nature portfolio

Peer Review File

MODULAR IN VIVO ANTIBODY–ADC CLICK TO REVERSE DRUG RESISTANCE IN TUMORS

Corresponding Author: Dr Patrícia Pereira

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
• Summary of key results

In this manuscript Simó et al describe the development of an application for the use of click chemistry through precise biorthogonal reactions involving well established anti-cancer therapeutic antibodies that target epitopes on independent tumor antigens, covalently linked to each other in vivo. They utilized a tetrazine (Tz)-transcyclooctene (TCO) cycloaddition reaction. As stated by the authors, this approach aims to overcome the co-localization challenges that limit passive antibody combinations and eliminates the spatial proximity requirements that constrain bispecific antibodies. They elegantly demonstrate the successful conjugation, binding the immunoconstruct to different tumor cells, as well as pre-clinical in vivo distribution, toxicology and anti-tumor efficacy, utilizing a mouse model. They went on to show that panitumumab/TCO or pertuzumab/TCO combined with T-DXd/Tz (clicked) rescued mice whose tumors had been initially resistant to regular unclicked T-DXd. Finally, authors demonstrated that when site specific conjugation is utilized, there is lower liver accumulation of Abs in the click group, which could have clinical relevance relative to treatment tolerance. The results support the conclusion that this strategy is potentially feasible and effective for therapeutic development across multiple disease contexts.

• Originality and significance:

There has been substantial advance in recombinant DNA technology allowing the production of a variety of immunoconstructs that are undergoing clinical development, including bispecific antibodies and their creative variations, such as biparatopic bispecifics, crossmabs, trifunctional antibodies, multi-specific engager proteins, dual affinity re-targeting (DART), diabody, bispecific T cell engager (BiTE) and bispecific killer cell engager (BiKE). Many bispecific antibodies are already approved by regulatory agencies for clinical use or are in clinical development. There are a few Bispecific ADCs on late stages of development on ongoing pivotal trials, for instance BL-B01D1 (EGFR x HER3).
The main difference here that makes this an original contribution is that the immunoconstruct is created in vivo through the inverse electron demand Diels–Alder (IEDDA) reaction, that follows the sequential administration of relevant components, in this case 2 well established and clinically used anti-tumor antibodies, each conjugated with TCO or Tz.

• Validity:

No major flaws identified. Presented data has high quality. The conclusions are supported by the results.

• Suggested improvements

Main questions

1) Could the observed colocalization be an exclusive result of the inverse electron demand from Diels-Alder reaction without the need for molecular recognition between Trastuzumab and HER2? Is the presence of HER2 close to EGFR required to the IEDDA reaction? If not, please consider redrawing Figure 1E in which both EGFR and HER2 appear to be on the same cell (?), in the contrary to what is shown in Figure 1a and suppl fig 1.

2) Regarding site-specific conjugation:
- In the site-specific conjugation, are there any concerns about location of linker and cytotoxic payload, specifically when ADCs have higher DAR such as T-DXd?
- Has the site-specific conjugation interfered with clicked antibodies anti-tumor activity in any tested model?

3) The authors are invited to include as a supplement the results of mass spectrometry/MALTI-TOF of the IgG-TCO and of other conjugated mAbs shown in Figure 5d.

4) The methodologies of synthesis should be detailed to guarantee reproducibility.
- Please add details about synthetic procedures, as the molar proportions are insufficient. For instance, site-specific conjugation, stains, agitation time, mass, temperature, etc.

5) Pre-clinical toxicity: how was the dose of ADCs for use in mice selected? The mouse dose of T-DXd-Tz of 20mg/Kg (Suppl Fig 12) has a Human Equivalence of approximately 1.62mg/Kg, which would be a subtherapeutic dose. A mouse dose of T-DXd-Tz 5mg/Kg (line 334) would have a Human Equivalence of approximately 0.4 mg/kg.

6) Discussion: The changes to liver distribution are potentially critical for better toxicity profile and could have a very positive impact on clinical development. The liver data would deserve a couple of sentences articulating this in discussion (related to information on pages 19-20).

Minor remarks:

7) Methods section:
- On page 29, line 650, were cells washed after second Ab trastuzumab-Tz?
- What is the batch size of production of Abs conjugated with TCO or TZ?

8) Could the authors explain why the NCI N87 cell line shows a more intense fluorescence from panitumumab binding than to T-DM1 (trastuzumab)? Would this be an illusion, or could this be quantified and added to figure 1"h"?

9) On line 323, suggest mentioning "the preclinical safety of the antibody…"

10) Suggest removing sentence on line 124 "Furthermore, no bispecific ADCs have yet achieved clinical approval (17), which highlights the need for strategies that can effectively target multiple antigens without these spatial constraints." to prevent premature obsolescence of the manuscript.

11) Figures

- Figure 1 E is confusing. The figure shows the anti-EGFR mAb panitumumab/TCO (green) and an anti-HER2 mAb Trastuzumab ADC/Tz in red. The corresponding text states (line 242) that 2 anti-HER2 naked mAbs/Tz (trastuzumab or pertuzumab) were used with panitumumab/TCO.

- Which anti-HER2 mAb was used? If an ADC, please correct the text to state T-DM1 or T-DXd. If naked anti-HER2 mAb, please correct the figure and remove the orange oval circle representing the cytotoxic payload.

- Figure 2a/2b is confusing: The text on line 255 mentioned trastuzumab or trastuzumab-Tz conjugated with pHrodo. However, the figure represents a trastuzumab ADC. Is this naked trastuzumab or T-DM1 (or T-DXd)?

- Extended file Figure 2d represents naked trastuzumab Ab/Tz, but text describes T-DXd/Tz. We suggest adding the representation of cytotoxic payload on T-DXd representation.

- Suppl Figures 4 and 19 do not have values on scale bars on panel "f" and "b", respectively. Please add the scale bar values on Figure or legend (value nm).

- On extended file Figure 2 panel "e", it is not possible to identify the survival curves for T-DXd, pertuzumab or saline. Is there a perfect overlap (if so, please clarify on legend), or should these be deleted?

Referee #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)
The authors propose to address current limitations of antibody-drug conjugates (ADC) and bispecific antibody approaches with a strategy in which two antibodies that recognize different antigens or epitopes are each chemically modified with a complementary click chemistry group (here tetrazine or TCO). The authors propose that this will allow for dual epitope

targeting, as the two modified antibodies will covalently bind to each other on the cancer cell surface. The authors perform a number of in vitro and animal studies to examine this concept, and demonstrate significantly increased effects in several tumor models.

The main criticism of the manuscript is that the authors do not convincingly demonstrate that the findings in their animal studies relate to dual epitope targeting (which is the main claim in abstract, introduction and throughout the manuscript). In the animal studies, the authors infuse an antibody to a known antigen/epitope on the cancer cells, and subsequently introduce an ADC that typically does not have specificity to antigen on the cancer cells. While the authors demonstrate this leads to a significantly enhanced anti-cancer response, these effects may simply relate to the covalent coupling of the ADC to the firstly bound antibody – and a subsequent anti-cancer effect as the drug is not present in the tumor. Is an ADC even required for this effect – instead of an ADC, could the introduction of the drug molecule with the relevant click chemical group lead to the same effect? The particular click chemistry pair chosen here has very high and fast reactivity, as the authors note, and are likely to react with each other independently of any binding of the antibody to the cell. It is unclear the second antibody is bound to the cancer cells, and indeed is likely simply a carrier for the drug molecule to the firstly bound antibody. If the main mechanism is the latter, there are many examples of that type of strategy already in the literature.

Along these lines, all quantification of antibody colocalization and internalization studies in culture studies is performed with just one of the antibodies (Fig. 1g, h; Fig. 2 – Trastuzumab only). It is unclear whether internalization of the EGFR targeting Ab is also increased? If the impact in these studies is only to enhance Her2 Ab internalization (which this antibody binds), could one accomplish the same effect in vitro with a greater concentration of that antibody alone? Or, could increased internalization simply be due to formation of a complex of antibodies on the cell (both antibodies have multiple functional groups, and so could form covalently linked clusters), and not relate to any ligand binding capacity of second antibody?

