NCL News

Nanotechnology Characterization Laboratory

Accelerating the transition of concepts to clinical applications

Volume 1, Issue 2

NCL Facilitates Product Development:

A Case Study in Nanotechnology Characterization

A number of companies developing nanotechnology products to detect and treat cancer are making use of NCI's Nanotechnology Characterization Laboratory (NCL) to help them characterize the physical and biological properties of their products, and to conduct a number of tests in preparation to submit an investigational new drug (IND) or investigative device exemption (IDE) application. The NCL assists in the evaluation of nanomaterial product quality and safety with resources and expertise that many small companies lack.

The following article describes the NCL's evaluation of nanoparticles for Dendritic Nanotechnologies (DNT; recently acquired by StarPharma Holdings Ltd. of Australia). The product that DNT submitted to the NCL for characterization was a polyamidoamine (PAMAM) dendrimer/contrast agent complex. The complex would potentially enable DNT to create imaging agents that could better target tumors simply by modifying the surface of the dendrimer. (See side bar for more information on dendrimers).

Getting Started

The NCL accepts proposals for the characterization of nanomaterials from academia, industry and government. Product sponsors are encouraged to contact the NCL once a cancer therapeutic, diagnostic or image contrast agent using nanotechnology is formulated and initial biological in vitro or in vivo studies indicate efficacy.

The first step in initiating a collaboration with the NCL is submission of an application in the form of a three to four page white paper describing the product concept along with initial characterization and efficacy data. Within 45 days after submitting the white paper, the NCL will inform the sponsor as to whether it will advance the application to the next stage – a phase II proposal. The phase II proposal is a ~10 page document that expands upon the concepts presented in the white paper and includes additional criteria for evaluation, such as assessment of the inherent toxicity of the nanomaterial, and its amenability to scale-up.

Following the selection of DNT's application by the NCL, the collaboration began with an intense download of information from DNT on what they knew about their product. "It's difficult to become fluent with a concept just by reading a proposal or a white paper, so we had several interactions with DNT via teleconference to make sure that we understood the nuances of their strategy. We became very familiar with their

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In This Issue:

Dendrimers

At Dow Chemical in the 1980s, DNT founder Don Tomalia, Ph.D., invented a new type of branching polymer that grew in tiny spherical structures he termed "dendritic polymers" or dendimers. Today, there are over 100 compositional families of dendrimers with unique physical and chemical properties. Each class of dendrimers can either attach or encapsulate other chemical groups and materials, yielding a number of products with potentially useful medical applications.

At least two dendrimer-based products are on the market, including an *in vitro* diagnostic kit made by Dade Behring and another developed by Qiagen that is used as a vector for DNA transfection in genetic engineering applications. But there are no dendrimer products on the market for internal use by humans. "The big challenge is getting dendrimers well enough characterized to take them through the next steps … and to define the safety and the risk margins well enough so that one can begin to start thinking about human clinical trials," said Dr. Tomalia. ■

View The NCL technical report on the DNT dendrimers at: http://ncl.cancer.gov/120406.pdf

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preliminary data in order to leverage what they had already done," said Dr. Scott McNeil, Director of the NCL.

Nanoparticle characterization began with a limited number of assays performed by the NCL for prescreening, to confirm the identity of the material sent by DNT, and to make sure that the material was not contaminated. The dendrimer material was then moved into a concurrent set of tests for physical and *in vitro* characterization using the NCL's assay cascade. Parameters measured included size, size distribution and stability, while *in vitro* studies looked at biocompatibility and cytotoxicity.

Getting to the bottom of certain issues, whether it's product stability or assay interference, requires a true collaboration. According to Dr. McNeil, "The NCL doesn't simply take a sponsor's material and approach them a year later and say 'Here's your data.' It's collaborative in that if we find something that we don't understand, we can engage them and ask: 'Have you seen this before? Is it an effect of assay conditions? Is it an effect that's unique to that nanoparticle platform?'"

NCL and DNT scientists interacted repeatedly to analyze the data and ensure that the data were interpreted correctly. "We really felt like we were in each other's laboratories and we could see each other's faces, see their data and ask questions," said Don Tomalia, Ph.D., founder of DNT.

