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Developing Treatment, Treatment Validation, and Treatment Scope in the Setting of an Autism Clinical Trial

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14. ABSTRACT

The first year of the project has been used to obtain IRB approval from the UMDNJ-RWJMS IRB office. We obtained IRB approval on December 7th, 2009. Our approved protocol and supporting documents have been submitted to the Human Research Protection Office (HRPO) Office of Research Protections (ORP) of the DOD for review. We are also in the process of applying for a Certificate of Confidentiality from the NIH as per our IRB requirements. No Human Subjects Work will be done until we receive either a COC or a letter indicating we do not need one. Once we have approval from the Human Research Protection Office (HRPO) Office of Research Protections (ORP) of the DOD we will continue work on Tasks 2 -3. Work on Tasks 4 – 5 will not begin until subject recruitment and treatment has occurred. Please see initiating project W81XWH-08-1-0728 and partnering projecW81XWH-08-1-0730.

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Introduction:

This project is to test to see if DHA treatment can beneficially affect excretion of urinary biomarkers of oxidative stress and the autism clinical phenotype. In addition polymorphic variants of genes of certain enzymes that synthesize and metabolize docosahexaenoic acid (DHA) may contribute to the phenotype of some autism cases. We will test to see if any of these genes are risk factors for autism. We will also measure changes in excretion of the polyunsaturated fatty acid (PUFA) derived biomarkers of oxidative stress (isoprostanes and neuroprostanes) together with the changes in production of anti-inflammatory lipid mediators. We will test these biomarkers to see if we can monitor and validate effectiveness of DHA therapy. We will also test the genotypes of key DHA-metabolizing enzymes can predict which patients will respond to therapy Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730.

Body:

PROJECT #2: PI T.P. STEIN, PhD, PARTNERING PI, W81XWH-08-1-0729

Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730.

Unless otherwise stated, tasks are divided between the synthetic core (directed by Dr. B.W. Spur) and the Analytical core (directed by Dr. T.P. Stein).

Task #1 Obtain IRB approval (Drs. Stein and Spur).

The first year of the project has been used to obtain IRB approval from the UMDNJ-RWJMS IRB office. The major points in the timeline follow. In the course of this timeline numerous meetings took place with the IRB office, the Chairpersons of the three PI's of this project, and the Research Dean in order to facilitate the process. In addition we consulted with a number of persons with expertise in the field.

Our initial submission to our IRB office was submitted on July 18th 2008. We received a de-briefing memo from our IRB on August 4th 2008 with twenty one suggestions and recommendations.

We responded to this memo on September 15th 2008. The IRB requested clarification to one of our responses. This clarification was sent in on November 5th, 2008. Our IRB offices were moved in the month of October 2008 and their review was not completed until November.

We received a "Notice of Approval with Stipulations" from the IRB on November 26th, 2008 and we received the stipulations themselves on December 5th, 2008. We responded to all four stipulations and sent the responses to the IRB on December 22nd, 2008. An expedited review was scheduled for January 9th, 2009. The reviewer decided that our response to the first stipulation (related to simplification of the language in the consent form) should be reviewed by a full committee. The committee met on January 30th, 2009 and we received the memo on February 6th, 2009. At this meeting our four responses were tabled, and 24 suggestions /

recommended changes were sent to us, most of them completely new. One of these changes requested was that we obtain an IND for the use of DHA.

We responded to this by sending our IRB copies of documentation to support that an IND was not needed because 1.) an FDA letter dated May 17, 2001 to Martek (manufacturers of the DHA to be used) designated their DHA as "Generally Regarded as Safe" (GRAS) (please see http://www.cfsan.fda.gov/~rdb/opa-g041.html) and 2.) documentation from the FDA on their website www.clinicaltrials.gov (search for MARTEK and DHA) shows that none of the 10 current or completed studies that used Martek's DHA had had an IND including one with subjects with autism (showing that our use was not a new indication). We responded to the other 23 new questions and submitted all this for review on February 27th, 2009.

