August 8, 2014

Sephorah Zaman, PhD

Editor, *Journal of Visualized Experiments*

Dear Dr. Zaman,

Attached please find our revisions to the manuscript we have submitted entitled “*Harnessing the Bioorthogonal Inverse Electron Demand Diels-Alder Cycloaddition for Pretargeted PET Imaging.*” Thank you very much for your consideration of our work and your careful review of the manuscript. We would also like to thank the three reviewers for their enthusiasm about the work and their very positive and thoughtful commentary. We have made a number of modifications based on the critiques offered by the work’s reviewers. Below we have listed the reviewers’ comments (in *blue italics*), our responses to said comments, and the changes we have made. In addition, we have listed the changes we have made to the manuscript requested by the editors.

***Editorial Changes:***

*“All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.”*

Thank you! We have downloaded that version and are using it as the starting point for the revised manuscript.

*“Prior to peer review, the length of the Short Abstract is exactly at our 50 word limit. If, in response to peer review comment, changes are made to the Short Abstract, please ensure that the final length does not exceed 50 words.”*

As none of the reviewers had any problems with the Short Abstract, we have not changed it. It remains exactly at the 50 word limit.

*“Editor removed the highlighting from step 4.1, because as agreed, this step lacks sufficient detail for filming and will therefore, not be filmed. Highlighting was also removed from all steps describing anesthesia (step 4.7 and part of step 4.8).”*

This is fine with us!

*“In the Reference List, some journal names are abbreviated, while others are not. Please be consistent in either abbreviating or not abbreviating journal names.”*

We have changed the Reference List such that none of the journal names are abbreviated. We apologize for the error.

*“Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.”*

We have thoroughly proofread the revised manuscript.

*“If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."”*

Figures 1, 3, and 5 are original. Figures 2, 4, 6, and 7 are modified from the *Journal of Nuclear Medicine* publication on which this article is based. The *Journal of Nuclear Medicine* allows authors to re-use their own figures in subsequent review publications. On the *JNM* website, one can read that “*JNM* authors retain the following nonexclusive copyrights, to be exercised only after the article has been published in final format in the print version of *JNM* ... (a) Reprint the article in print collections of the author’s own writing ... (f) Reuse original figures and tables in future works created by the author.” (<http://jnm.snmjournals.org/site/misc/permission.xhtml>)

In accordance with *JNM* recommendations, the figure captions have been altered to read “This figured is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthgonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013;54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.”

***Reviewer #1:***

*“Manuscript Summary:* This manuscript provides a detailed method for using a previously developed "click" reaction for in vivo pretargeting. Overall the authors provide a clear description of the methodology used.*.”*

We thank the reviewer for their careful reading of our manuscript.

*“*Step 1.8 mentions that this is a stopping point. How long is the compound stable in the given storage conditions (-80, dark)?”

This is a good point. Step 2.11 has been modified to include the line “The completed Tz-Bn-NOTA precursor is stable for at least 1 year under these conditions.”

*“Step 2.11 same question as above.”*

While we do not know *exactly* how long the mAb-TCO conjugate is stable in storage, we have stored it for multiple months at -80 in the dark. As a result, Step 1.8 has been modified to include the line “The completed mAb-TCO conjugate should be stable for at least 3 months under these conditions.”

“Step 3.4 What is the composition of the 64Cu solution (pH, volume) the researchers used? How tolerant is this reaction to increased volume?”

This is a great point. We have added the following to the discussion of Step 3.4: “Typically, [64Cu]CuCl2 is supplied in a small volume (<30 μL) of 0.1 N HCl, and thus only small volumes (< 10 μL) of this stock solution are needed for the radiolabeling reaction. If larger volumes of the [64Cu]CuCl2 stock are needed, the radiolabeling reaction is tolerant of increasing the overall reaction volume. However, the pH of the radiolabeling reaction solution should be monitored carefully to ensure that it does not fall below pH 4.0.”

*“Step 4.1 How many cells were implanted?”*

We have altered Step 4.1 to read, “In a female athymic nude mouse, subcutaneously implant 1 x 106 SW1222 colorectal cancer cells and allow these to grow into a 100-150 mm3 xenograft (9-12 days after inoculation).”

“Typo: Line 83 "both are have" should be "both have"”

This typo has been corrected. Thank you for noticing it!

