to allow scientists to compare activity; as a result, these metrics are convenient and broadly used. We have expanded our discussion of the hook effects we observed to bring it to the reader's attention. The possibility of a hook effect is always a consideration for PROTACs and the only option is for scientists to be cautious and use the minimal concentrations required to obtain desired results to avoid the possibility of a hook effect. We note this is a general good practice, as solubility is also a consistent concern (not just for PROTACs).

8. Since no POI-degradation in the dark was observed for PHOTAC-I-3 by the Trauner group, the PI would be infinite and thus **8** would not have "the largest PI value for any photo-PROTACs reported". Maybe a different metric/statement would highlight the advantage of **8** over PHOTACs in terms of its light-controlled activity window more accurately.

Our goal was to provide as accurate a comparison as possible while recognizing that we are limited by the data shown in the literature. Unfortunately, the percent of degradation at each concentration or the PI value for PHOTAC-I-3 were not provided in DOI: 10.1126/sciadv.aay5064, so we estimated this value to the best of our ability, using the ratio of the highest concentration tested in the dark (10 μ M) and lowest light concentration with strong BRD4 degradation (0.3 μ M). We carefully phrased our text about the Radar Chart in Figure 4d as a qualitative: *"We note that this is qualitative, rather than a precise and quantitative evaluation, as different experimental conditions were used for the three systems, but the relative comparison of features is instructive."* We can only utilize the data provided in the original report, and there is no WB data for the compound above 10 μ M.

However, it is important to consider the DC₅₀ for protein degradation in the context of the resulting biological activity. The authors did report the cytotoxicity EC₅₀ values for their compound in the dark and light (0.631 and 0.088 μ M, respectively). This gives a cytotoxicity PI of 7.1, which the authors reported in the body of their paper. If it is assumed that the cytotoxicity of PHOTAC-I-3 comes from on-target activity in both the dark and light, the expectation should be that the compound is inducing protein degradation in the dark at concentration values close to the EC₅₀ and there is thus a similar PI for protein degradation (i.e., close to 7). The only other interpretation consistent with the cytotoxicity data is that the PHOTAC-I-3 has undesired off-target toxic effects in the dark, but we are loath to raise this possibility without any evidence to support it. As a result, we are comfortable with our statement based on the reported data in this paper and the others that we extensively reviewed.

9. Fig. 3b: Please also include data for 10,000 (and 30,000) nM after red-light irradiation to make it comparable to **3a** and **S23** to see if there is a hook-effect/where it is.

We have performed and added data for 10,000 nM after red-light irradiation and this is now shown in Supplementary Figure 26C. No hook effect was observed.

10. Fig. 3c: How was the PI for BRD2 calculated? From **3a+b** it appears to be larger (~100).

PI values for all proteins were calculated as a ratio of DC₅₀ in the dark and light. All DC₅₀ values are provided in Supplementary Figures 32 (dark) and 33 (light). The quantification was performed using Image Lab 6.1 (Bio-Rad), as detailed in the Methods section. While visual inspection leads to an impression of a higher PI, we report here the one provided by the software in order to maintain consistency between all WB data analysis.

11. The molecular docking does not seem relevant and could be moved to the supporting information, as it does not provide much value. While it is used to postulate that the Ru-complex brings “steric/hydrophilic interference” for the CRBN-BRD4 complex, this remains a hypothesis and looking at Hill slopes in Fig. 3d, the Hill slope is higher in the dark, which indicates that the Ru-complex could promote cooperativity (increasing the Hill coefficient) at this similar binding site.

The reviewer’s concern may stem from the fact that we did not leverage the docking and structural analysis as effectively as we could, and we have now improved this section. The new discussion provides a plausible rationale for the different activities observed for the RuPHOTAC with the different BRD proteins, and as a result, we feel it is very important information. We also added docking of control compounds in Supplementary Figure 35. This docking demonstrates why the control compounds are not able to induce ternary complex formation between BRD4 and CRBN. This finding is consistent with our new experimental data now shown in Supplementary Figures 29 and 30.

12. P13, Fig. 3b: How do you explain that 8+Light does degrade PIM1, whereas 3, which should be formed after irradiation, does not according to Fig. S24? (I assume exposure/image settings in S24 are not ideal.)

We do not have a conclusive answer for this question. PIM1 is a challenging system, where the expression levels and subcellular localization (which can be in the plasma membrane, nucleus, nucleoli, cytoplasm, and mitochondria) is highly variable depending on cell line and cellular health / functions. Our hypothesis is that the Ru(II) center aids in localization within the subcellular region where the (majority of the) PIM1 resides, and that PROTAC **3** does not partition similarly within the cell, so the organic PROTAC does not have the same level of activity as **8**.