Making Use of the Findings

Findings from the characterization studies may enable a sponsor to initiate an IND or IDE application or may indicate the need to re-engineer the submitted nanomaterals in order to address any issues that were revealed. Data generated during characterization are used by the NCL to continually evolve a set of assay protocols for use with nanomaterials.

The NCL researchers were hard pressed to find any toxic effects from the dendrimers, even at high doses. "We found it to be fairly benign. Under our *in vitro* assays we didn't see any adverse effects as far as immunotoxicity or cytotoxicity were concerned," remarked Dr. McNeil. These results suggested that DNT had a technology platform with a good safety profile, and a sound basis for the development of derivative products.

The second discovery was, at first glance, not as exciting. Apparently, the FDA-approved contrast agent was leaking out from the dendrimer interior, and the rate of exiting could affect the product's efficacy as a "blood pool" agent. (Blood pool agents or intravascular contrast agents remain in the blood for a prolonged time compared with conventional contrast agents which diffuse quickly into the interstitial space.) Previously, DNT had conducted their stability evaluations in water, but the NCL assays were conducted in a medium that simulated human serum and measured the effect at a level of precision that DNT had not been able to achieve on their own. This evaluation revealed some flaws, but that kind of knowledge is necessary, particularly when it is revealed early in the development of a product. "It [the results] told us that we are probably going to have to go one more step to make sure it [the imaging agent] stays associated with the dendrimer before we inject it into a human patient," said Dr.Tomalia. "Serum contains a lot of electrolytes which can change the ionic character of the dendrimer interior and affect its ability to associate with the contrast agent," he said. "We have some ideas that we are looking at to slow down the exiting or to keep it totally intact within the interior." In fact, DNT won a fast track Small Business Innovation Research (SBIR) grant from the NIH in September to improve the efficacy of such related dendrimer conjugates.

The NCL drew some important lessons from the collaboration as well. NCL found that the dendrimers could interfere with standard assays for bacterial contamination, and that the design of the assay had to be modified to account for this interference. Interference, whether inhibiting or enhancing signals, is likely to be a recurring theme as the NCL establishes a standard set of assays for characterization. "The DNT material was one of the first nanoparticles that we received formally and that helped calibrate all of our instrumentation and all of our assays," said Dr. McNeil. "We found a large number of those assays were very sensitive to the nanomaterials, which could interfere with the analysis. It gave us a huge understanding and appreciation for how nanomaterials are different from small molecules in normal drug development."

Benefits to the Sponsor Company

Collaboration with the NCL provides a more thorough understanding of the physical characteristics and safety profile of the submitted nanomaterial at no cost to the sponsor.

Collaboration with the NCL provided DNT with several clear benefits. First and foremost was a more precise knowledge of the physical characteristics of their product, some of which led to timely reconsideration of its design to improve performance. "That gave us a lot of confidence back in the synthesis lab and with the manufacturing people. It gave us a lot of information about the safety and physical characteristics of these materials

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that we really didn't have before," said Dr. Tomalia.

DNT also sees working with the NCL as a way to gain access to knowledge and expertise that would have been prohibitively expensive if they sought characterization services from a private contractor. "Being a small startup company as we are, this is an unbelievable resource. If we had to have it done [by a CRO] it would have been a very expensive proposition. We probably could not have done it," said Dr. Tomalia. "If we [the NCL] can help a company avoid spending that money or at least ensure that they place their bets on concepts that are more likely to be winners, I think that is appreciated by the company. The tangential outcome of this particular project with DNT is that we were able to help them understand that their platform is certainly a viable solution for other concepts in that it is benign, while the specific formulation might have to be redesigned for efficacy," said Dr. McNeil.

Benefits to the Nanotechnology Community

Part of NCL's mission is to use submitted material as an opportunity to develop and refine protocols for nanotechnology characterization and then disseminate those protocols to the entire research community.

Dr. McNeil notes that "the NCL has two customers. One is the sponsor that gives us the material, but the other customer is the nano-biotechnology community as a whole. We want to make sure that we are contributing to national research, not just research that is unique to one specific nanotechnology strategy, or one company. The intended audience are researchers who can use the data and protocols to help further their particular concepts into clinical development."