***Reviewer #2:***

*“Manuscript Summary:* The manuscript is written clearly and the things you should take into account when you would like to apply the IEDDA to your own system are well explained. In the experimental procedure some details are missing, which I summarize hereafter. these should be addressed because otherwise researchers can not reproduce their procedure.

Thank you very much for your careful reading of our manuscript. We apologize for leaving the relevant details out of the protocol, and we have endeavored to add them where they are needed.

*“*Line 84: are should be removed”

This typo has been corrected.

*“*What is the molarity of the NaHCO3 solution? Is a centrifuge tube necessary? Can this also be done in other types of tubes?”

We accidentally omitted the bicarbonate molarity, and we agree that the reaction can be done in other types of tubes. Step 1.1 has been altered to read “1.1) In a small reaction vessel, dissolve 7 mg NH2-Bn-NOTA (1.25 x 10-2 mmol) in 600 μL NaHCO3 buffer (0.1 M, pH 8.1). Check the pH of the solution. If needed, adjust the pH of the solution to 8.5 using small aliquots of 0.1 M Na2CO3.”

*“*1.2 Is Tz-NHS a solid? Weighing 0.5 mg is pretty challenging”

We have never had trouble weighing out 0.5 mg of Tz-NHS on an analytical balance, but just in case using a stock solution is easier, we have amended Step 1.2 to read “1.2) Add the NH2-Bn-NOTA solution to 0.5 mg Tz-NHS (1.25 x 10-3 mmol) in a 1.7 mL microcentrifuge tube. The Tz-NHS can either be weighed out dry or from a stock solution in dry DMF or DMSO (< 50 μL).”

*“*1.4 What is the retention time of unreacted NH2-Bn-NOTA? what is the wavelength at which the compounds should be visible? What is the flow of the HPLC purification? Can the whole product be purified in one run? Or must this be done in multiple runs? Details are missing!”

“1.8 Are there any analytical measures that can be taken to be sure that the product is pure? NMR, HPLC, TLC, MS?”

While we agree that the details are missing, we believe that the HPLC purification of a compound is highly dependent on the equipment set up of the lab. We have given example retention times for the compounds, and have added the wavelength at which the compounds can be monitored, but the flow rate and whether the compound can be purified in a single run or multiple runs is dependent on the column that the end user possesses. Step 1.4 has been revised to read, “1.4) After 30 minutes, purify the product using reversed-phase C18 HPLC chromatography to remove unreacted NH2-Bn-NOTA. The NH2-Bn-NOTA can be monitored at a wavelength of 254 nm, while the Tz-NHS and Tz-Bn-NOTA are best monitored at wavelength of 525 nm. The retention times and flow rates are obviously highly dependent on the HPLC equipment setup of each laboratory (pumps, columns, tubing, *etc.*). However, to present an example, if a gradient of 5:95 MeCN/H2O (both with 0.1% TFA) to 95:5 MeCN/H2O over 30 minutes and an analytical 4.6 x 250 mm C18 column is used, the retention times of Tz-Bn-NOTA, Tz-NHS, and NH2-Bn-NOTA will be around 10.5 min, 9.5 min, and 6 min, respectively. The product can be purified from the other reaction components in either single run or multiple runs (depending on the loop) using a semi-preparative or preparative C18 HPLC column. 1H-NMR, analytical HPLC, and ESI-MS are all methods that can be used to verify the purity of the completed Tz-Bn-NOTA precursor.11”

*“2.4 What is the max. volume of DMF that can be used?”*

This is an excellent point. Step 2.4 has been amended to include “Do not exceed 5% DMF by volume in the solution.”

*“2.8 Purification of the immunoconjugate is not completely described. Suggestion for addition: rinse the size exclusion column as described by the supplier to remove any preservative present on the column. Apply the reaction mixture to the size exclusion column, rinse with xx mL 0.9% NaCl and collect the product hereafter with xx mL 0.9% NaCl.”*

We completely agree with this point by the reviewer. However, as we were not allowed to refer to any specific products (*i.e.* GE PD-10 size exclusion columns), we felt it was not appropriate to list the steps we’d take if we were using such products. However, now that the reviewer has pointed this out, Step 2.8 has been modified to include the following passage: “First, rinse the size exclusion column as described by the supplier to remove any preservatives present on the column during storage. Then, add the reaction mixture to the size exclusion column, rinse the column with 1.5 mL 0.9% NaCl, and subsequently collect the product using 2 mL of 0.9% sterile saline as the eluent. Note: This step will yield the completed huA33-TCO as a 2 mL solution. “

*“2.8 How can other researchers check whether there is no unreacted TCO present in the immunoconjugate?”*

While there is no reliable way to check if there is any unreacted TCO present in the immunoconjugate mixture, it is extraordinarily unlike that the TCO-NHS moiety (MW = 267 Da) will elute from the size exclusion column along with the mAb-TCO construct (MW > 150,000 Da). Therefore, no modifications to the manuscript have been made in response to this query.