The quantification of WB for dBET1 and compound **3** show a ~50% reduction in PIM1 levels with a slight hook-effect (see Supplementary Figures 22 and 23), consistent with a prior publication that we cite. This previous report ([DOI: 10.1126/science.aab1433](https://doi.org/10.1126/science.aab1433)) also showed a ~50% reduction of PIM1 upon dBET1 and JQ1 treatment, but used a different cell line (MV4;11) which could influence protein levels. Those authors did not have an explanation for why dBET1 and JQ1 impacted PIM1 levels, but raised the possibility of secondary transcriptional effects.

We note that we did not achieve an apparent dose response curve for PROTACs dBET1 and **3** against PIM1, but a dose response was found for **5** and **6**. Notably, both **5** and **6** can be doubly protonated. This could possibly lead to similar physiochemical properties to **8**, which contains the +2 Ru center. This might alter their subcellular localization, so that **5**, **6**, and **8** (before photo-release of **3**) are all present in the same subcellular region with the majority of the PIM1, and this enhances their ability to target PIM1. However, it is not possible for us to experimentally test this hypothesis, so we have not commented on it outside of this *Response* to the reviewer’s question.

13. Supporting Information:

Synthetic intermediates are characterized with different level of detail: Sometimes ¹H, ¹³C, MS, sometimes only ¹H and sometimes no analytical data. In the synthesis of **2** there was certainly enough material present to support ¹³Cs. Further, yields are missing in some cases S8, S9. NMRs spectra as supp. figures for intermediates should also be provided.

The intermediates were characterized and the requested analytical data were added to SI.

14. Number of Protons assigned in NMR data sometimes exceeds the number of protons in the molecules (for example compound 5, 50 assigned, 49 in molecule) or is missing protons (for example S4: Only 23H of 24H), in other cases signals appear to be missing (e.g. ^{13}C of S2, S4).

The proton NMRs were revised and corrected and carbon NMRs were revised, with all signals listed. In some cases, the number of signals can be fewer than the number of carbons when the atoms are equivalent. These carbons have the same chemical shift, resulting in the taller peaks. In addition, we note that NMR characterization of these large molecules is exceptionally challenging and can be misleading. To aid the reader, we provide a discussion of this point in the Supplementary Information, and provide many references so the interested reader can review a number of published reports of PROTACs where the NMR spectra have these issues. The inability to conclusively characterize these large molecules by NMR is a reason to rely on HRAM and HPLC, which we discuss.

15. Please also provide original uncropped western blot images as an extra supporting file (I assume they are not included in the reviewer data).

This file is provided in the Source Data file for review.

16. In summary, this is an important scientific project and I support publication once the issues are addressed. There are no doubts about the validity of the results, but some claims should receive stronger support by data. I would also like to encourage all authors to perform a thorough proofreading of the SI to ensure this section matches the high quality of the main manuscript.

We sincerely thank the reviewer for the positive feedback and the recommendation for publication. We have carefully proofread and edited the SI to improve the writing.

Minor issues:

17. P3 L55-57: (2) is not true. For the PHOTACs by Carreira and Crews, the stable isomer is actually the active form (which is a strong drawback by itself) but the isomers are also highly stable (half-lives of days).

If a half-life of ~9h for the Trauner PHOTACs is a “low thermal stability” is debatable. Maybe it would be best to make this point about the difficult incorporation about photoswitches into the PROTAC structure that can yield dark-active PROTACs.

We thank the reviewer for bringing this to our attention so that we could correct this statement. The text has been significantly modified.

18. P4 L81-82: Since the production of potentially toxic byproducts is mentioned as drawback, are the Ru-complexes here truly non-toxic, the corresponding controls are not included in the data.

We agree that this is important information. We now include the analysis of three negative controls, including **9** (the key released byproduct) and controls Ru-thalidomide (**S20**) and Ru-JQ1 (**S19**), all of which demonstrated no cytotoxicity at 10 μM concentration.

19. N P6, L109 : Should be S20, 21 instead of S19.

P10 Fig 2a: The net +2 charge could be indicated in the structure, as it is a key feature of 8 over 7.

Thank you, these corrections were made (but please note the figure numbers have changed again due to the addition of several figures).

20. P10 Fig. 2e: One more data point for the cytotoxicity would be so interesting to see if **8** becomes more toxic in the dark than after irradiation or if it behaves the same, but I understand that solubility might be at the limit.