The NCL makes its findings publicly available to the nano-biotechnology community in the form of a formal report that's very similar to the one submitted to DNT or any other sponsor. Dr. McNeil reassures potential sponsors that, prior to publication of the final NCL report, "we give the company a preview of the report and ask them to identify items that might be proprietary or items that they may not want to be publicly disclosed." The NCL removes proprietary information and then publishes the report on its website (see the DNT report at: http://ncl.cancer.gov/120406.pdf.) The NCL does not release proprietary company information.

The report also provides other researchers in the nanotechnology community with an opportunity to interact with the sponsor. In the case of DNT, this interaction is particularly important because the multifunctional nature of its platform technology means someone else may have a better targeting, therapeutic or imaging agent that DNT might want to incorporate into its dendrimer nanoparticle.

What's Next?

DNT plans to advance its PAMAM dendrimer platform to enclose oncology drugs like cisplatinum or methotrexate, as a way to deliver potentially toxic cancer treatments safely to tumors without exposing healthy cells. They will also seek ways to ensure these drugs and imaging agents remain associated, perhaps combining them in a single particle with a targeting agent to provide a signal that reveals exactly where the therapy is going, including to remote metastasized tumors. The company has also submitted another nanoparticle with a very different chemical architecture, called Priostars, for preliminary analysis and characterization by the NCL. Dr. Tomalia's overall impression of DNT's collaboration with the NCL is positive: "We are very excited about this and feel we really advanced quickly. We could never have done it on our own or moved this far this fast." ■

NCL Connections

The NCL - FDA Interface

Nanotechnology is unquestionably on the move. Government R&D investment is expanding, with funded research programs in the U.S., Japan and the EU each receiving about $1 billion. Industry investment in nanotechnology R&D has matched these numbers and will very soon exceed them. Applications already exist in medicine and consumer products, including drugs, medical implants, sunscreens, cosmetics, and more. But along with the increasing prevalence of nanotechnology in our lives, there has come a pressing need to regulate these products for safety.

The U.S. Food and Drug Administration (FDA; http://www.fda.gov/) has a great interest in developing mechanisms for regulating products incorporating nanotechnology. Currently, these products are reviewed through conventional regulatory channels, based on their properties and intended use. But how does the FDA review and regulate a "multifunctional" nanoparticle that contains an imaging agent, a therapeutic agent, and a targeting agent combined? If the properties of the particle are highly dependent on its size and surface characteristics, what sort of quality

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control is needed to ensure consistency in manufacturing? These are questions that are likely to come up frequently as the FDA grapples with this new type of product. For its part, the NCI Nanotechnology Characterization Laboratory (NCL) is working with the FDA to find answers to these questions.

Scott McNeil, Director of the NCL, emphasizes that "NCL is not directly involved in the regulatory process. Instead, we generate rigorous preclinical characterization data that informs the regulatory process." In other words, the NCL is helping to increase the base of knowledge at the FDA so they can make more informed decisions on nanotech-based products.

Regulation of any drug or device used in humans requires a comprehensive knowledge of its properties and how those properties might affect the product's safety and efficacy. Conventional drugs are evaluated for their chemical composition, their solubility, and their stability. But therapeutics using nanotechnology may need special instrumentation to evaluate these and other properties. Anil Patri, Ph.D., Senior Scientist, NCL, provides an interesting example: "A regulator might require information on the size of the particle, which would have to be very well defined, if there were a strong correlation between its size and its therapeutic or toxic effects. Everyone involved has to agree what is meant when one asks: What is the size?" Sound like a simple question? Think again. Dr. Patri tells us, "Size can be defined in 20 different ways at the nanometer scale," and small differences in measurement can have big consequences. A two nanometer increase in size, for example, is enough to change the way nanoparticles are cleared from the body: from the kidneys to the liver.

Size can be measured using scattering methods such as photon correlation spectroscopy, multiangle light scattering, small angle x-ray scattering and small angle neutron scattering. The NCL can use all of these methods to define size, as well as microscopy techniques such as atomic force microscopy and scanning electron microscopy. Each method provides some information about the particles' size, and size distribution. To make a product with consistent, predictable behavior, there must be consensus not only on the acceptable range of sizes, but also on how those sizes are measured.