We received the de-briefing memo on March 6th, 2009. The memo requested that we needed to get an IND from the FDA for the project and it contained 2 new additional requirements. First, our IRB wanted us to create a tissue bank for the storage of the samples. Second our IRB wanted us to apply for a Certificate of Confidentiality for this project before they would give full approval.

We convened a meeting with the Chair of the IRB, the IRB director and the PI's on May 8th, 2009 to discuss each of their requirements.

The key outcomes of the meeting and our responses were as follows;
First, even though it is already considered General Regarded as Safe (GRAS) in children, we would nonetheless need either an IND for the use of Martek's DHA or a letter from the FDA saying that one was not needed. We completed the application for the IND and we submitted it on July 16th, 2009 (available upon request, 298 pages). We received a letter from the FDA August 4th, 2009 exempting us from needing an IND.
Second, even though to our knowledge there is no federal or state rule or regulation requiring us to create a tissue bank and there was no university policy in place, a Tissue Bank Application, Protocol and Manual along with supporting documents would have to be submitted for this project. Upon receipt of the March 6th memo asking us to set up a tissue bank we decided to take two parallel tracks. The first was to write a Tissue Bank Application. The second was to present to the IRB that we would destroy the samples after the termination of the project, and if at that time there was future scientific use for the samples and a tissue bank was available we would amend the protocol and place the samples in a bank. We submitted a Tissue Bank application on May 24th 2009. We have since completely re-written the Tissue Bank Application, Protocol, Manual and supporting documents and are preparing to re-submit.

Third, our IRB told us that we must apply for a Certificate of Confidentiality (COC) before they would give us full approval. Prior IRB approval is a NIH requirement for submitting a COC application.

Additional submissions, responses and amendments.
The following dates represent requests for changes or requests for clarifications (to either the consent form, assent form, protocol, application or other supporting documentation) and subsequent replies by us;

Memo from the IRB	May 29, 2009	Replied to on July 10th, 2009
Memo from the IRB	September 1, 2009	Replied to on September 9th, 2009
Memo from the IRB	September 30th, 2009	Replied to on October 19, 2009
Memo from the IRB	November 20th, 2009	Replied to on December 5th, 2009

In addition there were 3 modifications to the protocol, all related to changes in study personnel between the time the grant was submitted and the time the study was approved.

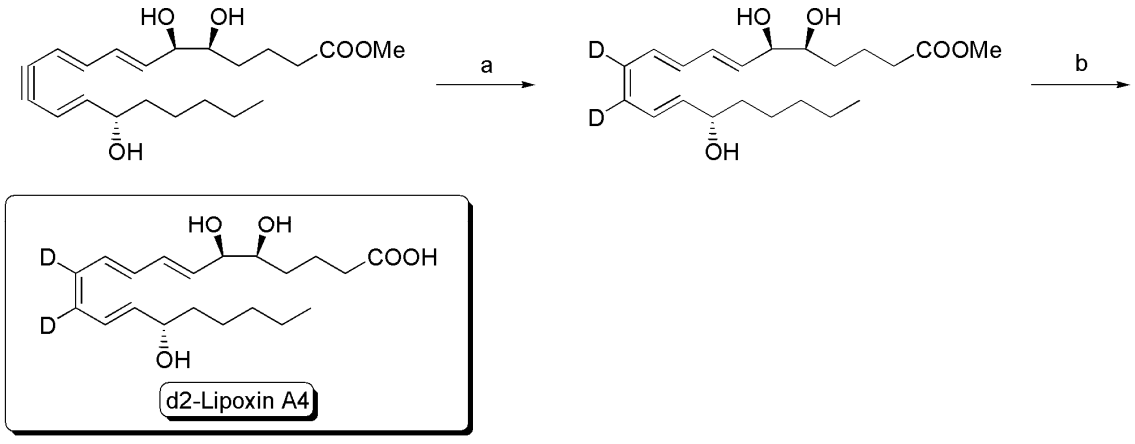
We obtained IRB approval on December 7th 2009. Two important points. First our IRB office accepted the FDA's letter stating that we do not need an IND. Second, our IRB office also accepted our proposal to destroy the samples at the end of the project. We are continuing with the Tissue Bank application and once approved we will amend the protocol for this project to allow us to keep the samples by placing them into the Tissue Bank.

