Cambridge Isotope Laboratories, Inc.
isotope.com

Isotope Days 2024- Andrew Hinck, PhD
Synthesis of ^{13}C-Methyl-Labeled Amino Acids and Their Incorporation into Proteins in Mammalian Cells

Kelly Andrade (00:00):

Good morning, everybody. We'll give people a minute or two just to start rolling in.

(00:15):

Good morning. Looks like we still have some people rolling in, so we'll give it another minute or two.

(00:46):

Alrighty. Good morning, everybody. Thanks for hopping on this morning. We're really excited for today's webinar. Before we get started, we want to take a moment just to recognize all of those in Florida, those that are affected by the hurricane, those that have passed away. Our hearts and prayers really go out to all of those that have been affected, their families, and we hope that everyone can remain safe and are able to recover quickly from the events that they've been having down in Florida.

(01:21):

I would like to welcome you all to the second session of a series of Isotope Days featuring cutting-edge science facilitated by stable isotopes. Today's session focuses on NMR and other related techniques and technologies. My name is Kelly Andrade, and I am going to be your host for today's event.

(01:40):

There's a couple goals we hope to achieve in today's webinar. I hope that we're all able to explore the cutting-edge applications of stable isotopes, learn something new from the scientists in the NMR field, the products that CIL offers and the role that we play in the research field, that you all are able to strengthen your understanding of these products in the area, and most importantly, that everyone's able to stay engaged and have fun throughout.

(02:07):

In case you missed it, we also had our mass spec portion of Isotope Day last week, and next Thursday, we will have the environmental session as well. Most of the recordings will be posted on our website along with today's session in the coming weeks.

(02:24):

Our agenda today is outlined here, and following this brief introduction we have a wonderful set of panelists today who are going to speak on a variety of different scientific applications, approaches, all utilizing stable isotopes and NMR, as well as other related techniques and how they use this all to help answer their research questions. Each talk is going to be about 15 minutes with three to five minutes of Q&A afterwards. Again, as I mentioned, most will be recorded and disseminated after the fact, and if you have any questions throughout the duration of the presentations, please utilize the Q&A box to submit your questions for the Q&A session.

(03:06):

Before we get started, we have a brief three-question poll, which will help us get an understanding of who is in the audience today, what your areas of research are, and the application interest that you have, so I'll give people about a minute to answer the survey that should be on your screen.

(03:48):

All right, I'll give another 20 seconds or so.

(04:15):

Another 10 seconds just to finalize the poll that you guys have on your screen.

(04:27):

All righty, so we can go ahead and close out the poll now. Without further ado, we can jump into our scientific talks. First up, we have Dr. Andrew Hinck, professor and deputy chair at University of Pittsburgh. Dr. Hinck received his Ph.D. from the University of Wisconsin-Madison, where he was trained as a biochemist in the lab of Dr. John Markley. In 1994, Dr. Hinck joined Dr. Dennis Torchia's lab at the NIH. There he collaborated with the Roberts and Sporn Group.

(05:06):

My apologies. There he collaborated with the Roberts and Sporn Group at the National Cancer Institute to determine the structure of TGF-B1, labeling it with 15N and 13C by expression in mammalian cells and utilizing NMR spectroscopy. In '97 he moved to the University of Texas where he began building up his lab and making inroads toward understanding molecular mechanisms of receptor complex assembly in the TGF-B superfamily.

(05:36):

In 2015, Dr. Hinck moved his lab to the University of Pittsburgh where he continues to apply NMR to study structure-function relationships of difficult-to-produce TGF-B family proteins produced in mammalian cells. His talk today is titled, "Synthesis of 13C-Methyl Labeled Amino Acids and Their Incorporation into Proteins in Mammalian Cells." Without further ado, Andy, the floor is yours.

Dr. Andrew Hinck (06:13):

Okay. Well, thanks, Kelly, for the invitation, and I'm excited to present the work we've done in terms of producing proteins in mammalian cells that are labeled with C-13. As Kelly mentioned in my bio, I actually have been trying to produce proteins in mammalian cells for NMR for some time, and I'm excited to tell you about these most recent developments.