The NCL is an attractive partner for FDA because it serves as a clearinghouse of sorts for a wide variety of nanoparticles, including quantum dots, liposomes, dendrimers, fullerenes, and gold nanoparticles. With all of this material submitted by laboratories and manufacturers across the country, the NCL gains a better understanding of how to get reliable answers to important questions that regulators will want to know. For example, how does a surface charge influence toxicity, clearance, and biocompatibility? "That's the type of information in which the FDA is very interested. We are helping them understand trends in compatibility and toxicity; the data that's generated will help inform reviewers as to what to monitor in IND applications," said Dr. McNeil.

The interaction between NCL and the FDA goes beyond the exchange of data. "We also interface with the FDA at the programmatic level. Multiple FDA staff members sit on our advisory board, they participate in our technical reviews, they help us in shaping where the NCL is heading," said Dr. McNeil. "Yet another level of collaboration between NCI and FDA exists on the nanotechnology subcommittee under the auspices of the Interagency Oncology Task Force (IOTF)," said Dr. Wendy Sanhai, Senior Scientific Advisor, Office of the Commissioner, FDA and FDA chair of this subcommittee. "It is clear that priorities under FDA's Critical Path Initiative and NCI's Nano Alliance are aligned. FDA is determined and committed to working with NCI and multiple stakeholders to clinically translate potential lead compounds and medical products from preclinical, through clinical development, manufacture, FDA submission and product launch. (All FDA Critical Path reports are available at http://www.fda.gov/oc/initiatives/criticalpath/. See also the FDA Follow-Up Report listing more than 40 projects FDA initiated or helped initiate in 2006). "We feel that tools such as the Exploratory IND guidance will be quite helpful in facilitating this process. There are some good research tools and tests available and multiple efforts are underway to develop more of these as well as standards in this evolving field. However, it is also clear that no one entity can do all this. The key is to effectively leverage expertise and resources from multiple stakeholders —share the risks and share the benefits.".

So while the wave of nanotechnology advances, the NCL and the FDA are working together to put the evaluation of nanotechnology products on a firm and rational scientific foundation.

NCI Alliance for Nanotechnology in Cancer

The NCL is part of the NCI Alliance for Nanotechnology in Cancer. For more information on the Alliance, please visit http://nano.cancer.gov

NCL Protocols

Kidney and Liver Cytotoxicity Assays

Failure to detect toxicity in preclinical studies contributes to drug candidate failure during clinical phase testing. It is estimated that twenty percent of all drug candidate failures result from unacceptable levels of toxicity[1]. *In vitro* cytotoxicity assays are generally used as an initial evaluation of a material's biocompatibility, in the selection of a drug candidate from a series of molecules and to explore toxic mechanisms. *In vitro* assays can also assist in identification of potential toxicities *in vivo*. Specialized target organ cell lines, such as kidney and hepatocyte, are used in *in vitro* screening programs for this purpose.

A number of methods have been developed to study cell viability in the presence of drug candidates. As part of their assay cascade, the NCL has adapted two standard cytotoxicity test methods for evaluation of nanomaterials. The test methods, described below, are applied to both Hep G2 human hepatocarcinoma and LLC-PK1 porcine proximal tubule cells. As a further assessment of potential cytotoxicity, NCL also measures the degree of caspase-3 activation in the presence of nanomaterials. The caspase-3 activation assay, a measure of caspase-dependent apoptosis, assists in identification of the type of cytotoxicity present – necrotic, apoptotic, or both.

Measure of cell viability

The reduction of tetrazolium salts is frequently used as a measure of cell viability. The yellow tetrazolium salt (MTT) is reduced by mitochondrial enzymes in metabolically active, intact cells to form a purple, water-insoluble formazan salt. Cells are then lysed and formazan crystals solubilized with detergent with the resulting color quantified spectrophotometrically at 570 nm. The amount of color produced is directly proportional to the number of viable, intact cells.