Our approved protocol and supporting documents have been submitted to the Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD for review. We are also in the process of applying for a Certificate of Confidentiality from the NIH as per our IRB requirements. No work will be done on the recruited subjects until we receive either a COC or a letter indicating we do not need one and we have approval from the Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD.

Task #2. Task #2 pertains to the synthetic core (Dr. Spur). To chemically synthesize the protective lipid metabolites: lipoxin A4, 15-hydroxy-eicosatetraenoic acid (pre-cursor of lipoxin A4), resolvin E2, 17-hydroxy-docosahexaenoic acid (precursor of D series resolvins) with and without a deuterium label.

SYNTHESIS OF di-deutero-Lipoxin A4

Dr. Spur is responsible for step (1), synt hesis of resolvins wit h and without deuterium labels to be used for methods development. Since the last progress report, Dr. Spur has completed the synthesis of di-deutero-Lipoxin A4. An outline of the synthesis is described below.

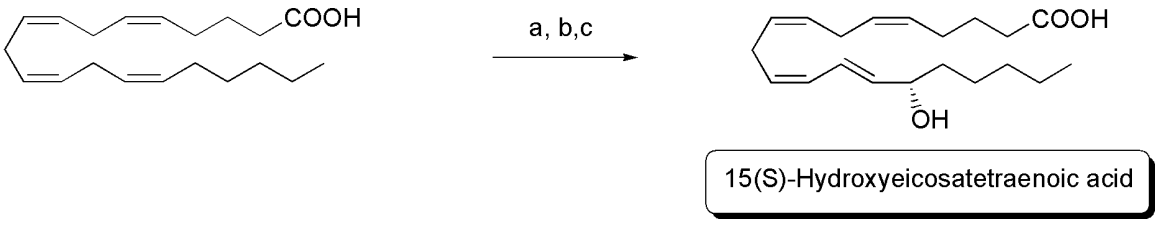


Reagents and conditions: (a) Zn(Cu/Ag), d4-MeOH, D_2O, 40 °C; (b) 1N LiOH, H_2O, THF, 0 °C, then H^+ (NaH_2PO_4 saturated), 0 °C.

The synthesis of the di-deuter o-Lipoxin A4 was achieved star ting from the triple bound precursor as outlined above. The triple bond precursor was synthesized from 2-deoxy-d-ribose to obtain the chiral centers in 5 and 6 positions. The chiral center in the 15-position uses S-octynol as the starting materi al. Reduction of the triple bond precursor with D_2O in d4 methanol with the freshly pr epared Zn/Cu/Ag alloy gave di-deutero Lipoxin A4 methyl ester. Mild ester cl eavage with 1N Lithium hydroxide in Water/ Tetrahydrofuran at 0 °C followed by acidification with a saturated solution of NaH_2PO_4 in the presence of Ethyl acetat e gave the required d2-Lipoxin A4. (Characterized by H-NMR, C-NMR, UV, MS)

Notable aspects of the synthesis – which will have relevance to the synthesis of other deuterated analogs, were: The mild introduc tion of deuterium has been achieved avoiding by products. Palladium (Lindla r) over-reduction and isomerization has been successfully eliminated. The method became now a standard procedure that allows cis specific reduction of conjugated en-yne systems that is import ant in the total syntheses of many natural products including the resolvins and analogs. In the same way Hydrogen and Tritium labeling can be perfo rmed avoiding Hydrogen and Tritium gas. The stability of the final product compar ed with the classical methods showed a dramatic increase at room temperature. Other aspects of our synthesis include the chiral reduction with the Noyori transfer ca talysts in water (green chemistry with high enantiomeric excess (ee). Sodium or ammonium formates are the reducing agents.

