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Bing Wu, Ph.D.

Review Editor, *JoVE*

Dear Dr. Wu,

Attached please find our revisions to the manuscript we have submitted to *JoVE* entitled “Pretargeted Radioimmunotherapy Based on the Inverse Electron Demand Diels-Alder Reaction” (JoVE59041). We would like to thank you and the reviewers for your consideration of our work and your careful review of the manuscript. We have made a number of critical modifications to the manuscript based on the critiques offered in the review. As requested, we have addressed these changes on a point-by-point basis. Below we have listed the comments of the Reviewers 1, 2, and 3 (in **bold**) along with our responses to said comments and the changes we have made (in normal text). For the sake of clarity, any addition or modification to the text in the manuscript are listed below in *purple italics*.

**Editorial Comments:**

**Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

We have fully proofread the manuscript.

**Please revise lines 50-52, 154-156, 192-194, 289-291, 315-318 to avoid previously published text.**

These lines have now been revised.

**Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a \*.doc or \*.docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”**

All of the figures in this manuscript are either original or have been modified from our own work that was recently published in *Molecular Pharmaceutics*. Therefore, according to the rules of the American Chemical Society, permissions are not required. However, we have added the appropriate citations for the original manuscript to each of the figure captions.

**Please shorten the Summary to no more than 50 words.**

The summary has been shortened, it now reads: *“This protocol describes the synthesis and characterization of a trans­-cyclooctene (TCO)-modified antibody and a 177Lu-labeled tetrazine (Tz) radioligand for pretargeted radioimmunotherapy (PRIT). In addition, it details the use of these two constructs for in vivo biodistribution and longitudinal therapy studies in a murine model of colorectal cancer model.”*

**Please spell out each abbreviation the first time it is used.**

We have corrected all instances of this error.

**JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: ZevalinTM, BexxarTM, *etc.***

All commercial language has been removed from the manuscript.

**Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).**

We have removed personal pronouns where appropriate, but we have kept personal pronouns in places in which we think their use improves the pedagogical framework of the article. Please do not edit further.

**Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.**

We have made the necessary revisions.

**Please specify the volume of this solution to be prepared.**

We have added the necessary information.

**Does the antibody solution refer to the huA33 solution? Please specify.**

We have added the necessary information.

**What temperature is set during rotary evaporation?**

We have added the necessary information.

**Please specify the amount of [177Lu]LuCl3 added.**

We have added the necessary information.

**How to confirm that tumors are of sufficient size?**

We have added the necessary information.

**Please specify the specific step where the huA33-TCO solution is prepared, as Protocol Section 1 has many steps.**

We have added the necessary information.

**Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”**

We have added the necessary information.

**Please specify the dose of radioligand.**

We have added the necessary information.

**After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.**

The appropriate sections have now been highlighted.

**Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.**

These directions have been followed.

**Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.**

These directions have been followed.

**As Table 1 shows NMR and MS assignments, please indicate in the protocol that the organic compounds are characterized with NMR and MS.**

This has been added.

**Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.**

This has been done where appropriate.

**Please reference Figure 3 and Figure 4 in the manuscript.**

These references have been added

**Figure 3: Please change “30 m” to “30 min” and “60 m” to “60 min” if the “m” is intended for time unit.**

These changes have been made.

**Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text. Please remove the embedded table(s) from the manuscript. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text. Please remove embedded Table of Materials from the manuscript. Please remove trademark (™) and registered (®) symbols.**

All embedded tables and figures have been removed from the manuscript.

**Reviewer #1:**

**The current manuscript by Membreno and Zeglis, builds on the extensive work published by their group and others on the in vivo pretargeting approach based on the inverse electron demand Diels-Alder reaction. This is a high impact topic for the scientific community, with potential and imminent clinical applicability for the circumvention of the long residence times of radiolabeled antibodies. This is a very thorough methods article, which is well written, correctly organized and that describes the modification of the huA33 antibody with a TCO moiety, radiosynthesis of 177Lu-labeled tetrazine and in vivo evaluation of this system. Only a minor revision is recommended.**

We thank the reviewer for their thorough critique of our work.