The approach taken here to conjugate the click groups to the antibodies is non-specific, potentially leading to conjugation at ligand binding sites or other key portions of the antibodies. A site-specific conjugation would be greatly preferable – and the authors demonstrate in the last figure of the manuscript an approach to achieve. As the degree of modification is likely much different for antibodies modified in this manner, and that could dramatically impact the results, it is unclear why all of the studies in the manuscript are not performed with this chemical modification strategy? On a related note, the authors describe this second chemical approach as site-specific, but in the text indicate “covalent linkage occurred predominantly through the Fc regions of the antibodies (Figure 5c, Supplementary Figure 19)” – if some of the conjugation is not at the desired site, it is not site-specific.

Minor points:
Supplemental figures cited out of order, which made difficult to follow.

Manuscript is quite long, and written in a conversational tone in places – making it difficult to follow at times.

In the Introduction, the authors state “This approach is advancing towards clinical translation21 (clinicaltrials.gov identifier: NCT05737615)” to support the strategy. However, the clinical trials website indicates this trial has been withdrawn, so this is a bit confusing.

The use of click chemistry, and this particular click partner (tetrazine and TCO) has been widely used and validated, and so data demonstrating these react with each other is confirmatory and not needed in main figures.

Referee #4

(Remarks to the Author)
This manuscript details a protocol using tetrazine bioorthogonal chemistry in situ/in vivo to construct antibody-antibody conjugates. The general concept is to target two epitopes, either on the same or different antigen using two different antibodies that themselves are modified with tetrazine or trans-cyclooctene bioorthogonal groups. The idea as presented is that the coupling would occur on the cell surface and enables more targeted delivery of payloads and avoids issues with simple monoclonals or bispecifics. The authors than present in vivo data to backup these claims.
The general concept is intriguing and builds on a lot of prior work that has been done over the last ~17 years on the use of tetrazine chemistry to achieve pretargeted chemistry. One key novelty here is, while nearly all previous approaches use small molecules to chase the pretargeted antibody, here they use an entirely different antibody. There are likely some benefits to in situ/in vivo assembly of complex antibody-antibody conjugates. However, after reading the paper carefully, I do not feel the authors numerous claims and assertions are backed up by convincing data. I also think that while some of the effects that they observe are interesting, they are not due to the mechanisms proposed as I elaborate below. More convincing data and likely significant rewriting would be needed to improve the scholarly presentation and give evidence for the claims before reevaluation.
Specific Comments/Concerns:
Page 6 line 122: the authors try to discuss the drawbacks of bispecifics and the requirement of close spatial proximity. However, for this presente approach, close spatial proximity is also needed. So I do not understand the proposed benefit. Is it that it doesn’t need to be as close as bispecific? Great explanation here is warranted.

Page 6 line 128: The authors describe sequential administration of bioorthogonal tetrazine/dienophiles but this strategy is broadly known as pretargeting and has been discussed extensively. The authors have also left out key references from Weissleder and Robillard that were some of the first demonstrations of pretargeted tetrazine chemistry (see https://doi.org/10.1021/bc8004446, https://doi.org/10.1073/pnas.1113466109, 10.1002/anie.200906294)
Page 9 line 183: similar to previous comment. The authors state that antigen levels are different in different regions of a tumor and this is one reason why bispecifics fail. However, I have no understanding as to why their approach would be theoretically superior or how distances related to the regions of a tumor would be bridged.
Page 10 line 213: Here another fundamental issue is observed. The authors state that the antibodies must bind and then react with one another due to proximity. Its explicitly stated this happens in the tumor. However, no direct evidence is provided and instead its far more likely, given the very long half life of monoclonal antibodies, that these reactions are actually happening with circulating antibodies in the bloodstream. I suspect that the authors are creating essentially bispecifics in the blood and this could be responsible for the observed effects. It is interesting if this is true, but the manuscript would require rewriting and would be significantly strengthened with additional evidence that the reactions are actually happening in vivo.
-In general some of the data for the “clicked” antibodies vs. controls would benefit from a bispecific control if the authors are claiming benefit over bispecifics. However, the authors might consider toning it down and instead claim this is a modular and straightforward way to create bispecifics in situ without have to do extensive engineering.
-in some experiments the authors wait significant time (1 day) before subsequent injection of the second antibody. What are the internalization kinetics of the 1st antibody? These targets are well known to internalize when bound to antibodies. If the antibody is internalized, than theoretically it is unlikely for the tetrazine reaction to occur due to the barrier of the cell membrane. There observed effects despite the delay time further suggest that the reaction is actually happening in the bloodstream.
Page 21 line 457: discussing the Nobel prize here seems unnecessary. Its also conflating click/bioorthogonal/tetrazine and while the literature does this often already, there are actual mechanistic differences between all these concepts and reactions.
In general the microscopy data needs to be higher quality to draw the conclusions the authors make. Currently its hard to make out subcellular distributions and the images are quite blurry.
Many times data is presented without error bars.
I find the stability data in figure S4a surprising. While these kinds of tetrazines are quite stable, I would expect much more degradation over 2 days in serum. How exactly was this stability data performed. Did the authors actually test the functional stability of the tetrazine?

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
Thank you for submitting the revised manuscript and thoroughly addressing the reviewers’ comments. The authors’ concerted effort to strengthen the work is evident. Key clarifications and additions have produced a clearer and more cohesive manuscript.

Several previous concerns have been satisfactorily resolved, particularly those related to the modular ADC-antibody click strategy and the presentation of the in vivo efficacy data. The revised figures and expanded methodological details enhance both clarity and reproducibility.

The data are of high quality, and the conclusions are well supported. The authors have comprehensively addressed all the reviewers’ prior comments, and the manuscript is now suitable for publication.

The statistical analyses appear appropriate for the study design, and the error bars and probability values are accurately described in the figure legends.

Overall, this is a compelling study. Addressing the remaining minor points outlined below will further strengthen the work.

Specific comments on the updated version of the manuscript:

1) Page 11, line 200: “The antibody click formation could be blocked with an excess of each click moiety (Supplementary Figure 5c).”
Could the authors please specify on text that this is related to “the presence of an excess of the unconjugated TCO or Tz.” – as said in the supplementary figure legend?

2) For comparison across cell lines, using a consistent y axis is generally preferred. It allows clearer visual assessment of relative treatment effects. If different scales are necessary, this should be clearly indicated in the figure and legend. This would apply to Figure 2e, and Supplementary Figures 10 and 27.

3) There are three instances in Supplementary Table 2 where the term “aganocal” appears. I suspect this is a typographical error and should read “agonal.”

4) Page 21, line 427: “Additionally, site-specific antibody click led to improved antibody conjugate stability (Supplementary Figure 25) and a favorable safety profile (Figure 5e, Table 2). Would this be “Supplementary Table 2”?

Referee #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)
The authors have adequately addressed my earlier concerns.

Referee #4

(Remarks to the Author)
I have read the revised manuscript by the authors. overall I think they did an excellent job responding to the my questions and concerns. I think the revised manuscript has improved substantially in readability and quality of the cell images. I think there are still some questions about mechanistically what is happening (is antibody coupling happening in the tumor vs outside and to what extent and also how does internalization affect the second reaction) but this is quite a complex problem and can be subsequently addressed in future studies. I will make one suggestion which is the authors might want to introduce the structure of the Tz and TcO at least once in the main figures to guide the reader. most readers will not know what these designations mean chemically, and they are very easy to draw. Even just a simple Tz= and Tco= would be helpful. Otherwise, I think the strategy they have presented is an extremely important variant of in vivo tetrazine chemistry and is timely given the rising interest in applying such chemistry in vivo, especially in the ADC field.

Reviewer 1

In this manuscript Simó et al describe the development of an application for the use of click chemistry through precise biorthogonal reactions involving well established anti-cancer therapeutic antibodies that target epitopes on independent tumor antigens, covalently linked to each other in vivo. They utilized a tetrazine (Tz)-transcyclooctene (TCO) cycloaddition reaction. As stated by the authors, this approach aims to overcome the co-localization challenges that limit passive antibody combinations and eliminates the spatial proximity requirements that constrain bispecific antibodies. They elegantly demonstrate the successful conjugation, binding the immunoconstruct to different tumor cells, as well as pre-clinical in vivo distribution, toxicology and anti-tumor efficacy, utilizing a mouse model. They went on to show that panitumumab/TCO or pertuzumab/TCO combined with T-DXd/Tz (clicked) rescued mice whose tumors had been initially resistant to regular unclicked T-DXd. Finally, authors demonstrated that when site specific conjugation is utilized, there is lower liver accumulation of Abs in the click group, which could have clinical relevance relative to treatment tolerance. The results support the conclusion that this strategy is potentially feasible and effective for therapeutic development across multiple disease contexts.