*“2.10 Apparently huA33 can be concentrated. Maybe good to mention that it can be troublesome for other mAbs and aggregation can occur. Other researchers should be aware of this problem. Not every antibody can be concentrated endless.”*

This is a good point. We have added the following passage to Step 2.10: “It is important to note that while huA33 and a variety of other well-known antibodies (*e.g.* bevacizumab, trastuzumab, cetuximab, and J591) are very tolerant of being concentrated, in other cases, aggregation and precipitation can occur upon concentration. Researchers attempting this procedure with a new antibody should trust the literature or their own knowledge of the antibody in question with regard to whether or not to concentrate the antibody.”

*“3.4 64Cu in ?? Please add details about the acid in which it is supplied. What is the maximum volume of 64Cu allowed? The pH is important for the radiolabeling, so there should be limitations on the volume and concentration of acid in which 64Cu is supplied.”*

This is a great point, and Reviewer #1 made a similar comment. We have added the following to the discussion of Step 3.4: “Typically, [64Cu]CuCl2 is supplied in a small volume (<30 μL) of 0.1 N HCl, and thus only small volumes (< 10 μL) of this stock solution are needed for the radiolabeling reaction. If larger volumes of the [64Cu]CuCl2 stock are needed, the radiolabeling reaction is tolerant of increasing the overall reaction volume. However, the pH of the radiolabeling reaction solution should be monitored carefully to ensure that it does not fall below pH 4.0.”

*“Line 212: A h is missing after 12.7”*

The “h” has been added in the manuscript.

*“3.6 see comments to 1.4 regarding details that are missing.”*

Again, we believe that the exact details regarding the retention times of the radiolabeled 64Cu-Tz-Bn-NOTA will be highly dependent on the HPLC set-up of each laboratory. We have added additional detail to Step 1.4 in response to the reviewer’s concerns. To Step 3.6, we have added that “... free, uncomplexed 64Cu will elute with the solvent front (TR ~ 2-4 min)”. We do not believe that the inclusion of additional details here will be particularly helpful to researchers trying to repeat this work. Step 3.6 now reads, “3.6) After 10 minutes of incubation, purify the product using reversed-phase C18 HPLC chromatography. The retention times and flow rates are obviously highly dependent on the HPLC equipment setup of each laboratory (pumps, columns, tubing, *etc.*). However, to present an example, if a gradient of 5:95 MeCN/H2O (both with 0.1% TFA) to 95:5 MeCN/H2O over 15 minutes is used, the retention time of 64Cu-Tz-Bn-NOTA should be around 9.7 min while free, uncomplexed 64Cu will elute with the solvent front at around 2-4 min.”

*“4.6 maybe good to add the amount of 64Cu-Tz-Bn-NOTA in nmol.”*

This is a great point. It is dependent on the specific activity of the 64Cu-Tz-Bn-NOTA, but we have adjusted Step 4.6 to read “4.6) Inject 200 μL of the 64Cu-Tz-Bn-NOTA radioligand solution (300 μCi; 11.1 MBq; 1.6 nmol of 64Cu-Tz-Bn-NOTA with specific activity of 6.7 MBq/nmol) into the tail vein of the xenograft-bearing mice.”

*“Line 261: the IEDDA can be tested using equimolar amounts of mAb and TCO. Can is the ideal concentration for this? This information is missing.”*

There is no “ideal” concentration for testing this reaction, as it works over a wide range of concentrations. However, we did omit example concentrations in this discussion. Therefore, we have added the following passage, “This is done by mixing the radiolabeled tetrazine (100 μCi; 0.55 nmol, assuming a specific activity of 6.7 MBq/nmol) with a slight excess of huA33-TCO (50 μg; 0.66 nmol).”