**Line 2: Although the title of this manuscript is very appealing, it seems generic also. There are several other pretargeted radioimmunotherapy approaches than the one described here by the authors. This is only one of them, using the IEDDA reaction. Please make the title more concise and informative.**

This is an excellent point, we have added the phrase “*Based on the Inverse Electron Demand Diels-Alder Reaction*” to the title in order to be more specific and distinguish this approach from other pretargeting methodologies (i.e. streptavidin/biotin, oligonucleotides, bispecific antibodies, etc.).

**Lines 78-80: To circumvent the problem of long residence times of the antibodies in the blood several strategies are presented. Recent findings using radiolabeled nanobodies emphasize their potential as therapeutic tools in cancer treatment. However, the authors fail to mention the nanobodies in their list of available alternative strategies.**

This is a good point. We did not mean for list to be exhaustive. We have now added “*nanobodies*”.

**Line 102: The claim that "TCO is more stable in vivo than its Tz partner" should be rephrased. This is not always true. Physiologically stable radiolabeled tetrazines have been developed by different labs, including the one of Hannes Mikula in Vienna. Also, other authors have attempted the inverse strategy (Tz-modified antibody and a TCO-bearing radioligand) with success. Reformulate accordingly.**

Great find! The wording of the section has now been modified to suggest that there are other methodologies to IEDDA pretargeting. We have elected not to include more discussion of these alternative tetrazine/TCO formulations, as it would be beyond scope of this protocol. That said, while there is certainly some interesting chemistry being evaluated, very little of it has been tested *in vivo*.

**Line 150, 1.2: Were normal 1.7 mL microcentrifuge tubes used or low protein binding tubes? The latter could help minimize protein sample loss during sample manipulation.**

For these experiments, we actually found that low protein binding tubes were not necessary for high-yield! The text has been modified accordingly.

**Line 223, 2.6: For purification of the reaction, was the reaction diluted? Or was it injected directly on the HPLC?**

Great catch! The reaction was diluted in water prior to purification. This change has been made to 2.5 and 4.5.

**Line 230: I assume the mobile phase was used without any (acid) additive to prevent removal of the Boc protecting group. It would state the importance of this in the text, to be clear to the readers as well.**

We had previously stated in Step 2.5 that the HPLC should be run without additive. However, we have updated the text to note that, “*Solvents without acid should be used to prevent the premature removal of the Boc protecting group*.”

**Line 234, 2.7: Could you provide an alternative to using liquid nitrogen to freeze the sample with before placing it in the lyophilizer? Some labs do not have access to it.**

An excellent point. We have updated the protocol to include alternatives.

**Lines 230-232 and 258-260: What was the HPLC flow rate used to get the retention times mentioned in the text? Please clarify.**

We have clarified in both sections: a 1 mL/min flow rate is used.

**Line 314, 5: For the radiolabeling of Tz-PEG7-DOTA, is it important to work in metal-free conditions? For example, for the preparation of the ammonium acetate buffer.**

Interestingly, across dozens of radiolabeling experiments, we have found that these procedures do not require metal-free buffers or solvents. At the beginning of Section 5, however, we have noted that this is a good practice!

**Lines 330-332, 5.6: Please explain with more detail the procedure to alternatively purify the labeled [177Lu]Lu-DOTA-PEG7-Tz using a C18 cartridge. The way it is written now is too much simplified.**

Thank you for bringing this to our attention! We have now expanded this section to include more detail.

**Line 343, 6.1.1: How do you identify the mice in the biodistribution experiment if they do not receive ear tags. You need to know which ones are euthanized at the different time points.**

We have now updated this section to include our use of tail markings.

**Lines 343-369: Sections 6.1.1 and 6.1.2 contain mostly the same information. The information could be mentioned in one section only, for both biodistribution and therapy studies.**

We agree that the inclusion of tumor growth information was redundant, and this has been condensed accordingly. However, we have elected to keep sorting of animals separate for each, as the rigor needed for ensuring highly homogeneous tumor distributions for a therapy experiment is greater than that needed for a biodistribution experiment.