(06:44):

Just a little bit of background, I think most people in the audience know that uniform labeling with N-15 and C-13 and full assignment of the proton, carbon, and nitrogen signals has been highly impactful for studying proteins at the molecular level using NMR, particularly very strong for studying smaller sized proteins. You can go up to about 25 kilodaltons with just N-15, C-13 labeling. Although beyond that, it begins to become challenging due to rapid T2 relaxation that limits the application to larger proteins.

(07:28):

You can extend that by deutering the proteins and using sensitivity enhancement approaches to improve the line widths and extend the application of these methods. Here on the right side of the slide,

I show this for a 40 kilodalton receptor of the TGF-B family that we studied. This protein is deuterated and N-15 labeled, and we're using Trosy techniques. We get a pretty nicely resolved proton-carbon correlation spectra, and pretty good sensitivity on the spectra to enable assignments across the peptide bond. Although you can see for the relevant cross peaks that give the correlations to enable the assignments, they're starting to get a bit weak at this molecular size, even with deuteration and even with sensitivity enhancement. While, again, this technique's been very powerful, it does have limitations in terms of the sizes of proteins to which it can be applied.

(08:38):

Another limitation of the, I would say, aside from molecular weight limitations of uniform labeling with N-15 and carbon is that it can only be applied to proteins that can be labeled that way so uniformly, so that largely relegates this to being used for proteins that can be expressed in either bacteria or yeast.

(09:07):

Now, to extend the methodology, Lewis Kaye's group made some really important advances in this area almost 25 years ago where they showed that if you look at the sidechain methyls, you can observe NMR signals in quite a bit larger proteins with pretty good sensitivity and resolution. This is due to the fact that the sidechain methyls have favorable T2 relaxation properties due to picosecond rotation around the methyl bond axis.

(09:46):

You also have increased signal intensity due to the chemical equivalence of the methyl protons. You have insensitivity to pH, which for a lot of larger, more complicated proteins is necessary. A lot of proteins tend to be more stable in the pH seven to eight range, so you don't have problems with loss of signal due to solvent exchange. Most importantly, I would say is Lewis' lab, but then other labs, Jerome Boisbouvier's lab in Grenoble, develop methods for robust labeling of methyl groups in proteins based on incorporation of simple, C-13-based, metabolic precursors such as alpha-ketoisovalerate to give labeled methyls of valine and leucine or alpha-ketobutyrate to give methyl labeling at the delta position of isoleucine.

(10:43):

Boisbouvier's group showed that if you go a few steps earlier in the metabolic pathway and label with C-12 acetolactate, you can also get labeling of valine and leucine. Although, one advantage of doing it that way is that the labeling can be stereospecific.

(11:00):

All these approaches are very powerful, I would say, well-developed, and really extensively applied and, I think, really has transformed applications of NMR to larger and more complex proteins than could be studied with just carbon and nitrogen labeling alone based on E. coli expression.

(11:24):

But the issue is that while the NMR technology is pretty well-developed, it's unfortunately, really only applicable to proteins that can be expressed in native form in bacteria. On the other hand, a lot of larger

human proteins such as highly disulfide-bonded receptors, which is a big focus in my lab, or membrane proteins such as GPCRs, they cannot be produced in a natively-folded form in bacteria.

(11:57):

You can think about using yeast to make some of these proteins, and that certainly is possible for some of these, but I would say not all. For example, a lot of the GPCRs, while there's been a few examples of successfully producing homogeneous GPCRs for structural work in yeast, the number of examples are actually pretty limited, and actually most GPCRs cannot be readily made using yeast expression.

(12:28):

There's also some disadvantages of the methyl labeling in yeast. The labeling efficiency is not as high and the extent of labeling that you can get compared to bacteria is not as high. Now, in terms of going to ... If you want to think about studying some larger, more complicated proteins and neither bacteria nor yeast is possible, you can start to think about using insect cells or mammalian cells. These cells are the cell types that normally produce these complicated proteins, and they have a lot of chaperones. They have sophisticated secretion systems. They're really made to produce these proteins in a native form.

(13:19):

The challenge though, from an NMR perspective is you cannot simply use metabolic precursors in insect cells or mammalian cells for producing methyl-labeled proteins because those cells lack the requisite biosynthetic machinery. So, you have to think about other approaches if you want to do methyl labeling for proteins produced in insect cells or mammalian cells, and one attractive approach is using direct incorporation of C-13 labeled amino acids. One of the challenges with that, I would say, is the lack of low-cost synthetic routes to the common methyl-bearing amino acids substituted with carbon-13 at the methyl position, so that's where we were trying to make some impact.