*“line 273: how many mice are used?*

We have now included this information in the following sentence: “Both acute biodistribution (n = 5 per time point) and PET imaging (n = 12) experiments reveal that the pretargeting strategy is capable of delineating the colorectal tumor growth with excellent image contrast and high tumor-to-background activity ratios (***Figure 6***).”

“*Additional Comments to Authors:* It would be interesting to get some information on how to determine whether a mAb can be used for IEDDA. Clearly it should be non-internalizing. But how should this be determined and what is an acceptable internalization rate?”

This is an excellent point. Unfortunately, however, because the technology is so new, neither us nor anyone else knows what the exact parameters for the antibody should be. As the technology develops, we are confident that we will determine these parameters, but currently, they do not exist, and therefore we cannot discuss them in the manuscript. We have, however, modified a sentence to confer this, “While the ideal kinetic parameters have yet to be determined, the antibody ⎯ and the reactive *trans*-cyclooctene it carries ⎯ must remain on the outside of the cell, for the internalization and sequestration of the antibody prior to the injection of the radioligand would dramatically decrease the number of *in vivo* click reactions.”

***Reviewer #3:***

“Page 9, 388 ff Tz-Bn-NOTA is not a radioligand, it is a precursor, please reword. Explain what biological half-life 6-8 hrs) means in this context; is it blood clearence (sic)? If yes, please discuss why the clearence is so slow, protein binding? Also discuss how to improve this; PEG spacer? glycation? This tetrazine-based radioligand does not appear to be suitable for clinical translation. Is the slow clearance (via feces) also due to circulating TCO-Mab?? Would a clearing agent be necessary?"”

These are excellent points. The “biological half-life” to which we refer is not blood clearance but rather clearance from the body as a whole. We agree that this tetrazine ligand is not ideally suited for clinical translation, and we are indeed working on developing novel tetrazines with faster clearance properties as the reviewer suggests. To answer the final question, the slow clearance (via the feces) is *independent* of the mAb-TCO conjugate and seems to simply be a property of the tetrazine radioligand. To address these concerns, this paragraph of the Discussion has been amended to read as follows:

“Finally, the pharmacokinetics of the tetrazine-based radioligand must be considered when choosing a suitable radioisotope. In the system described here, the radiolabeled Tz-Bn-NOTA moiety is excreted from the body via the gut with a biological half-life of approximately 3-4 hours, making 64Cu the positron-emitting radioisotope with the most complementary physical half-life. Unfortunately, the biological half-life of the tetrazine moiety is too long for it to be compatible with either the more rapidly decaying radiometal 68Ga (t1/2 = 68 min). In this case, any radioactivity in the tumor would decay through several half-lives before the excess radioligand finished clearing from the body. As a result, images would have to be acquired at early time points, when tumor-to-background activity ratios remain low.36 Ideally, future generations of tetrazine radioligands would be engineered ⎯ perhaps via PEGylation, glycation, or other means ⎯ to excrete from the body more quickly. This would allow for radiolabeling with more rapidly decaying radioisotopes such as 68Ga and 18F which would in turn further enhance the dosimetric benefits of the pretargeted imaging strategy. Ultimately, as researchers adapt this technology for use with other radioisotopes for imaging (*e.g.* 124I, 111In, 18F, 89Zr, 68Ga, etc.) or therapy (*e.g.* 89Y, 177Lu, 225Ac, 125I etc.), new tetrazine-based ligands will need to be developed to incorporate different chelators or radiolabeling prosthetic groups. The thorough investigation of the pharmacokinetics of these novel constructs will be essential to ensuring advantageous matches between the clearance properties of the ligands and the physical half-life of the radionuclides.”

“Typo: Line 83 "both are have" should be "both have"”

This typo has been corrected. Thank you for noticing it!

*“Page 7, line 300 why is too long an injection interval a problem, this antibody appears to stay on the cell surface for very long”*

This is a great point. Overly long interval times are less of a problem than overly short ones, but they still may lead to decreased tumor uptake. To explain this further, we have added the following sentences to the work, “Conversely, if too little antibody is used, the amount of uptake in the tumor will naturally suffer. Similarly, overly long interval times may also reduce levels of tumor uptake as a result of slow antibody internalization, transcyclooctene isomerization, or antibody/antigen shedding.”