Response 1.1: We are very grateful to the reviewer for the positive overview of the manuscript and constructive suggestions to improve the paper.

Originality and significance: There has been substantial advance in recombinant DNA technology allowing the production of a variety of immunoconstructs that are undergoing clinical development, including bispecific antibodies and their creative variations, such as biparatopic bispecifics, crossmabs, trifunctional antibodies, multi-specific engager proteins, dual affinity re-targeting (DART), diabody, bispecific T cell engager (BiTE) and bispecific killer cell engager (BiKE). Many bispecific antibodies are already approved by regulatory agencies for clinical use or are in clinical development. There are a few Bispecific ADCs on late stages of development on ongoing pivotal trials, for instance BL-B01D1 (EGFR x HER3).

The main difference here that makes this an original contribution is that the immunoconstruct is created in vivo through the inverse electron demand Diels–Alder (IEDDA) reaction, that follows the sequential administration of relevant components, in this case 2 well established and clinically used anti-tumor antibodies, each conjugated with TCO or Tz.

Validity: No major flaws identified. Presented data has high quality. The conclusions are supported by the results.

Response 1.2: We thank the reviewer for the positive feedback on the originality, significance, and validity of our work.

Main questions

1) Could the observed colocalization be an exclusive result of the inverse electron demand from Diels-Alder reaction without the need for molecular recognition between Trastuzumab and HER2? Is the presence of HER2 close to EGFR required to the IEDDA reaction? If not, please consider redrawing Figure 1E in which both EGFR and HER2 appear to be on the same cell (?), in the contrary to what is shown in Figure 1a and suppl fig 1.

Response 1.3: Thank you for requesting more information on this important point. To clarify, the IEDDA click reaction between TCO and Tz is a bioorthogonal reaction, and on its own, it does not require recognition between the antibody and the target for the click reaction to occur. Because antibodies have long circulation times, a fraction of ligation occurs systemically before tumor accumulation (**Extended Figure 1a**), while additional ligation can occur at the tumor site after antigen binding. The chemistry-related aspects of our approach are illustrated in **Figure 1b**. The experimental validation of the click reaction after mixing the two antibodies (TCO- and Tz-modified antibodies), which also shows that antibody-antigen recognition is not required for the TCO-Tz ligation to occur, is shown in **Figures 1b, 1d, 5a-c; Extended Figure 1a; SI Figures 5, 22**. We have also included new data to show that the click reaction occurs when the two antibodies are mixed in the presence of serum (**Figure 1c**).

Additionally, the click reaction between the two antibodies does not strictly require HER2 to be physically close to EGFR on the same cell membrane in order for the IEDDA chemistry to occur. In the system described here, the click reaction can take place when the two clickable antibody components come into sufficient proximity after systemic administration or cell incubation. Additionally, the click reaction *in vivo* is a key aspect of our approach. When the antibodies were pre-clicked prior to administration, the resulting antibody complex showed increased accumulation in the liver and lower tumor accumulation when compared with the *in vivo* click (**Figure 5f and Extended Figure 5**).

Although the IEDDA reaction enables ligation of the two antibodies *in vivo*, the click reaction alone does not account for the biological effects observed. Instead, these effects are a result of a combination of bioorthogonal ligation and antigen-directed antibody targeting, which will ultimately lead to tumor localization, receptor engagement, and internalization. For example, our data in **Figure 2e** and **SI Figure 10** show that the internalization benefit from the click reaction is not observed in HER2-negative and EGFR-negative cancer cells. To further support this, we included additional data with multiple IgG control antibodies lacking target specificity (**Figure 5 f,g; Extended Figures 3c,d, and 5**; **Supplementary Figure 10, 11, 18, 26**), and these controls did not show biological responses compared with the HER2- and EGFR-targeting antibodies. Our results show that the observed biological effects depend on the combination of bioorthogonal chemistry and target-directed antibody recognition. The IEDDA reaction leads to the ligation of the two antibodies *in vivo*, and antigen binding plays a key role in the biological outcome.

We have also revised our schematics as suggested by the reviewer.

2) Regarding site-specific conjugation:

- In the site-specific conjugation, are there any concerns about location of linker and cytotoxic payload, specifically when ADCs have higher DAR such as T-DXd?

- Has the site-specific conjugation interfered with clicked antibodies anti-tumor activity in any tested model?

Response 1.4: We agree with the reviewer that additional optimization may still be warranted for clinically translating high-DAR ADCs such as T-DXd. In our study, the site-specific modifications were made through Fc glycosylation sites, which are separated from the Fab antigen-binding region and from the location where the drug is attached in the ADC (**Figure 5a and Supplementary Figure 20**). In both cases of the ADCs T-DM1 and T-DXd, the payload attachment sites are distinct from the Fc glycosylation sites where the clickable handle was added. With the site-specific approach, we generated only 2-4 TCO/Tz moieties per antibody (**Supplementary Figures 20 and 23**) and preserved formation of the clicked higher-molecular-weight products (**Figures 5a-c; Supplementary Figure 22**), reduced antibody aggregation and improved stability (**Supplementary Figure 25**) while also reducing liver uptake (**Figure 5d**) relative to the random conjugation.

Regarding the anti-tumor activity of the site-specific antibodies (which was also a comment made by the other reviewers), we included new therapy data. The site-specific click conjugation did not interfere with the activity of the clicked antibodies (**Figure 5h; Supplementary Figures 14, 15**). The site-specific click retained biologic function and showed anti-tumor efficacy *in vivo* in EGFR-high/HER2-low or HER2-negative tumor models.

3) The authors are invited to include as a supplement the results of mass spectrometry/MALTI-TOF of the IgG-TCO and of other conjugated mAbs shown in Figure 5d.

Response 1.5: Thank you for making this suggestion to us. We included MALDI-TOF data for all the conjugates in **Supplementary Figure 23**.

4) The methodologies of synthesis should be detailed to guarantee reproducibility.

- Please add details about synthetic procedures, as the molar proportions are insufficient. For instance, site-specific conjugation, stains, agitation time, mass, temperature, etc.

Response 1.6: Thank you for pointing this out to us for inclusion. We revised the methods section to include more details.

5) Pre-clinical toxicity: how was the dose of ADCs for use in mice selected? The mouse dose of T-DXd-Tz of 20mg/Kg (Suppl Fig 12) has a Human Equivalence of approximately 1.62mg/Kg, which would be a subtherapeutic dose. A mouse dose of T-DXd-Tz 5mg/Kg (line 334) would have a Human Equivalence of approximately 0.4 mg/kg.

Response 1.7: Thank you for this comment and for asking for clarification. The doses used in our preclinical studies were selected based on commonly used dosing ranges for ADCs in preclinical tumor models, including previously reported studies with trastuzumab emtansine (T-DM1; doi: 10.1038/s41467-022-30142-9, 10.3892/or.2013.2547) or trastuzumab deruxtecan (T-DXd; doi: 10.2967/jnumed.122.265172, 10.1038/s41467-025-58266-8), rather than by direct human-equivalent dose scaling.

Mouse dosing in the efficacy studies reported here was selected within ranges that allow evaluation of tumor growth inhibition while maintaining tolerability. The doses in the range of 5-20 mg/kg have been extensively used in preclinical studies that evaluate T-DXd and other HER2-targeting ADCs in mouse xenograft and syngeneic models. The goal of our experiments was to establish proof-of-concept for the antibody-ADC click under conditions comparable to prior preclinical ADC studies.

To clarify this point, we have added information to the manuscript to indicate that the doses were selected based on commonly used preclinical dosing ranges for ADC studies, including those reported for T-DXd in mouse tumor models.

Kindly see Page 55-Methods: "Once tumor volumes reached 100-2500 mm^3, ADC treatments were initiated, and doses were selected based on commonly used

preclinical dosing ranges for ADC studies, including those reported for T-DXd in mouse tumor models[4,5,61,62]."

6) Discussion: The changes to liver distribution are potentially critical for better toxicity profile and could have a very positive impact on clinical development. The liver data would deserve a couple of sentences articulating this in discussion (related to information on pages 19-20).