(14:16):

What we did was we collaborated with two chemists here at the University of Pittsburgh, Matteo Borgini and his advisor Peter Wipf, and Peter and Matteo developed a scheme that was based on to enable synthesis of two so far, C-13-methyl labeled amino acids very efficiently and at pretty low cost as I'll explain in a moment. Basically, I have done some chemical synthesis, but I am not a synthetic chemist, so this was really the work of Matteo and Peter.

(14:54):

Basically, what they did is they basically start with alanine, L-alanine. They add a protecting group on the N-terminus and a, what they call, a directing group on the C-terminus, and then they use that to then essentially build up the sidechain. The C-13 that's going to give rise to the C-13-methyl is introduced in the form of C-13-iodomethane, which is a relatively low-cost reagent.

(15:34):

Basically, using this approach, they were able to synthesize isoleucine, which notably has two chiral centers, one at the alpha position, but one at the beta position as well, so this is a stereospecific

synthesis. The synthesis is possible in terms of achieving high stereoselectivity at the beta position due to this directing group that's attached on the C-terminus. That basically coordinates metal, and that leads to high stereospecificity for addition of the methyl group at the beta position. Then, too, once the compound's been synthesized, you have to be careful about the deprotection to not erode the stereospecificity.

(16:19):

In any case, this amino acid, C-13-isoleucine labeled at the gamma-2 position was successfully synthesized in good yield and, again, at low cost. Peter and Matteo also used a similar approach to synthesize C-13-valine that was labeled at both of gamma-1 and gamma-2 positions. Again, this was done very efficiently and also with low cost. This is a less challenging synthesis because, in this case, there's just the one stereocenter at the alpha position.

(17:00):

These amino acids were then incorporated into mammalian, incorporated into some of the receptors that we study in my lab. These are relatively not very large. They're only about 20kDa, but they do have multiple disulfide bonds. You can express these in E. coli, but it's actually difficult to refold them and recover natively-folded receptor. They have either three or four disulfide bonds.

(17:32):

We used two of them as model systems, one called BGZPC and another one called R3like. We produced the proteins in suspension-cultured, HEK293 Freestyle cells, and we use Hyclone Trans Fx medium, so this is commercial medium without amino acids. Then, we add to that all of the amino acids that we don't want to label, an unlabeled form, and then along with the synthesized amino acid with the C-13-methyl label.

(18:06):

The medium that we reconstructed using this procedure is not quite as good as full commercial medium for these HEK293 Freestyle cells, but yield is about half of full commercial medium for these cells. It's not perfect medium for protein production on these cells, but it does work reasonably well.

(18:34):

In this table here, it shows them the amounts of the methyl iodide that we used in our syntheses and the amount of corresponding amino acid that we're able to obtain. With about two grams, either one or two grams of C-13-methyl iodide, which is relatively inexpensive, two or $300, you can get between about 100 and 200 milligrams of the corresponding amino acid. Then with those amounts of amino acids that's shown here below, you can produce, using relatively small culture volumes, on the order of a milligram of protein.

(19:11):

With just two or $300 worth of methyl iodide, you can get to about a milligram of protein. We're looking at the methyl signals, which is very sensitive. These amounts of protein are actually enough for seven or eight NMR samples, so it's really only about $30 or so per NMR sample. It's actually pretty low- cost.

(19:39):

That's how we produce the proteins, and then we, of course, examine them to look at the labeling in terms of its efficiency and whether it's what we expect for the amino acids that were incorporated. This is for BGZPC. This protein has five methionines and 13 valines. You can see it in the amino acid sequence here, and this is exactly what we see in the spectrum.

(20:06):

This is the isoleucine methionine labeled spectrum. We see the five isoleucine signals. I'm sorry, the six isoleucine signals and the five methionine. These are the 13 valine signals. In this case, there's 26 signals because it was both gamma-1 and gamma-2 labeled, and again, the five methionine signals. So this looks really exactly as expected. The signals are exactly where we expect in terms of their chemical shifts, and then there's the exact number of signals that we expect.