We agree this could be interesting, but as the reviewer anticipated, we are limited to 30 μ M concentrations by solubility.

21. Fig. 3a: Concentrations do not display properly for me (windows).

Thank you for bringing this issue to our attention. This was corrected.

22. P14: The discussion of the Hill-coefficient and the urge to use it more in the development of optical tools is very important, but maybe it could be condensed a bit, since the majority of cases reports worse compounds with low PI, but there are also better compounds and the $PI > 100$ is only for protein degradation, not toxicity.

We appreciate the reviewer's point. We believe that this is such an important topic for the field that we have presented it in a way to make sure it is very clear and understandable to scientists including those at early career stages or experience in this scientific area, as we would like to catalyze others to consider this feature very carefully. This required the use of a full paragraph.

23. Figure 3a,b : Ideally both would be run on the same immunoblot. E.g. BRD2 seems to have different image settings here which makes it more difficult to compare both.

Unfortunately, this would require the purchase of a 26 lane gel system, which we are not in a position to do. Please note that we are comparing dose responses, not the band intensity values for protein bands at individual concentrations. We are looking for the concentration midpoint values, and these can be observed and compared across gels and experiments. Running samples on a single gel vs. two gels does not improve the quantification of a dose response.

24. Fig. 5c: If you were to run this experiment again, also include a dBET or JQ1+Light control. (However, I see limited value in the treatment of xenograft cells in a transparent animal, so this is explicitly not a request to rerun any of those animal experiments).

Thank you for the clarification that this is not a request to rerun the animal experiments, as we are not in a position to repeat them. We do note that there have been no reports in the literature of either dBET1 or JQ1 being photoreactive, and we observed no changes in the HPLC of these molecules with irradiation, so we did not perform this study.

If the reviewer is concerned that the light treatment of **8** results in any photodegradation of the released PROTAC **3**, the HPLC and HRAM data confirms that that released PROTAC is not damaged by red light (the retention time, UV/Vis, and HRAM are unchanged).

25. P21, L390-393: A therapeutic window with a $PI > 100$ was not completely reached with the Cytotox-PI of 87, so I would not state it as a prominent goal here which could make it read like this great study failed its goal.

We appreciate the reviewer's thoughtful comment. We also understand that our focus in the text on providing a $PI > 100$ can make it seem like we did not reach our goal if the objective was the cytotoxicity PI value, rather than the PI for targeted protein degradation. However, it is well known that the cytotoxicity for "dirty" compounds, including many PROTACs based on JQ1, results from a combination of effects from JQ1's many targets, for which JQ1 has different affinities. We believe that a cytotoxicity $PI > 100$ would be readily achieved with a "clean" and selective PROTAC, but wanted to be able to provide a rigorous proof-of concept of RuPHOTACs with the best studied system (i.e., JQ1-based PROTACs). We hope that our simulations and data, along with our

discussion in the text, provide a compelling case for why a value of ~100 is needed in most cases (assuming that the Hill coefficient is not exceptionally high). We consider the study to be a success but also hope to raise this important issue to the attention of the field.

26. P23, L435-437: HaloTag-fusions could provide the same level of information, by labelling with a HT-dye (e.g. red) and then washing out unbound dye (and possibly washing in a second HT-dye (green) to label newly synthesized protein). I am not sure if it has been used in this context though.

That is an interesting idea, but not one we have investigated, and it requires several more steps and manipulations than the system we have presented.

27. P28, Line 556: Please state final DMSO concentrations.

Thank you for bringing this omission to our attention. This information is now provided in the **Methods** section, and we used "0.1% final DMSO concentration".

28. Supporting Information:

Page S6: Number of ^{13}C signals exceeds number of carbons in 6 by far, please correct or explain this observation.

This is due to the fact that all PROTACs were synthesized as a mixture of diastereomers, which include an enantiopure (+)-configuration at the JQ1 stereocenter and a racemate at the IMiD stereocenter. Inclusion of the tertiary amide group results in different chemical shifts for the carbons in the different rotamers in the ^{13}C NMR. This has been previously reported in the literature. Examples include compounds 22-26 published by Pirali (<https://doi.org/10.1021/acs.jmedchem.2c01218>). A similar excess of carbons signals was published by Pan (<https://doi.org/10.1021/jacs.9b06422>; <https://doi.org/10.1016/j.ejmech.2023.116062>). This is an unfortunate complication for these large molecules, which we address in the text of the Supplementary Information (see above).

29. Synthetic procedures are written somewhat inconsistent, sometimes amounts are stated as either mg or mmol, sometimes both is given (which I would prefer).