Response 1.8: We thank the reviewer for this suggestion. We agree that the observed changes in liver distribution may have important implications for future translation. In response, we have performed additional serum chemistry and histology analyses of the site-specific click conjugates (**Supplementary Figure 14, Supplementary Table 2, Figure 5e**) and we added additional text in the Discussion.

Kindly see Page 25-Discussion: "Additionally, site-specific non-stochastic click modifications reduced liver accumulation compared to stochastic tertiary-amino directed conjugates. These results are particularly relevant for clinical translation, as hepatic uptake is a key determinant of off-target toxicity and tolerability for ADCs."

Minor remarks:

7) Methods section:

- On page 29, line 650, were cells washed after second Ab trastuzumab-Tz?

Response 1.9: Thank you for this question. For our immunofluorescence assays, our standard protocol is to incubate the first pair (panitumumab-TCO) 30 min at 4°C, followed by 1 wash with PBS (containing Ca^{2+} and Mag^{2+}). Then, we incubate the cells with the second pair (trastuzumab-Tz) for 37°C for 3 or 24 h. After incubation with the second Ab, the cells are washed with PBS (containing Ca^{2+} and Mag^{2+}) prior to fixation.

To clarify this point, we have added more information to the manuscript regarding how we perform our immunofluorescence assays (**see Page 49-Methods)**

- What is the batch size of production of Abs conjugated with TCO or TZ?

Response 1.10: Thank you for this question. The conjugation reactions used to generate TCO- and Tz-modified antibodies were performed at laboratory scale for the purposes of this study. Our typical batch sizes for animal and cell studies range from 0.5 mg to 5 mg. Our workflow involves standard antibody conjugation conditions and purification workflows (e.g., desalting columns/size exclusion), which are also techniques commonly used in ADC preparation and antibody modification protocols. Following conjugation and purification, we typically recover 70-90% of the antibody conjugate.

The chemistry used for conjugating TCO or Tz uses well-established bioconjugation methods, and therefore, larger batches can be produced using the same reaction conditions and purification strategies that are routinely used for antibody conjugates and ADC manufacturing. We do not foresee any problems in scaling up the production of these antibody conjugates.

8) Could the authors explain why the NCI N87 cell line shows a more intense fluorescence from panitumumab binding than to T-DM1 (trastuzumab)? Would this be an illusion, or could this be quantified and added to figure 1"h"?

Response 1.11: **Figure 1h** is now **Figure 1g**. We have redone our images using confocal microscopy for higher-quality images, and we show IF images of NCIN87 in both **Figure 1g** and **Supplementary Figure 8**. We quantified total fluorescent signal (panitumumab click with trastuzumab) using plate reader measurements (**Figure 1f**) to show that binding is higher in the click when compared with the no click. We have also included binding data in **Supplementary Figure 6**; the signal is lower for panitumumab compared with trastuzumab in NCIN87 cancer cells. We hope this additional data addresses the reviewer's comment.

9) On line 323, suggest mentioning “the preclinical safety of the antibody…”

Response 1.12: Thanks for this comment. We have added in the manuscript more information on the safety of our antibody-ADC click for both random and site-specific modifications by including histophatological analysis, animal weight and blood serum chemistry analysis (**Figure 5e, Supplementary Figures 14, 15 and Table 2**)

Kindly see Page 16-Results: “The safety of the antibody-ADC click was supported by histopathological analysis, and the absence of weight loss or other clinical signs of toxicity in treated animals (Supplementary Figures 14,15; Supplementary Table 2).”

10) Suggest removing sentence on line 124 “Furthermore, no bispecific ADCs have yet achieved clinical approval (17), which highlights the need for strategies that can effectively target multiple antigens without these spatial constraints.” to prevent premature obsolescence of the manuscript.

Response 1.13: We agree with the reviewer and removed this sentence. As suggested by the other reviewers, we removed comparisons with bispecific to avoid confusion.

11) Figures

- Figure 1 E is confusing. The figure shows the anti-EGFR mAb panitumumab/TCO (green) and an anti-HER2 mAb Trastuzumab ADC/Tz in red. The corresponding text states (line 242) that 2 anti-HER2 naked mAbs/Tz (trastuzumab or pertuzumab) were used with panitumumab/TCO.

Response 1.14: We revised our schematics and the text. To clarify, we tested our approach preclinically with pertuzumab-TCO plus T-DXd-Tz (**Extended Figures 1a-b, 2, 4c-d**) and panitumumab-TCO plus T-DXd-Tz or plus T-DM1-Tz (**Extended Figures 1a&c, 3, 4a, 5; Figures 3-5**) to show the potential of antibody click using antibody pairs that target different HER2 domains or different receptors.

- Which anti-HER2 mAb was used? If an ADC, please correct the text to state T-DM1 or T-DXd. If naked anti-HER2 mAb, please correct the figure and remove the orange oval circle representing the cytotoxic payload.

- Figure 2a/2b is confusing: The text on line 255 mentioned trastuzumab or trastuzumab-Tz conjugated with pHrodo. However, the figure represents a trastuzumab ADC. Is this naked trastuzumab or T-DM1 (or T-DXd)?

Response 1.15: Thank you for these suggestions. We revised our figures and text accordingly.

- Extended file Figure 2d represents naked trastuzumab Ab/Tz, but text describes T-DXd/Tz. We suggest adding the representation of cytotoxic payload on T-DXd representation.

Response 1.16: Thank you for these suggestions. We revised our text accordingly. To clarify, the IF was acquired using naked antibodies, and ADCs were used for therapy studies.

- Suppl Figures 4 and 19 do not have values on scale bars on panel "f" and "b", respectively. Please add the scale bar values on Figure or legend (value nm).

Response 1.17: Thank you for these suggestions. We revised our figures to include the scale bar values.

- On extended file Figure 2 panel "e", it is not possible to identify the survival curves for T-DXd, pertuzumab or saline. Is there a perfect overlap (if so, please clarify on legend), or should these be deleted?

Response 1.18: We agree that the survival curves were confusing and overlapping. To simplify, we replotted our data as a fold in tumor volume for easier visualization (**Extended Figure 2f, Supplementary Figure 16**).

Referee #2

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Response 2.1: Thank you for your thoughtful input during the review of this manuscript.

Referee #3

The authors propose to address current limitations of antibody-drug conjugates (ADC) and bispecific antibody approaches with a strategy in which two antibodies that recognize different antigens or epitopes are each chemically modified with a complementary click chemistry group (here tetrazine or TCO). The authors propose that this will allow for dual epitope targeting, as the two modified antibodies will covalently bind to each other on the cancer cell surface. The authors perform a number of in vitro and animal studies to examine this concept, and demonstrate significantly increased effects in several tumor models.

Response 3.1: We thank the reviewer for the thorough comments. We appreciate the time and effort invested in reviewing our work, and the suggestions made that have significantly improved the manuscript.

The main criticism of the manuscript is that the authors do not convincingly demonstrate that the findings in their animal studies relate to dual epitope targeting (which is the main claim in abstract, introduction and throughout the manuscript).

Response 3.2: We thank the reviewer for raising this important point. We agree that the original text may have overstated the interpretation that the *in vivo* findings were only due to dual epitope targeting. In response to this comment, we have revised the Abstract, Introduction, Results, and Discussion to clarify this point.

In addition, we have included new data showing that the observed effects are associated with bioorthogonal antibody click combined with antigen-directed dual-targeting, which together enhance antibody localization, retention, and receptor-mediated internalization in cancer cells. These results indicate that the biological effects observed in our animal studies arise from the combination of click-mediated antibody ligation and antigen recognition, rather than from dual epitope targeting alone.

The relevant sections of the manuscript have been revised accordingly, and we have also added more information to the questions below to further explain the new studies we conducted.

Additionally, spatial analysis of HER2 and EGFR expression in tumor specimens showed limited overlap, which shows that these receptors occupy largely distinct tumor regions. This spatial heterogeneity provides a biological rationale for the antibody–ADC click strategy, as targeting complementary receptors may expand ADC delivery across heterogeneous tumor compartments. The antibody–ADC click strategy reduces the dependence of ADC uptake on the expression of a single antigen (e.g. HER2), as the ADC can be redirected through interaction with the complementary antibody targeting a different receptor (e.g. EGFR).