*“100 ug A33-TCO per mouse means about 400 mg per human?? Any concern of an immune reaction? TCO conjugated to a slow clearing protein?? 10 MBq per mouse, what activity to patients? 40 GBq??, please discuss.”*

The reviewer makes an interesting point. However, it is important to note that this PET imaging system, like all radiopharmaceuticals, will not be scaled from mice to humans based simply on mass or surface area. Radiopharmaceuticals (*e.g.* 89Zr-DFO-mAbs) are typically tested in animals using 5 – 10 MBq. As the reviewer points out, when this is scaled to humans based only on mass/surface area, it results in 20 - 40 GBq. As it turns out, the typical dose for a human in the clinic is 10 mCi (370 MBq). In the case of this pretargeting system, the most likely human doses are 10 mg of A33-TCO and 10 mCi (370 MBq) of 64Cu-Tz-Bn-NOTA.

All that said, this protocol *does not* discuss any clinical matters and thus represents simply a guide to the preclinical approach. Therefore, we do not think discussing these translational matters is within the scope of the protocol, and we have not made any changes to the manuscript. If the editors believe it to be important to include this discussion

*“The legends to Fig.6, 7 need to be improved. What does the reader see?”*

Excellent point. The legends have been improved. They now read:

“**Figure 6:** PET images of 64Cu-Tz-Bn-NOTA/A33-TCO pretargeting strategy. Mice bearing subcutaneous SW1222 xenografts (100–150 mm3) were administered A33-TCO (100 μg) via tail vein injection. After 24 h, the same mice were administered 64Cu-Tz-Bn-NOTA (10.2–12.0 MBq [275–325 μCi]) via tail vein injection and subsequently imaged 2, 6, 12, and 18 h after the administration of the radiopharmaceutical. Transverse (top) and coronal (bottom) planar images intersect the center of the tumors. High levels of uptake in the gut at early time points (*i.e.* 2 and 6 h) largely clear by 12 h, leaving the tumor (white arrow) clearly delineated from all non-target tissue by 12 and 18 h post-injection. This figured is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthgonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013;54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.”

“**Figure 7:** PET images of suboptimal pretargeting experiments. (A) Mice bearing subcutaneous SW1222 xenografts (100–150 mm3, arrow) were administered A33-TCO (100 μg) via tail vein injection. After 12 h, the same mice were administered 64Cu-Tz-Bn-NOTA (10.2–12.0 MBq [275–325 μCi]) tail vein injection. (B) Mice bearing subcutaneous SW1222 xenografts (100–150 mm3, arrow) were administered A33-TCO (300 μg) via tail vein injection. After 24 h, the same mice were administered 64Cu-Tz-Bn-NOTA (10.2–12.0 MBq [275–325 mCi]) tail vein injection. In both cases, the mice were imaged 12 h after the injection of 64Cu-Tz-Bn-NOTA. In both panels, transverse (top) and coronal (bottom) planar images intersect the center of the tumors. While the pretargeting strategy clearly delineates the tumor in both cases, the results in both of these images are sub-standard compared to those displayed in Figure 6. In both cases, there is a significant amount of background activity uptake in the heart. Under the conditions of Figure 7A, this is most likely the result of the huA33-TCO construct not being given enough time to localize in the tumor. Under the conditions of Figure 7B, this is likely a consequence of injecting too much huA33-TCO and having excess immunoconjugate still circulating in the blood even 24 h after injection. This figured is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthgonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013;54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.”

*“Legend to Fig.6, 326, it is 275-325 microCi not mCi, see also line 335. The imaging time point is missing in the legends.”*

This typo has been corrected, and the imaging time points have been added to the legends. Thank you for noticing it!

*“Page 6, line 259, please give specific activity as MBq/nmoles not per µg. Without having the molecular weight it has little meaning.”*

As we discussed in the response to Reviewer #2, the specific activity values have now been changed to MBq/nmol.

“Page 7, line 265, what is a radioactive plate scanner; please be more precise, Phosphor imager?”

We were somewhat limiting in defining the radioactive plate scanner because we were not allowed to mention name brands (*i.e. Bioscan*). To clarify things somewhat, we have amended the text to read “radioactive TLC plate reader”.

In addition to all of these changes, we have made a few other minor changes to correct typographic errors found during our subsequent proof-reading of the manuscript. These changes have been noted in the revised manuscript file using “Track Changes”.

Thank you again, and please let me know if you require any more information.

Respectfully,



Brian M. Zeglis, Ph. D.