**Lines 395, 396, [6.2.1.2](http://6.2.1.2/" \t "_blank): Is this a critical step? Is it to prevent aggregation? Please clarify.**

We have added the following clarification: “*NOTE: PBS treatment is necessary to prevent nonspecific binding of the antibody to the walls of the syringe.”*

**Line 405, 6.2.2: This section is lacking the description of the use of controls. I can see in Figures 6 and 7 (huA33-TCO only and radioligand only controls) that you have used them. Therefore, these should also be mentioned in the text as well. Did you consider using a tumor which does not express A33 or only expresses it at low levels as negative control? It is not clear to me if the mAb is also injected in the biodistribution experiment. Please specify what is the injection sequence for the biodistribution experiment and the therapy study.**

To address the reviewer’s excellent points in order:

1. We have added the following passage following Line 768: “*It is also recommended that appropriate controls be run in tandem, including but not limited to the use of an antigen-negative cell lines, antigen blocking with an excess of unconjugated antibody, and the injection of isotype control immunoconjugates to determine non-specific accumulation at the tumor.*” We have also added emphasis to the use of key controls for the therapy study in Line 533.
2. Comparisons with an A33 negative cell line have been investigated in previously published work. Interestingly, comparing different cell lines in an experiment like this would be quite difficult, as they would likely have different growth rates, morphology, and responses to therapy. A blocking study could certainly be an option, but it would be much easier to run as a part of a biodistribution experiment.
3. The sequence of injections has now been addressed in Section 6.2 “*NOTE: The sequence of injections for both biodistribution experiments and therapy studies proceeds as follows: huA33-TCO is injected first, followed by [177Lu]Lu-DOTA-PEG7-Tz after a predetermined interval.”*

**Line 431, 6.3.2: How and when is the blood collected? Is it using cardiac puncture? Is it before the mouse is euthanized? Please specify.**

We have added “post-euthanasia” to remove any confusion. The exact method will be left to the reader based on institutional guidelines and group-specific methodologies.

**A data analysis section is missing on the "PROTOCOL" section. Statistical analysis methods should also be specified.**

While we have not added a data analysis section, we have included additional steps in the protocol (6.4.4 and 6.3.5) with suggestions on the presentation of data as well as basic statistical tests that can be utilized. In addition, the “Representative Results” should give readers an appropriate level of initial guidance in their analysis.

**As 177Lu can be used for SPECT imaging, together with biodistribution experiments, radiation dosimetry for this radiotracer could be determined. Why was this not performed?**

Radiation dosimetry was calculated from biodistribution experiments and published in our *Molecular Pharmaceutics* manuscript! The data was not included in this protocol, as its calculation was deemed beyond the scope of this work.

**Line 489: According to the latest nomenclature rules for radiotracers (cfr. Coenen et al., 2017. Nuclear Medicine and Biology. Consensus nomenclature rules for radiopharmaceutical chemistry — Setting the record straight.) instead of "specific activity", molar activity should be mentioned on the text since it is the measured radioactivity per mole of compound. Please reformulate this here and in the different parts of the text where "specific activity" is mentioned.**

This is an excellent point. We have amended the manuscript accordingly.

**Line 508: A small typo: "xenogarfts" should be xenografts instead.**

Great catch! We have corrected this typo!

**Table 1: The calculated m/z for Tz-PEG7-DOTA is missing two decimal digits.**

Another great catch! We have corrected this typo, too!

**Lines 795, 796: The use of the inverse strategy (Tz-modified antibody and a TCO-bearing radioligand) should also be discussed.**

In this protocol, we have elected to only address the more established mAb-TCO/Tz-radioligand strategy. While the inverse strategy has shown some potential, the efficacy of PRIT based on this approach has yet to be demonstrated. Furthermore, we would never want to step on the toes of these authors if they were to want to publish a protocol in *JoVE*.