In the animal studies, the authors infuse an antibody to a known antigen/epitope on the cancer cells, and subsequently introduce an ADC that typically does not have specificity to antigen on the cancer cells. While the authors demonstrate this leads to a significantly enhanced anti-cancer response, these effects may simply relate to the covalent coupling of the ADC to the firstly bound antibody – and a subsequent anti-cancer effect as the drug is not present in the tumor.

Response 3.3: We appreciate this important point. We have performed additional internalization studies to show that the enhanced internalization observed in antibody click requires antigen expression, not just antibody coupling. As an example, we included additional experiments in cancer cells that lack HER2 and EGFR expression (**Figure 2e** and **Supplementary Figure 10**). In these control studies that lack both HER2 and EGFR, we did not observe antibody internalization.

Kindly see Pages 12-13; Results – "Panitumumab–trastuzumab click resulted in significantly higher trastuzumab internalization versus no click antibody combinations in HER2-positive/EGFR-low NCIN87 ($p=0.0193$; Supplementary Figure 10) and HER2-negative/EGFR-high MIAPaCa-2 or MDA-MB-231 ($p<1x10-6$; Figure 2e) cancer cells, indicating improved internalization and trafficking to endolysosomes. The enhanced

internalization observed under click conditions was not observed in control cell lines lacking EGFR or HER2, or in control panitumumab-TCO plus non-targeting IgG-Tz-pHrodo (Figure 2e, Supplementary Figure 10)."

To further evaluate whether the observed effects were driven by nonspecific antibody coupling, we performed experiments using multiple IgG control antibodies that lack antigen specificity (**Figures 5f,g, Extended Figure 3c,d and 5, and Supplementary Figures 10, 11 and 26**). These control pairs did not reproduce the internalization, uptake, or anti-tumor effects observed with the HER2- and EGFR-targeting antibodies. If the primary mechanism were simply covalent coupling of the ADC to the firstly bound antibody, one would expect an enhanced therapeutic response when using a control IgG ADC-Tz. However, we do not observe such an effect. The panitumumab-TCO plus IgG ADC-Tz click had a therapeutic response similar to single IgG ADC or antibody combination (**Extended Figure 3d**).

In additional control experiments, we evaluated pre-clicked antibody constructs. In contrast with *in vivo* antibody click, pre-clicked antibodies showed increased liver accumulation and reduced tumor localization (**Figures 5f,g** and **Extended Figure 5**).). Our findings suggest that the enhanced tumor accumulation of the antibody click depends on both *in vivo* antibody ligation and antigen-directed tumor targeting, and we have revised the different sections of our manuscript to clarify.

> **Kindly, see Pages 9 - Introduction:** "In this context, the bioorthogonal ligation between the antibody and ADC is governed by click chemistry, whereas antibody–ADC-tumor receptor binding and trafficking influence retention, internalization, and therapeutic efficacy."
>
> **Pages 23/24 - Discussion:** "The observed therapeutic effects of the antibody–ADC click are a result of a combination of systemic and tumor bioorthogonal ligation, and tumor-specific biological targeting, which ultimately leads to enhanced ADC internalization."

We also want to clarify that, "HER2-negative" refers to the clinical classification and does not indicate complete absence of trastuzumab uptake. These tumors retain low uptake for trastuzumab as determined via PET imaging, which is insufficient for effective ADC monotherapy but still allows antibody engagement when delivery is enhanced through the antibody–ADC click strategy.

Is an ADC even required for this effect – instead of an ADC, could the introduction of the drug molecule with the relevant click chemical group lead to the same effect? The particular click chemistry pair chosen here has very high and fast reactivity, as the authors note, and are likely to react with each other independently of any binding of the antibody to the cell. It is unclear the second antibody is bound to the cancer cells, and indeed is likely simply a carrier for the drug molecule to the firstly bound antibody. If the main mechanism is the latter, there are many examples of that type of strategy already in the literature.

Response 3.4: We thank the reviewer for raising this important point. We agree that, in principle, a small-molecule payload bearing a complementary click handle could react with an antibody in circulation or already localized at the tumor site.

In our previous studies we observed approximately ~20% ID/g pertuzumab accumulation in NCIN87 tumors and ~10% ID/g remaining in circulation at 24 h post-injection. When a tetrazine small molecule was administered 24 h later in a pretargeting system, we observed only ~3% ID/g tumor uptake of the tetrazine probe (doi: 10.2967/jnumed.119.225813). In the event of the non-targeting small molecule tetrazine be conjugated with a payload, only about 3% would accumulate in the tumor. Therefore, we do not expect that "the introduction of the drug molecule with the relevant click chemical group leads to the same effect." In the present work, using the same pertuzumab–TCO antibody and tumor model, we observe that the complementary antibody partner (trastuzumab-Tz) reaches ~30% ID/g tumor uptake (**Extended Figure 1b**), which is substantially higher than what was observed with the small-molecule tetrazine probe.

These results further suggest that the enhanced tumor accumulation of the second antibody click pair is a result of click chemistry and dual-targeting.

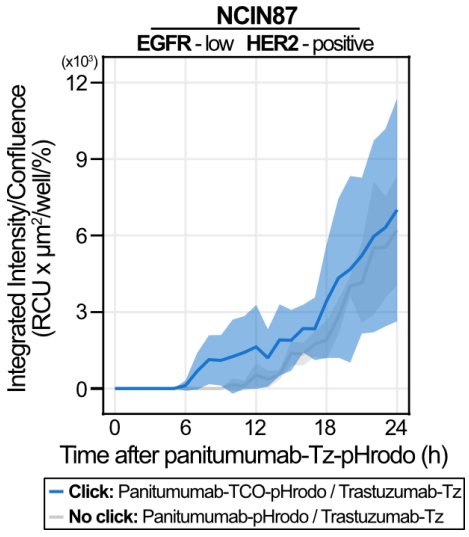
The goal of the present work was to evaluate a strategy in which two antigen-directed antibodies are brought together through bioorthogonal ligation, allowing the biological behavior of both antibodies (including tumor localization, receptor engagement, and internalization) to contribute to the observed effects. Several observations and control groups in our study support that the biological response is not simply the result of a drug molecule reacting with a previously localized antibody. First, we observed that the enhanced internalization is not present in HER2-negative or EGFR-negative cancer cells (**Figure 2e** and **Supplementary Figure 10**). Second, IgG control antibodies lacking antigen specificity do not reproduce the biological responses observed with the HER2- and EGFR-targeting antibodies (**Extended Figure 3c,d**, and **Supplementary Figures 11 and 18**). Third, additional experiments were done in which, after administration of panitumumab-TCO, an excess of tetrazine was administered to quench some of the available antibody-TCO groups prior to administration of T-DXd-Tz. Under these conditions, an heterogeneous reduction in therapeutic efficacy was observed (**Supplementary Figure 17**).

Along these lines, all quantification of antibody colocalization and internalization studies in culture studies is performed with just one of the antibodies (Fig. 1g, h; Fig. 2 – Trastuzumab only). It is unclear whether internalization of the EGFR targeting Ab is also increased? If the impact in these studies is only to enhance Her2 Ab internalization (which this antibody binds), could one accomplish the same effect in vitro with a greater concentration of that antibody alone?

Response 3.5: We thank the reviewer for this important comment. EGFR signaling has been shown to modulate HER2 trafficking and internalization, and EGFR/HER2 heterodimer formation can reduce HER2-ADC internalization. Targeting EGFR alongside HER2 therefore provides a biologically motivated strategy to enhance ADC uptake. The goal of this approach is

to leverage FDA-approved HER2 ADCs (T-DXd or T-DM1) as the therapeutic payload and enable their activity in tumor regions that may have very low to negative or heterogeneous HER2 expression by using a second antibody that targets a more highly expressed receptor (e.g., EGFR). In this context, the drug being delivered is the HER2 ADC, and trastuzumab-based ADCs therefore are the key therapeutic component of the system reported here. For this reason, trastuzumab internalization was selected as the primary readout in the internalization studies, and the study was designed and optimized to monitor trastuzumab.

In regard to the question of whether internalization of the EGFR-targeting antibody (panitumumab) is also increased, the experimental design of the assay makes this difficult to quantify directly. In the internalization experiments, the cells are washed after incubation with the first antibody (panitumumab) to remove unbound antibody present in the media before the second clickable antibody is introduced. The goal with those *in vitro* internalization assays is that trastuzumab clicks with panitumumab bound to the cell surface (please note that the incubation step with panitumumab is done at 4 °C). During the subsequent internalization period at 37 °C, trastuzumab remains associated with the cells is the most reliable marker for quantifying uptake. In contrast, monitoring the internalization of panitumumab is technically more challenging in this setting (see figure below) because the assay was designed to track the delivery and trafficking of the HER2 antibody/ADC component.