SYNTHESIS OF 15(S)-Hydroxyeicosatetraenoic-acid [15(S)-HETE]

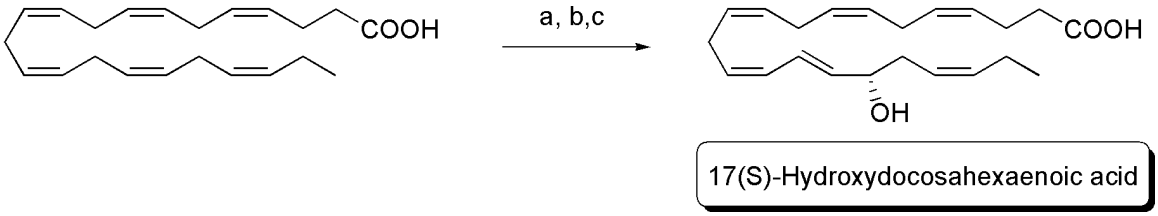


Reagents and conditions: (a) lipoxygenase (soybean type I), 0.1 M borate buffer (pH 9), O_2, 0°C; (b) Ph_3P, Et_2O, 0°C; (c) flash chromatography.

The synthesis of the Lipoxin precursor 15(S)-Hydroxyeicosatetraenoic acid was achieved in 3 steps starting from Arachi donic acid as outlined above. We have developed a new protocol that overcomes some of the limit ations using the enzymatic reactions. The literature describes this reaction using high dilution and only small amounts can be obtained. An im provement described the use of high pressure oxygen for this enzymatic reaction to obtain lar ger quantities. We used in step a commercial lipoxygenase from soybeans and introduced ox ygen gas through a fritted glass to disperse the gas very effici ently in the reaction at 0 °C. Under these conditions the reaction produces in a concentrated solution t he hydroperoxide very efficiently on large scale. The hydroperoxide is reduced with tr iphenyl phosphine in ether to produce 15(S)-

HETE directly. Purification by flas h chromatography gave the pure product. (Characterized by H-NMR, C-NMR, UV, MS, IR, optical rotation, chiral hplc)

SYNTHESIS OF 17(S)-Hdroxydocosahexaenoic-acid [17(S)-HoDHA]



Reagents and conditions: (a) lipoxygenase (soybean type I, 0.1 M borate buffer (pH 9), O_2, 0°C; (b) Ph_3P, Et_2O, 0°C; (c) flash chromatography.

The synthesis of the Resolvin D precur sor 17(S)-Hydroxydocosahexaenoic acid was achieved in 3 steps similar as described for the synthesis of 15(S)-HETE starting from Docosahexaenoic acid as outlined above. Using the same protocol gram quantities of 17(S)-HoDHA were easily available, The product was characterized by H-NMR, C-NMR, UV, MS, IR, optical rotation and chiral hplc.

Task #3 pertains to the analytic core (Dr. T.P. Stein). Part (i) is the development of isotope dilution LC-MSMS assays for lipoxin A4, 15-hydroxy-eicosatetraenoic acid (precursor of lipoxin A4), resolvin E1, 17-hydroxy-docosahexaenoic acid (precursor of D series resolvins) and Resolvins D1, D2, D4, D5 and D6 with and without a deuterium label. Part (ii) is setting up LC-MSMS methods to extend our published isotope dilution gc-ms assays for the isoprostane metabolites in urine in this population.

Dr. Stein is responsible for the analytical aspec ts. Please note that actual work on this project was precluded by the lack of IRB approv al. Any work done was done as part of the new equipment installation program using funds provided by the university and other grants.