All synthetic procedures were revised and corrected

30. Chemical formulas for HRMS ions should be corrected (chemical formula for [M] is written, but then stated as [M+H]⁺ with the mass for [M+H]⁺)

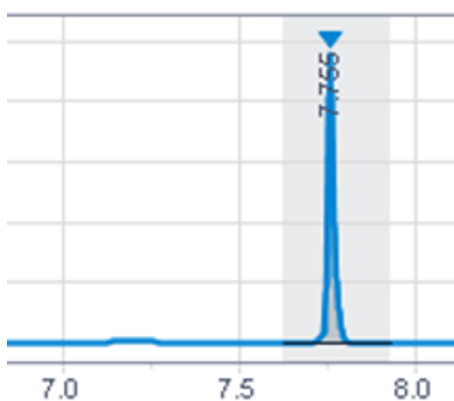
All chemical formulas were corrected

31. Page S13 Figure S23: BRD2 lane of 3, 3 uM should be rerun.

This immunoblot was repeated and the replicate data is now shown in Supplementary Figure 20.

32. Figure S19, the main text states "no degradation observed over 72 hours" but a small broad peak can be seen to form at ~7.2 min after 48 h in Fig. S19.

This small irregularity in the baseline of the chromatogram at ~7.2 min did not correspond to a UV/Vis spectrum with the features of a conjugated small molecule or a discernable mass spec. While we do not know what has caused this blip in the HPLC trace, we can conclude based on retention time, UV/Vis, and mass spec that compounds **3** and **9** were not formed in stability assay over 72 hours. For reference, an expansion of the chromatogram region is shown below.



33. Figure S21, cytotox concentration should probably be in uM not nM (else Table 1 is incorrect),

Thank you, this has been corrected and the figure is now Supplementary Figure 26 (A).

34. Western blot is missing indications of the concentration.

Thank you, this has been corrected in Figure 3a.

Reviewer 2 Comments to the Author

The authors seek to develop a novel RuPHOTACs platform to achieve light controlled degradation of target protein such as Brd4 as a novel anti-cancer approach. The authors gathered experimental evidence via various chemical, biochemical, and in vivo animal modeling approaches to demonstrate the potency of their novel platform for targeted degradation of target protein upon light induction as potential new tool to combat human diseases.

The manuscript is clearly written. However, the following minor concerns should be addressed.

We greatly appreciate the reviewer's positive remarks and have performed experiments and made changes to address each of the points raised. This is discussed in detail below.

1. Figure 3b, it will be nice for the authors to show if degradation of BRDs can be blocked by addition of free pomalidomide or free JQ1?

We thank the reviewer, and this data is shown in Figure 3i and j, and Supplementary Figure 37. As the reviewer anticipated, addition of JQ1 strongly reduces targeted protein degradation. The result was consistent with our findings with addition of the proteasome inhibitor bortezomib, also shown in these figures.

2. Figure 3a-b, it will be nice to demonstrate if these effects can be observed in different cell lines and whether different Brd isoform degradation kinetics differ in different cell lines

We concur with the reviewer, the cell line dependence is an important feature. The data from the SF8628 cell line is shown in Supplementary Figures 39, 40, and Supplementary Table 2. Additional studies for BRD degradation data were obtained in the HEK293 cell line, which is shown in the Dendra2 (D2) imaging studies. We performed a BRD3 protein degradation time course study, which is shown in Supplementary Figure 36.

3. Figure 3c, it will be nice for the authors to monitor changes in Brd isoform mRNAs.

There are no reports we have found in the literature that indicate that PROTACs alter transcription of BRD isoforms, so this experiment was not performed. However, this reviewer's question gives us the opportunity to highlight another advantageous feature of the Dendra2 assay, as it reports on this feature. The D2 assay would reveal a significant loss of the green emission signal if the PROTACs (or any molecule) acted at the point of blocking transcription, as suppression of mRNA transcript levels means that new protein would not be synthesized. This was not observed. Instead, the addition of bortezomib or JQ1 both resulted in higher BRD protein levels, which is consistent with the expected targeted protein degradation mechanism (and not inhibition of mRNA production).

Notably, the PROTACs are more potent when monitoring the red emission signature. This result is consistent with the loss of the post-translational population of protein, rather than transcriptional or translational inhibition and degradation of newly synthesized protein, which is represented by the green emissive form. A compound that suppresses mRNA levels would appear more potent against the green emissive form of the protein, rather than the red emissive form.

Based on the reviewer's question, we now highlight this feature where the green emissive form of the D2 reports on the upstream processes that lead to protein synthesis in the body of the paper:

"...the green form (Figure 3g) reports on the combined effects of protein degradation and new protein synthesis, either through effects on transcription or translation."