While increasing the concentration of trastuzumab could potentially enhance internalization *in vitro* as suggested by the reviewer, this strategy would primarily increase drug delivery to cells that already express HER2 and would not address the key clinical challenge of tumor heterogeneity and HER2-negative or HER2-low tumor populations. In contrast, the rationale of our approach is to enable delivery of HER2 ADCs to tumor regions that may have low or heterogeneous HER2 expression by leveraging a second receptor with higher expression, such as EGFR. From a biological perspective, increasing trastuzumab alone could also create selective pressure favoring the outgrowth of HER2-negative tumor cells, which is a mechanism of resistance to HER2-targeted therapies. Additionally, EGFR signaling has been implicated as a compensatory pathway and mechanism of resistance to trastuzumab-based therapies, which further supports the rationale for engaging this receptor in the antibody click strategy. Additionally, from a clinical standpoint, simply increasing the dose of trastuzumab-based ADCs would not be desirable due to dose-limiting toxicities associated with these agents, including known systemic side effects of HER2-targeted therapies such as interstitial lung disease. The goal of the present approach is therefore not to increase overall antibody dosing but rather to improve spatial delivery of existing HER2 ADCs within heterogeneous tumors through antigen-directed antibody ligation.

We have clarified these points in the revised manuscript.

Or, could increased internalization simply be due to formation of a complex of antibodies on the cell (both antibodies have multiple functional groups, and so could form covalently linked clusters), and not relate to any ligand binding capacity of second antibody?

Response 3.6: We thank the reviewer for this thoughtful comment. Although multivalent click chemistry could in principle generate covalently linked antibody clusters, our results do not support nonspecific clustering alone as the explanation for the increased internalization. If this were the primary mechanism, similar effects would be expected with non-binding control

antibodies; however, IgG control pairs lacking target specificity did not reproduce the observed responses (**kindly see Extended Figure 3d and Supplementary Figure 10 as examples**), and the effect was also lost in HER2-negative and EGFR-negative cells (**Figure 2e**). These findings support that ligand binding by the second antibody is functionally important and that the observed phenotype depends on both click-mediated ligation and antigen recognition.

The approach taken here to conjugate the click groups to the antibodies is non-specific, potentially leading to conjugation at ligand binding sites or other key portions of the antibodies. A site-specific conjugation would be greatly preferable – and the authors demonstrate in the last figure of the manuscript an approach to achieve. As the degree of modification is likely much different for antibodies modified in this manner, and that could dramatically impact the results, it is unclear why all of the studies in the manuscript are not performed with this chemical modification strategy?

Response 3.7: We thank the reviewer for bringing this important point. We agree that site-specific conjugation strategies have advantages in terms of molecular homogeneity and control over the number and location of modifications done in the antibody. In the initial studies, we used random conjugation of the click handles to lysine residues on the antibody because this approach is synthetically straightforward and allowed us to evaluate the feasibility of the antibody-ADC click concept across multiple models and antibody formats.

In response to the reviewer's comment, we expanded the manuscript to include additional studies using site-specific conjugation through Fc glycan engineering, which introduces the clickable handle at the CH2 domain of each heavy chain of the Fc region . This modification is spatially separated from the Fab antigen-binding domains and therefore minimizes the possibility of interfering with ligand binding. The resulting antibodies contain a defined and lower degree of modification (typically 2 to 4 click handles per antibody) compared with random lysine modification. The new experiments with these site-specifically modified antibodies

demonstrate that the key findings of the manuscript are preserved when a controlled conjugation strategy is used. In the revised version of our manuscript, we show that site-specific modifications to introduce click moieties on the antibody:

- retain efficient click ligation between two antibodies (**Figure 5a-c**, and **Supplementary Figure 22**).
- preserve antigen-directed biological responses, including internalization and therapeutic activity in tumor models (**Figure 5h**, and **Supplementary Figure 27**).
- show improved biodistribution properties, including reduced liver accumulation relative to the stochastic conjugates (**Figure 5d**).

Our data show that the observed biological effects do not depend on nonspecific lysine modification and are instead driven by the underlying combination of antigen targeting and bioorthogonal antibody ligation *in vivo*.

We have revised the manuscript to clarify these points and to highlight the site-specific conjugation strategy as an important direction for further optimization of the platform, and future translation.

On a related note, the authors describe this second chemical approach as site-specific, but in the text indicate "covalent linkage occurred predominantly through the Fc regions of the antibodies (Figure 5c, Supplementary Figure 19)" – if some of the conjugation is not at the desired site, it is not site-specific.

Response 3.8: We thank the reviewer for this comment. To avoid potential misunderstanding, we have removed this sentence from the manuscript.

Kindly see Page 20-Results: "To achieve site-specific modification of antibodies with TCO or Tz, we employed an enzymatic conjugation approach to specifically attach an azido moiety to the heavy chains of an antibody51 (Figure 5a)"

Minor points:

Supplemental figures cited out of order, which made difficult to follow. Manuscript is quite long, and written in a conversational tone in places – making it difficult to follow at times.

In the Introduction, the authors state "This approach is advancing towards clinical translation21 (clinicaltrials.gov identifier: NCT05737615)" to support the strategy. However, the clinical trials website indicates this trial has been withdrawn, so this is a bit confusing.

Response 3.9: The manuscript was revised to cite figures in order, and to make the different sections clearer. We are also confused by the withdrawal of the clinical trial, and removed to avoid additional confusion.

The use of click chemistry, and this particular click partner (tetrazine and TCO) has been widely used and validated, and so data demonstrating these react with each other is confirmatory and not needed in main figures.

Response 3.10: We thank the reviewer for this suggestion. While the tetrazine-TCO click reaction is well established, we would prefer to maintain this figure because it serves as an important validation experiment within the context of our antibody system.

Referee #4

This manuscript details a protocol using tetrazine bioorthogonal chemistry in situ/in vivo to construct antibody-antibody conjugates. The general concept is to target two epitopes, either on the same or different antigen using two different antibodies that themselves are modified with tetrazine or trans-cyclooctene bioorthogonal groups. The idea as presented is that the coupling would occur on the cell surface and enables more targeted delivery of payloads and avoids issues with simple monoclonals or bispecifics. The authors than present in vivo data to backup these claims.

The general concept is intriguing and builds on a lot of prior work that has been done over the last ~17 years on the use of tetrazine chemistry to achieve pretargeted chemistry. One key novelty here is, while nearly all previous approaches use small molecules to chase the pretargeted antibody, here they use an entirely different antibody. There are likely some benefits to in situ/in vivo assembly of complex antibody-antibody conjugates.

Response 4.1: We thank the reviewer for reading our work and providing suggestions to improve it. We particularly appreciate the recognition that our approach builds on the established field of bioorthogonal chemistry while introducing the concept of *in situ* assembly of antibody-antibody conjugates using two targeting antibodies.

However, after reading the paper carefully, I do not feel the authors numerous claims and assertions are backed up by convincing data. I also think that while some of the effects that they observe are interesting, they are not due to the mechanisms proposed as I elaborate below. More convincing data and likely significant rewriting would be needed to improve the scholarly presentation and give evidence for the claims before reevaluation.

Response 4.2: We thank the reviewer for constructive suggestions. In response, we performed several additional experiments to address the reviewer's concerns and have extensively revised the manuscript to better support the conclusions drawn from the data. We have responses to the comments and suggestions made, listed in response to the reviewer's comments below.

Specific Comments/Concerns:

Page 6 line 122: the authors try to discuss the drawbacks of bispecifics and the requirement of close spatial proximity. However, for this present approach, close spatial proximity is also needed. So I do not understand the proposed benefit. Is it that it doesn't need to be as close as bispecific? Great explanation here is warranted.

Response 4.3: We thank the reviewer for pointing this out to us to correct, and agree that the discussion comparing the antibody click with bispecific antibodies was confusing. While our intention was to highlight the conceptual differences between the approaches, we recognize that the wording in the original manuscript may have implied a direct comparison that was not our intention or necessary to explain the rationale of our platform.