Task 3- (i)

As pointed out in our previ ous progress reports there has been a major change in how steps 2 and 3 will be executed. We origina lly proposed we intended to do all of the mass spectrometric analyses by using an ol d Agilent 5970 quadrupole gc-ms. This is technically difficult but we do have the exper tise to accomplish this task. All other investigators in the fi eld use the more modern and more expensive liquid chromatograph-mass spec/mass spec methods . The University of Medicine and Dentistry of New Jersey has very generous ly given us the funds to purchase LC-MS/MS. This generous offer will have a major im pact on this project. Specifically there will be no question about us being able to do the a ssays necessary for this project. This project involves the analysis of compounds for which there are some existing assays

and others that need to be developed. For the existing assays we will no longer have to use as the starting point published LC-MS/MS method and try and adapt it to our older and less sensitive gc-ms instrument, we will only have to reproduce a published LC-MSMS method. For the compounds where there is currently no useable published assay, we will now have a state of the art instrument to develop the methodology. By upgrading our capabilities to LC-MS/MS removes the risk for some of the proposed analyses being beyond the range of our present instrumentation.

The instrument has been delivered, installed and is operating. It is located in Dr. Stein's laboratory. Dr. Stein and his technician's have been familiarizing themselves with the LC-MSMS and are now reasonably confident that they can start on using this instrument to accomplish their part of the overall project (sample analyses). As reported earlier, Masoodi published a paper describing a generalized methodology for detecting some of the compounds of interest in biological fluids (1). Our appraisal of this paper was that it could form the basis of developing a generalized LC-MSMS method for the simultaneous measurement of all of the resolvins, protectins and leukotrienes required for this project.

Most importantly they have published a more recent specific step by step description of how to proceed. We are in the process of verifying their methodology using a combination of purchased standards and samples already available from Dr. Spur (2). Our work is still very much in the preliminary phases, we have focused on understanding the capabilities of the instrument and examining the performance of the available standards.

Three important pieces of information have been identified. Firstly, the published LC conditions are not optimal for the compounds of interest to us. The resolvins D1 and E1 elute from the column too early for there to be any effective separation. To correct for this we have increased water content of the water/acetonitrile mobile phase and increased the run time to 45 minutes. Secondly, fragmentation and collision energies for optimal multiple reaction mechanisms (MSM) peaks vary with instruments; Rodriguez et al. used a Thermo-Fisher instrument where collision energies and fragmentation voltages tend to run significantly higher than with our Agilent instrument (1, 2). We are in the process of compiling reference libraries for when we actually analyze study urine for samples of interest. Thirdly, we have taken a preliminary look at what can be detected in urine. Without isotope dilution, most of the compounds are undetectable in urine. We are very fortunate that we have as a team member an expert in synthesizing deuterium labeled PUFA metabolites.

Task 3- (ii)

The other major change in the proposed analyses is with the isoprostane analyses. Isoprostanes are products of PUFA oxidation and are excreted in the urine. A very recent paper by Song, Rokach et al recommended focusing on the isoprostane metabolites are 8,12 iso-iPF3 α-VI and 5-epi-8,12 iso-iF3 α-VI (3) rather than 2,3 dinor-5,6 dihydro-PGF2t and iPF4α-VI as originally proposed. We will make this modification.

Dr. J. Rokach has very kindly provided us with these two metabolites plus their deuterium labeled analogs so we can incorporate them into our methods development. It needs to be emphasized that the most difficulty part of the analytical core program is the development and establishment of suitable methodologies . Once the methods are in place, the actual analyses should proceed smoothly.

Task 4
Use the newly developed assays to measure the new markers in the urine: lipoxin A4, 15-hydroxy-eicosatetraenoic acid, resolvin E, 17-hydroxy-docosahexaenoic acid, resolvins of the D series, including neuroprotectin. We anticipate 66 (placebo-treated) and 66 (DHA-treated) subjects; initially two urines pre-treatment and two at the end of the treatment phase will be analyzed (n=528) for each metabolite. This task will be started at the beginning of year 2 and continue until the end of year 3. We anticipate having to do multiple injections because while the sample preparation and derivitization will be common, gc conditions for resolution may well be different. We aim to do the analyses in not more than three batches (n=528 per batch), resolving several but not all of the compounds in each run (Stein, Leskiw).