(20:35):

Then for R3like, it also looked good, so we made an isoleucine methionine labeled sample, and we see the four expected isoleucine signals in the one methionine.

(20:51):

We wanted to know what was the labeling efficiency. Are these amino acids being incorporated with high efficiency? So, what we did was we recorded the spectrum of the same receptor, but with natural abundance C-13, and then we compared the signal intensities of either the valine or the isoleucine relative to methionine. Methionine is known to be very well incorporated with 100% efficiency. By comparing these ratios with the protein produced in HEK293 cells, or in the natural abundance sample, we can determine whether the incorporation efficiency was high or not. As far as we can tell, statistically, it looks like the amino acids are being incorporated with near 100% efficiency.

(21:42):

We also wanted to know about the line widths. The one thing about this that you need to keep in mind is that the protein that we're producing in mammalian cells is not deuterated, so we incorporate the C-13-methyl labeled amino acid, but it's not possible to deuterate to any significant extent the mammalian cells. The cells, unlike bacteria, the mammalian cells are not as tolerant to high D2O concentrations in the medium.

(22:11):

So, we actually expected the line widths of these methyl signals labeled in this way to be broader than you would get from normal isoleucine, leucine, valine labeling from metabolic precursors for protein produced in bacteria with background deuteration. What we did was then compare the line widths in our mammalian-produced methyl signals of valine versus the same signals but in a sample that we produced and managed to refold not very well. That's why the spectrum looks a little scraggly, but we were able to produce a small amount of native protein by bacterial expression and refolding and purification.

(22:53):

We compared the valine methyl carbon line widths in this ILV, bacterially-produced, deuterated sample relative to the same methyl signals in the mammalian, and here's the plot of the carbon line widths. You can see that relative to valine or relative to methionine in the HEK293 mammalian-produced protein where there's no deuteration, the valine line widths are indeed larger than the methionine line widths. Whereas in the E. coli protein where we have full background deuteration, you can see the valine line widths are more comparable to the methionine line widths.

(23:32):

So, while incorporation of C-13 labeled amino acids using mammalian expression systems seems to be very high. The line width, however, is not as narrow, what you can achieve with methyl labeling in bacteria with background deuteration, so that is one limitation of the method.

(23:55):

These are my conclusions now. We've developed an efficient synthetic route to C-13-methyl labeled amino acids, at least valine gamma-1, gamma-2 labeled and gamma-2 labeled isoleucine. We show high incorporation efficiency using mammalian cell expression. We see little to no scrambling, and we can produce these samples in quite a cost-efficient manner. It's pretty simple to do this actually. It's actually not technically that challenging, and we think that this can enable studies of larger, more complex human proteins than would be possible using just either bacterial expression or yeast expression.

(24:44):

I did point out the limitation of the method in terms of the broader line widths due to the lack of background deuteration. I think that's an area for future investigation. I think one possible approach there is to modify the synthesis to install deuterons at positions directly adjacent to the C-13 to minimize dipolar relaxation. Those nearby protons are going to be the ones that most strongly drive relaxation, and replacing deuterons should reduce that.

(25:22):

I should say, my group is not the only group doing this. Haribabu Arthanar at Harvard has also been playing around with these types of approaches, and they've also made similar observations to us and are also working to do exactly what I just described in terms of installing the deuterons to improve the line widths.

(25:43):

That's what I have for my presentation. Thank you for your attention. I just want to acknowledge very quickly the people that did the work. This work is primarily done by a postdoc in my lab, Lukasz Wieteska, and Cynthia Hinck, my wife who helped with a lot of the protein expression and purification. And these are chemists who we collaborated with, Matteo Borgini and Peter Wipf from the chemistry department at the University of Pittsburgh. Thank you again. Happy to take any questions.

Kelly Andrade (26:16):

Isotope Days 2024- Andrew Hinck, PhD
Synthesis of ^{13}C-Methyl-Labeled Amino Acids
and Their Incorporation into Proteins in Mammalian Cells

Wonderful presentation, Andy. Thank you so much. It's very exciting to see the work of methyl labeling being worked on in mammalian cells. Unfortunately, we don't have time to take the answers live for the Q&A, but we do have them in the question and A box. If you're able to type the answers in the chat, that would be greatly appreciated.

Dr. Andrew Hinck (26:38):

Okay, thank you.