In response to the reviewers' suggestions, we have revised the manuscript to remove the comparison with bispecific antibodies and instead focus on describing the mechanism and potential advantages of the antibody-ADC click strategy on its own terms.

Kindly, see Pages 7/8 - Introduction: "In parallel with advances in antibody combinations and multi-specific antibody formats, complementary modular strategies can be developed to enable flexible multi-target engagement *in vivo*."

"In heterogeneous tumors where HER2 expression is low or spatially variable, a co-expressed target such as EGFR may serve as a biological target to enhance the tumor uptake and internalization of HER2-targeting ADCs through in vivo bioorthogonal assembly."

Page 10 - Results: "FDA-approved antibodies and ADCs targeting HER2 and EGFR, which are receptor tyrosine kinases co-expressed in a panel of cancers35,36, were then conjugated with complementary bioorthogonal click moieties (Figure 1b) and administered sequentially to enable dynamic in vivo ligation. Antibodies or ADCs chosen here bind different epitopes of the same receptor (HER2/HER2) or co-expressed

receptors (HER2/EGFR, Supplementary Table 1). This approach can lead to antibody complexes that enhance lysosomal trafficking and intracellular payload delivery while leveraging tumor receptor biology to improve ADC distribution across heterogeneous tumor regions."

Page 6 line 128: The authors describe sequential administration of bioorthogonal tetrazine/dienophiles but this strategy is broadly known as pretargeting and has been discussed extensively. The authors have also left out key references from Weissleder and Robillard that were some of the first demonstrations of pretargeted tetrazine chemistry (see https://doi.org/10.1021/bc8004446, https://doi.org/10.1073/pnas.1113466109, 10.1002/anie.200906294)

Response 4.4: We thank the reviewer for this important comment and for highlighting the pretargeting strategies. We agree that sequential administration of tetrazine and dienophile components has been extensively explored in the context of bioorthogonal pretargeting, and we have added the suggested references (**kindly see, references 24-26**).

We would like to clarify that the approach described in this manuscript differs from classical pretargeting strategies. In most pretargeting approaches reported to date, a targeting antibody bearing one click component is administered first, followed by a small molecule carrying the complementary click partner, which is typically not itself target-specific. In contrast, in our approach, both click partners are conjugated to targeting antibodies, and the ligation occurs between two antigen-directed antibodies. To avoid potential confusion with the established pretargeting literature, we have revised the manuscript to clarify this distinction.

Kindly, see Pages 7/8 - Introduction: "Advances in bioorthogonal click chemistry have enabled highly selective reactions that allow two components to react in vivo after systemic administration18-20. In particular, the inverse electron demand Diels-Alder cycloaddition between transcyclooctene (TCO) and tetrazine (Tz) has been widely

explored in an approach termed pretargeting, in which an antibody bearing the TCO "click handle" is administered and accumulated in the tumor before injection of a non-targeted small molecule carrying the complementary Tz moiety19,21-26. Here, we introduce a modular strategy named antibody–ADC click, which uses full-length antibody–TCO and ADC–Tz as both targeting and reaction components, and enables the in vivo formation of functional antibody–ADC constructs that are directed against distinct domains of the same target or different tumor targets without requiring extensive antibody re-engineering."

Pages 23 - Discussion: "In vivo Diels–Alder chemistry has been successfully applied to radiochemistry and drug delivery22,24-26,54,55, including studies in large animal models23,56, most commonly making use of pretargeted antibodies and untargeted small molecule drugs. The antibody–ADC click approach uses tumor-targeting antibodies and ADCs that ligate together in vivo."

Page 9 line 183: similar to previous comment. The authors state that antigen levels are different in different regions of a tumor and this is one reason why bispecifics fail. However, I have no understanding as to why their approach would be theoretically superior or how distances related to the regions of a tumor would be bridged.

Response 4.5: We thank the reviewer for this comment and agree that the comparison with bispecific antibodies could be confusing in this context. Following the reviewer's suggestion and similar comments raised elsewhere, we have revised the manuscript to remove comparisons with bispecific antibodies to avoid potential misinterpretation.

In the revised text, we clarify the conceptual rationale of our approach. The goal of the strategy presented here is to leverage the therapeutic activity of FDA-approved HER2-directed ADCs (T-DXd and T-DM1) by enabling their delivery to tumor regions that may have low/negative or heterogeneous HER2 expression. This is achieved by using a second antibody

that targets a receptor that is highly expressed in those regions, such as EGFR. In this framework, the EGFR-targeting antibody helps localize the clickable HER2 ADC partner within EGFR-rich tumor areas. Thus, rather than bridging long spatial distances across tumor regions, the approach relies on local antibody distribution and antigen engagement within the tumor. We have revised the manuscript to better explain this concept and to avoid framing it in terms of bispecific antibody limitations.

Page 10 line 213: Here another fundamental issue is observed. The authors state that the antibodies must bind and then react with one another due to proximity. Its explicitly stated this happens in the tumor. However, no direct evidence is provided and instead its far more likely, given the very long half life of monoclonal antibodies, that these reactions are actually happening with circulating antibodies in the bloodstream. I suspect that the authors are creating essentially bispecifics in the blood and this could be responsible for the observed effects. It is interesting if this is true, but the manuscript would require rewriting and would be significantly strengthened with additional evidence that the reactions are actually happening in vivo.

Response 4.6: We thank the reviewer for raising this important point. We agree that, given the long circulation half-life of monoclonal antibodies and the high reactivity of the Tz-TCO pair, click reactions can occur not only at the tumor site but also in circulation. Our additional data support that antibody click can occur *in vivo*, including within the circulation, as visualized in the newly included experiments (**Extended Figure 1a**). We have revised the manuscript to clarify that antibody-antibody ligation is not restricted exclusively to the tumor microenvironment.

At the same time, our data indicate that antibody click in circulation is not the sole mechanism driving the biological effects observed. If the primary mechanism were simply the formation of bispecific-like antibody complexes in circulation, similar responses would be expected with a pre-clicked antibody or with clicking panitumumab-TCO with a control IgG

ADC-Tz. However, control experiments in our study demonstrate that the biological response depends on antigen-directed targeting and receptor biology. We have the main findings of our control experiments summarized below:

- Enhanced HER2-targeted antibody internalization under click conditions is observed in HER2-negative cancer cells expressing high or low levels of EGFR (**Figure 2e, Supplementary Figure 10**). However, enhanced internalization is not observed in HER2-negative/EGFR-negative cancer cells or in conditions where panitumumab-TCO is clicked with control IgG-Tz.
- Pertuzumab/HER2-ADC and panitumumab/HER2-ADC click leads to a higher reduction in tumor volume and improved animal survival when compared with control antibody combinations or with ADC alone (**Figures 3e-f, 4, 5h; Extended Figures 2f, 3, 4**). However, panitumumab-TCO click with a control IgG-ADC-Tz did show therapeutic efficacy (**Figure 3d**). If the click of panitumumab-TCO with IgG-ADC-Tz in circulation would lead to the delivery of a non-targeting ADC to the tumor, then we would expect therapeutic efficacy. However, this was not observed.

Our current data suggests that the therapeutic efficacy observed is a result of both click and tumor targeting. We have revised several sections of our manuscript to clarify:

Kindly, see Pages 9 - Introduction: "In this context, the bioorthogonal ligation between the antibody and ADC is governed by click chemistry, whereas antibody–ADC-tumor receptor binding and trafficking influence retention, internalization, and therapeutic efficacy."

Pages 23/24 - Discussion: "The observed therapeutic effects of the antibody–ADC click are a result of a combination of systemic and tumor bioorthogonal ligation, and tumor-specific biological targeting, which ultimately leads to enhanced ADC internalization."

-In general some of the data for the "clicked" antibodies vs. controls would benefit from a bispecific control if the authors are claiming benefit over bispecifics. However, the authors might consider toning it down and instead claim this is a modular and straightforward way to create bispecifics in situ without have to do extensive engineering.

Response 4.7: We agree with these comments and similar comments by this reviewer or the other reviewers. We revised our manuscript to avoid any comparisons with bispecifics.

-in some experiments the authors wait significant time (1 day) before subsequent injection of the second antibody. What are the internalization kinetics of the 1st antibody? These targets are well known to internalize when bound to antibodies. If the antibody is internalized, than theoretically it is unlikely for the tetrazine reaction to occur due to the barrier of the cell membrane. There observed effects despite the delay time further suggest that the reaction is actually happening in the bloodstream.