**Reviewer #2:**

**This methods article describes in detail the concept for pretargeted radioimmunotherapy (PRIT) in a very elaborate way. They use the inverse electron demand Diels-Alder click reaction between a radiolabeled tetrazine (Tz) and a trans-cyclooctene (TCO) coupled to an antibody. The TCO-antibody is injected and allowed to accumulate at the target tissue (slow process) for 24 hours before the radiolabeled-Tz is administered. The Tz then clicks to the antibody (quick process) and excess radioactivity is cleared quickly. This approach minimizes radiation to healthy tissue and increase radioactivity concentration within the target region. The theoretical background is thoroughly described in the introduction that gives the reader a good overview followed by a detailed protocol for how the technique should be executed and the results analyzed. I recommend to accept this article after minor revision.**

We thank the reviewer for their kind words about our manuscript.

**Line 147, page 5: The preparation of huA33-TCO: Please, specify which TCO is used or reference to "Table of materials".**

*Trans*-cyclooctene NHS ester (TCO-NHS) is listed in the Table of Materials. We have added the commonly used abbreviation for clarification.

**Some abbreviations should be added to the "Table of materials": For example, NHS, DMF, DMSO, MeCN or TEA. Please, check the whole manuscript.**

This is a great point. We have added all these abbreviations to the Table of Materials!

**Line 209, page 6 (2. The synthesis of Tz-PEG7-NHBoc): Please, specify what Tz is used.**

The tetrazine used is specified in the Table of Materials. We have added the abbreviation for clarification.

**Line 350, page 10 (6.1. Preparation of animals) "Once the tumors are of sufficient size". Please, indicate what this means. Are you referring to the size mentioned on 6.1.1.1 (100-150 mm2).**

Good catch! We have updated this for the sake of clarity, referencing 6.1.1.1.

**Lines 355-357 and 367-369, page 10 (6.1. Preparation of animals): These two paragraphs are repetitions of 6.1.1. Consider removing 6.1.1. or refer to it without repeating the text in these paragraphs.**

We have consolidated these paragraphs to minimize repetitions.

**Please, refer to GBq/mg when you use the term specific activity and GBq/µmol when molar activity is used. Recently, a Consensus nomenclature rules for radiopharmaceutical chemistry (2017) was published. Please, follow it.**

We have updated the nomenclature to reflect “molar activity” rather than “specific activity”.

**Please, use in the whole manuscript GBq or MBq and not mCi (for example, line 321, page 9).**

We have updated the manuscript accordingly.

**Reviewer #3:**

**In this manuscript the authors described the construction of a TCO-modified immunoconjugate of the huA33 antibody, the synthesis of a 177Lu-DOTA-labeled Tz radioligand, and the performance of in vivo biodistribution and therapy studies using this system in a murine model of colorectal carcinoma. The manuscript was comprehensive in the experiment procedures and provided adequate results. There are some minor revisions needed and are listed below:**

We thank the reviewer for their careful consideration of our work.

**In the step 4.3, since DOTA is not easily dissolved in organic solvent, should it be dissolved in DMSO first before adding to the reaction? Or is there a volume/concentration requirement for the reaction?**

This is an interesting point! We have found that pre-dissolving the DOTA in DMSO prior to its addition to the reaction mixture results in the formation of additional byproducts that are not found when the solid is directly dissolved as stated in the protocol. While vortexing is required, the DOTA does fully enter solution, ultimately leading to a cleaner product and higher yield. We have added a note that this vortexing may take a couple of minutes.