This task will not begin until subject recruitment, enrollment and treatment have been completed.

Task 5
Data will be collected and analyzed (6-36 months, S Buyske).

This task will not begin until subject recruitment, enrollment, treatment and analysis have been completed.

Task 6
Manuscripts prepared and submitted for publication (03 year, all investigators)

This task will not begin until subject data analysis has been completed.

Key Research Accomplishments

There have been two important research accomplishments to date: (i) the synthesis of di-deutero-Lipoxin A4 and (ii) the synthesis of the Lipoxin precursor 15(S)-Hydroxyeicosa-tetraenoic acid.

Reportable Outcomes:

There are no reportable outcomes at this time.

Conclusion:

A large amount of time was spent on getting IRB approval for this project. We received a conditional IRB approval on December 7th 2009. The condition is to apply to NIH for a Certificate Of Confidentiality (COC). We have written a COC and plan on submitting by the end of the week. We have also, submitted the approved protocol and supporting documents to the Human Research Protection Office (HRPO) Office of Research Protections (ORP) of the DOD for review. If the Human Research Protection Office (HRPO) Office of Research Protections (ORP) of the DOD requires no changes we will start recruiting subjects as soon as we have a COC. If changes must be made to the Protocol or supporting documents we will amend our protocol with our IRB and upon acceptance of the amendment by our IRB and receipt of the COC we will begin recruiting subjects. (Please see partnering project W81XWH-08-1-0730).

Now that we have an IRB approved protocol we plan on moving forward on a number of fronts. Firstly, Dr. Spur will complete the necessary syntheses (task #2). It is our hope that these will be completed within 18 months. This includes making the corresponding deuterated analogs. Most of these are new and original syntheses, but we believe our timeline for the syntheses to be realistic. Secondly by the end of year 1 Dr. Stein will be using the newly synthesized compounds to adapt mostly published methods for LC-MSMS analysis. We aim to complete methods development between months 12 to 18 of this project. Once the methods are in place it should be a relatively simple project to run the patient samples. Our intent, like those of the LC-MSMS papers we are following is to do all of the required analyses in a single run. This is a tremendous improvement over the GC-MS method proposed where the plan was to do multiple injections of a purified urine sample in order to collect the data for a single urine sample.

In summary, paradoxically the delay in obtaining IRB approval has served us very well. Had we started on time last year we would have been locked into to trying to modify state of the art analytical methodology (LC-MSMS based) to our older GC-MS methodology with no guarantee of success. Thanks to the generosity of the Dean of UMDNJ-SOM we now have state of the art instrumentation and this greatly increases the probability of success for this project.

References:

1. Masoodi M, Mir AA, Petasis NA, Serhan CN, and Nicolaou A. Simultaneous lipidomic analysis of three families of bi oactive lipid mediators leukotrienes, resolvins, protectins and relat ed hydroxy-fatty acids by liquid chromatography/electrospray ioni sation tandem mass spectrometry. *Rapid Commun Mass Spectrom* 22: 75-83, 2008.
2. Nicolaou A, Masoodi M, and Mir AA. Lipidomic Analysis of prostanoids by Liquid chromatography/electrospray ioni sation tandem mass spectrometry. *Methods Mol Biol* 579: 271-286, 2009.
3. Song WL, Paschos G, Fries S, Reilly MP, Yu Y, Rokach J, Chang CT, Patel P, Lawson JA, and Fitzgerald GA. Novel eicosapentaenoic acid-derived F3-

isoprostanes as biomarkers of lipid peroxidation. *J Biol Chem* 284: 23636-23643, 2009.

Appendices:

There are no appendices.