Response 4.8: We thank the reviewer for this important comment. We agree that HER2- and EGFR-targeting antibodies can undergo receptor-mediated internalization and that receptor trafficking dynamics are important to consider in sequential dosing experiments.

While these receptors can internalize, this process is dynamic rather than irreversible. Both HER2 and EGFR undergo continuous cycles of endocytosis and recycling. In addition, HER2 heterodimerization with EGFR and other biological processes has been reported to further stabilize HER2 at the plasma membrane and slow ADC-HER2 internalization, which can prolong the availability of HER2-bound antibodies on the cell surface.

Consistent with these trafficking dynamics, even with delayed administration of the second antibody, a fraction of the first antibody population remains accessible for bioorthogonal ligation. At the same time, we agree with the reviewer that the high reactivity of the Tz-TCO pair and the long half-life of antibodies make it possible for ligation to occur in circulation. Indeed,

our additional data (**Extended Figure 1a**) support that antibody ligation can occur in circulation. Importantly, several control experiments indicate that circulating ligation alone does not explain the internalization and therapeutic effects observed (**kindly see, Response 4.6**).

In our previous studies we observed approximately ~20% ID/g pertuzumab accumulation in NCIN87 tumors and ~10% ID/g remaining in circulation at 24 h post-injection. When a tetrazine small molecule was administered 24 h later in a pretargeting system, we observed only ~3% ID/g tumor uptake of the tetrazine probe (doi: 10.2967/jnumed.119.225813). In the present work, using the same pertuzumab–TCO antibody and tumor model, we observe that the complementary antibody partner (trastuzumab-Tz) reaches ~30% ID/g tumor uptake (**Extended Figure 1b**), which is substantially higher than what was observed with the small-molecule tetrazine probe. These results further suggest that the enhanced tumor accumulation of the second antibody click pair is a result of click chemistry and dual-targeting.

Additionally, if the primary mechanism were simply antibody-antibody ligation occurring in circulation followed by passive delivery of pre-formed complexes to the tumor, one would expect an enhanced therapeutic response when using a control IgG ADC-Tz. However, we do not observe such an effect. The panitumumab-TCO plus IgG ADC-Tz had a therapeutic response similar to single IgG ADC or antibody combination (**Extended Figure 3d**). We agree with the reviewer that a fraction of the antibody-antibody ligation may occur in circulation, as suggested by the new data (**Extended Figure 1a**). Our findings suggest that the enhanced tumor accumulation of the antibody click depends on both *in vivo* antibody ligation and antigen-directed tumor targeting, and we have revised the different sections of our manuscript to clarify.

Page 21 line 457: discussing the Nobel prize here seems unnecessary. Its also conflating click/bioorthogonal/tetrazine and while the literature does this often already, there are actual mechanistic differences between all these concepts and reactions.

Response 4.9: We have removed the mention of the Nobel prize in the manuscript. To try to homogenize our nomenclature, we have named our approach antibody click (for antibody-antibody ligation) and antibody-ADC click (for antibody-ADC ligation).

In general the microscopy data needs to be higher quality to draw the conclusions the authors make. Currently its hard to make out subcellular distributions and the images are quite blurry. Many times data is presented without error bars.

Response 4.10: We agree with the reviewer and acquired new IF images on confocal for higher quality and revised to include error bars.

I find the stability data in figure S4a surprising. While these kinds of tetrazines are quite stable, I would expect much more degradation over 2 days in serum. How exactly was this stability data performed. Did the authors actually test the functional stability of the tetrazine?

Response 4.11: We agree with the reviewer and have revised our manuscript. The stability data shown in the original **Figure S4a** was performed using ^{89}Zr-labeled trastuzumab-Tz incubated in human serum and analyzed by iTLC. However, this technique only provides information about the chelation stability of ^{89}Zr to the antibody and does not directly assess the stability of the Tz. Therefore, this previous figure was replaced with new data as described below.

To address the reviewer's comment, we performed additional experiments in which antibodies modified with TCO or Tz were incubated in mouse serum at different time points and subsequently reacted to determine whether antibody-antibody ligation could still occur (**Figure 1c**, **Supplementary Figure 5d**). These new data confirm that ligation between the two antibodies remains possible after incubation in serum.

Kindly see Page 48- Methods: "In vitro serum reactivity of antibody click pairs"

Referee #1

"Thank you for submitting the revised manuscript and thoroughly addressing the reviewers' comments. The authors' concerted effort to strengthen the work is evident. Key clarifications and additions have produced a clearer and more cohesive manuscript.
Several previous concerns have been satisfactorily resolved, particularly those related to the modular ADC-antibody click strategy and the presentation of the in vivo efficacy data. The revised figures and expanded methodological details enhance both clarity and reproducibility.
The data are of high quality, and the conclusions are well supported. The authors have comprehensively addressed all the reviewers' prior comments, and the manuscript is now suitable for publication.
The statistical analyses appear appropriate for the study design, and the error bars and probability values are accurately described in the figure legends."

Response: We are sincerely grateful to the reviewer for the careful evaluation of our work and for the constructive comments and suggestions, which have helped improve the quality and clarity of the manuscript.

"Overall, this is a compelling study. Addressing the remaining minor points outlined below will further strengthen the work.
Specific comments on the updated version of the manuscript:
1) Page 11, line 200: "The antibody click formation could be blocked with an excess of each click moiety (Supplementary Figure 5c)."
Could the authors please specify on text that this is related to "the presence of an excess of the unconjugated TCO or Tz." – as said in the supplementary figure legend?"

Response: We thank the reviewer for this suggestion. The corresponding sentence was revised to:

> The antibody click formation could be blocked in the presence of an excess of the unconjugated TCO or Tz (Supplementary Figure 5c).

"2) For comparison across cell lines, using a consistent y axis is generally preferred. It allows clearer visual assessment of relative treatment effects. If different scales are necessary, this should be clearly indicated in the figure and legend. This would apply to Figure 2e, and Supplementary Figures 10 and 27."

Response: Our studies were performed for comparisons across groups (*i.e.* click *vs.* no click). No comparisons were made across cell lines, and the figure legends were revised to clarify.

"3) There are three instances in Supplementary Table 2 where the term "aganocal" appears. I suspect this is a typographical error and should read "agonal."
4) Page 21, line 427: "Additionally, site-specific antibody click led to improved antibody conjugate stability (Supplementary Figure 25) and a favorable safety profile (Figure 5e, Table 2). Would this be "Supplementary Table 2"?"

Response: Thank you for putting this out to us for correction. The documents have been revised for typographical errors such as this one pointed out by the reviewer, and also for figure citations.

Referee #2

"I co-reviewed this manuscript with one of the reviewers who provided the listed reports."

Response: We appreciate the contribution of this reviewer to the review process and the time taken to provide thoughtful feedback on the manuscript.

Referee #3:

"The authors have adequately addressed my earlier concerns."

Response: We sincerely thank the reviewer for the positive evaluation and are pleased that the revised manuscript has adequately addressed the earlier concerns.

Referee #4:

"I have read the revised manuscript by the authors. overall I think they did an excellent job responding to the my questions and concerns. I think the revised manuscript has improved substantially in readability and quality of the cell images. I think there are still some questions about mechanistically what is happening (is antibody coupling happening in the tumor vs outside and to what extent and also how does internalization affect the second reaction) but this is quite a complex problem and can be subsequently addressed in future studies. I will make one suggestion which is the authors might want to introduce the structure of the Tz and TcO at least once in the main figures to guide the reader. most readers will not know what these designations mean chemically, and they are very easy to draw. Even just a simple Tz= and Tco= would be helpful. Otherwise, I think the strategy they have presented is an extremely important variant of in vivo tetrazine chemistry and is timely given the rising interest in applying such chemistry in vivo, especially in the ADC field."

Response: We sincerely thank the reviewer for the constructive evaluation of our work. We appreciate the reviewer's comments regarding the mechanistic complexity of *in vivo* antibody coupling and internalization dynamics, which we agree are very important areas for future investigation. In response to the reviewer's suggestion, we have now included the chemical structures of Tz and TCO in the main figures to better guide readers who may be less familiar with bioorthogonal chemistry terminology. We are grateful for the reviewer's recognition of the potential significance and timeliness of this strategy within the growing field of ADC development and multi-targeting approaches.