4. Figure 3, it will be nice to show the degradation of BRDs can be blocked by MLN4924 or MG132.

To address this suggestion, we performed several experiments to demonstrate that blockage of the proteasome function with inhibitors prevents degradation of the BRD proteins in the HEK cell line. This data, consistent with the reviewer's predictions, is shown now in Figures 3h, i, and j, and Supplementary Figure 37. Our studies led to an interesting observation of a different and unusual behavior in Ramos cells, where proteasome inhibitors reduced specific protein levels. This counterintuitive effect is being further investigated for a separate paper.

5. Figure 5: it will be nice for the authors to show if BRD4 protein abundances were reduced effectively in vivo.

We agree and attempted to perform this experiment. Unfortunately, the antibody used for all other experiments did not recognize the zebrafish BRD4 protein. We then performed a sequence alignment of the zebrafish and human BRD4, and identified and purchased an antibody that recognized an epitope in the most highly conserved region. (We were limited to the commercial providers of antibodies that report on the epitope location; many providers do not report the epitope region.) Unfortunately, this new antibody also did not recognize the zebrafish BRD4 protein.

We could not perform WB experiments on BRD4 degradation on the xenografted zebrafish, as it would have required a prohibitively high number of fish for each experiment. We relied instead on the cell counting as shown in Figure 5, as the Ramos cell viability has been demonstrated to be dependent on BRD protein levels. The results from the xenograft were all consistent with the cell-based studies.

Reviewer 3 Comments to the Author

The manuscript "RuPHOTACc Provide Photocontrol..." by Heidary, Glazer, and co-workers reports design and application of Ru-based PROTAC analogues - the design of a small molecule that attracts an ubiquitin ligase to the protein of interests, and results in its ubiquitinylation and degradation, has been enhanced with light-induced deprotection which can be achieved in situ with red light. A set of various molecular architectures has been tested with a Bet ligand linked to thalidomide (E3-ligand), and the ruthenium complex attached via diverse ligands. The authors tested their design using cell lines and in vivo with zebrafish embryos. They also designed a clever optical assay (the D2 assay) compatible with high-throughput screening, which shows the protein degradation activity in real time.

The manuscript is coherent, brings significant progress to the field, it uses a broad spectrum of analyses to support the claims, and introduces an original technology with sufficiently high phototherapeutic index, that it can be realistic to expect practical applications. The methodology and data analysis are sound and coherent. Enough details are provided to warrant reproducibility.

We thank the reviewer for their strongly supportive assessment.

The manuscript should be published with a minor revision:

- on the lines 188-195, authors discuss the advantages of ruthenium-caging vs. classical photocages which produce nitrosoarenes as side products which potentially contaminate the treated organism. Could the authors add a discussion here, what happens in vivo to ruthenium residues that are released upon their red-light-driven uncaging process? Are ruthenium salts toxic at such concentrations? Could they accumulate in the organism?

We greatly appreciate that the reviewer raised this point, allowing us to comment on another positive feature of the reported system. Our findings show that the uncaged Ru(II) compound **9** is nontoxic, and further, that it does not accumulate in vivo. We prove this new data and based on these results, have now added the following discussion to the paper in the section “**In vivo validation of RuPHOTACs**”, which is copied below for ease of review:

*The RuPHOTAC was also well tolerated as determined by studies in zebrafish embryos. The effects of **8** in the dark on zebrafish mortality and physiology matched the No Compound control, and the effects of **8** following irradiation was similar to what was observed with irradiation alone, as shown in Supplementary Figure 41. The RuPHOTAC was detected in vivo by ICP-MS (Supplementary Table S3), and the Ru(II) content for **8** in the dark is ca. 6-fold higher than the value when activated by light. This suggests that the photo-released **9** partitions out of the zebrafish and into the media, which is consistent with the higher hydrophilicity of **9** (Supplementary Figure 28). This could be an attractive feature, where the biologically innocuous Ru(II) complex released upon uncaging does not accumulate in the organism. These studies highlight the biocompatibility of the Ru(II) caging group, with low toxicity for both intact **8** and the released cage **9**. Notably, new PROTACs **5** and **6** also demonstrated no toxicity in zebrafish (Supplementary Table 4). This is in contrast to other PROTACs, suggesting that toxicity in vivo is not directly correlated to on-target PROTAC activity inducing degradation of BRD proteins, as **5** and **6** exhibited the most potent values for protein degradation. It also showcases an apparent absence of off-target toxic effects in vivo for these new systems.*