**In the step 5.3, how much of the Tz-PEG-DOTA is needed for each mCi of 177Lu?**

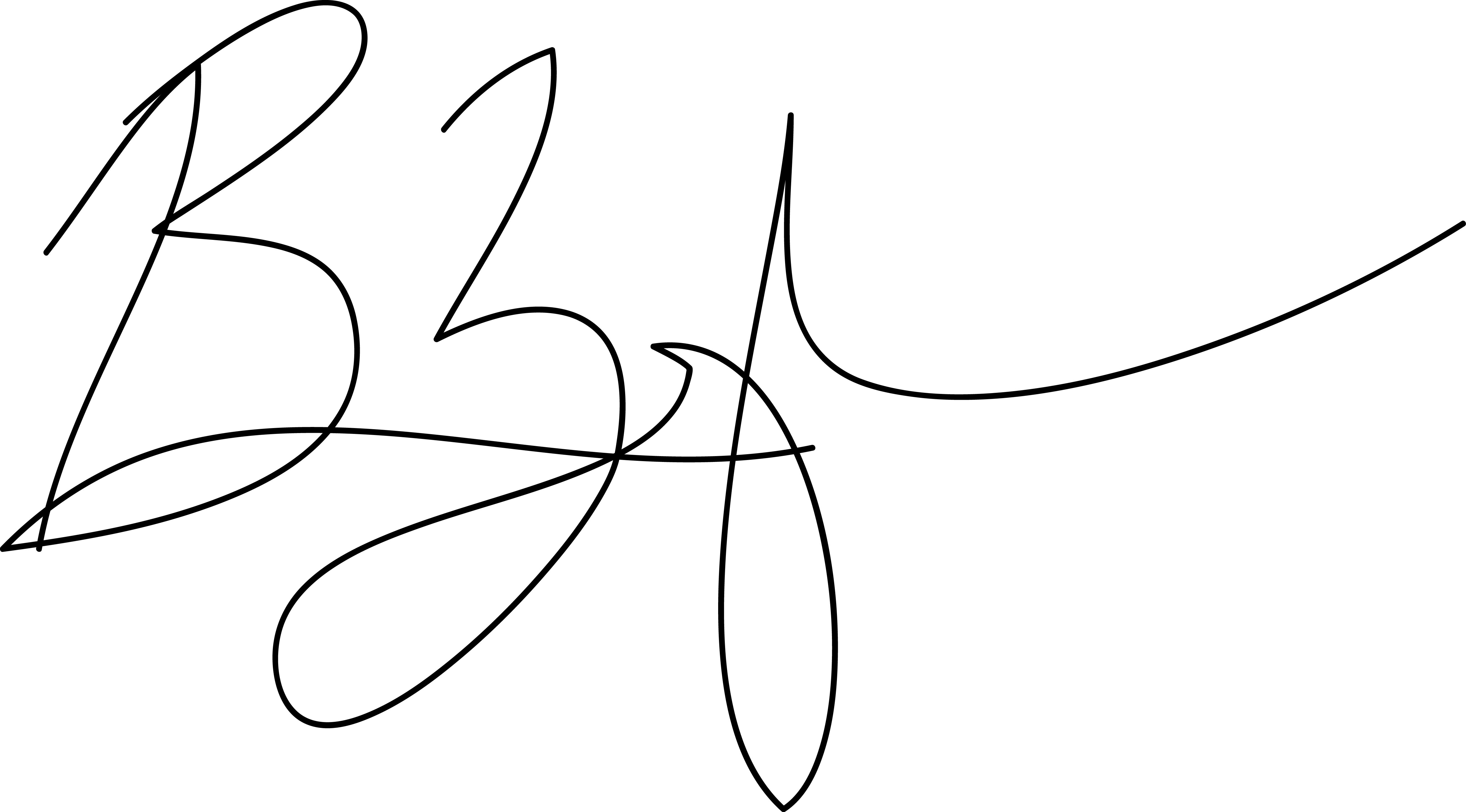
Our mistake! A reference has been added directing readers to Step 6.2.2.2, where more detail is given on the formulation of doses.

**The breakthrough of functionalized TCO was made by Joseph Fox’s group in 2008. The references should be cited.**

Great catch! This reference slipped through the cracks. A proper citation has now been included: reference 26.

Thank you for your time, work, and consideration. Please let us know if you require any more information.

Respectfully,



Brian M. Zeglis, Ph.D.