Measure of cytolysis

Cytotoxicity can be evaluated by quantification of plasma membrane damage through measurement of cytoplasmic enzyme activity released by damaged cells. The amount of enzyme activity detected in culture supernatant correlates to the proportion of lysed cells. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme present in all cells and is rapidly released into culture supernatant upon damage to the plasma membrane. NAD+ is reduced to NADH/H+ by the LDH-catalyzed oxidation of lactate to pyruvate. H/H+ is then transferred from NADH/H+ to the tetrazolium salt INT which is reduced to formazan. The amount of enzyme activity in the supernatant directly correlates with the amount of formazan formed. ■

Kidney and Liver Cytotoxicity Assays

Assay	*Measure of cell viability*	*Measure of cytolysis*
Mechanism	Reduction of yellow tetrazolium dye MTT to formazan by intracellular reductase enzymes in metabolically active cells.	Cytoplasmic lactate dehydrogenase, released upon cell lysis, catalyzes conversion of lactate to pyruvate, reducing NAD+ to NADH/H+. H/H+ is then transferred from NADH/H+ to the tetrazolium salt INT which is reduced to formazan.
Result	Amount of formazan produced is directly proportional to the number of viable, intact cells.	Amount of enzyme activity detected correlates with proportion of dead or plasma membrane-damaged cells.

Find the NCL protocols for analysis of cytotoxicity (NCL Methods GTA-1 and GTA-2)) at http://ncl.cancer.gov/NCL_Method_GTA-1.pdf and http://ncl.cancer.gov/NCL_Method_GTA-2.pdf.

[1] *Chemical and Engineering News. Improving efficiency. June 19, 2006.*

FAQs

Q: How does the NCL determine if an engineered nanoparticle is likely to be safe?

A: In short, the NCL examines the weight of the evidence from preclinical studies, looking for "consensus behavior". A recently published study[1] demonstrated interference of carbon nanotubes with the MTT assay (see "NCL Protocols" in this issue of NCL News for a discussion of the MTT assay). The study showed that carbon nanotubes have the ability to absorb formazan dye, giving the appearance of a reduction in cell viability. This phenomenon

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Upcoming Conferences

Conferences in which NCL staff will participate:

Toxicology Forum - Nanomaterials: Life Sciences and Regulatory Aspects
Location: Westin Embassy Row Hotel, Washington, D.C.
Dates: January 30 – February 2, 2007
Website: http://www.toxforum.org/html/winter_meeting.html

Informatics Needs for Nanomaterials Workshop
Location: Oak Ridge, Tennessee
Dates: February 8-9, 2007
Website: http://www.ornl.gov/adm/tted/

Materials Research Society Spring Meeting
Location: Moscone West / San Francisco Marriott, San Francisco, CA
Dates: April 9-13, 2007
Website: www.mrs.org/meetings

March 1, 2007: Next due date for submission of proposals to the NCL.

FAQs

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helped to explain the lack of consensus behavior of carbon nanotubes in the *in vitro* toxicology literature where groups were using different cytotoxicity endpoints and arriving at contrasting conclusions regarding carbon nanotube cytotoxicity.

Even if the previous toxic responses attributed to carbon nanotubes *in vitro* were the result of MTT assay interference, this does not mean that carbon nanotubes are necessarily safe, as often *in vitro* results are not predictive of *in vivo* responses due to the complexities inherent in the whole organisms (i.e. exposure-dose-response relationships, cell-cell interactions, etc.). As such, it is important to look at the total body of evidence.

[1] *Oops, they did it again! Carbon nanotubes hoax scientists in viability assays. Nano Letters 2006 June; 6(6):1261-8.*

Q. Is the NCL relationship with the nanomaterial sponsor considered to be a collaboration? How much control does the nanomaterial sponsor have during the characterization process?

A. The characterization of nanoparticles performed by the NCL is considered a collaboration between the NCL staff and the nanomaterial sponsor. Prior to the initiation of a project, the NCL staff and project sponsors engage in multiple interactions via teleconference, email and face-to-face meetings. Once the project begins, the sponsor receives an update at least once per quarter on the physical characterization, immunology and toxicology data generated from the NCL assay cascade.

The NCL and project sponsors jointly work through issues that might arise. For example, it is not uncommon for a nanoparticle formulation to have impurities in the first batches submitted to the NCL. In these situations, the NCL staff work with project sponsors to identify any contaminants; the NCL may then recommend methods to further purify the preparation.

Q. What are the deliverables provided to the sponsor by the NCL?

A. Updates and data reports on the progress of the nanomaterial characterization are provided to the sponsor at regular intervals – at least once per quarter. Once characterization of the nanomaterial is complete, a formal report of data from the NCL assay cascade is provided to the sponsor. Nanoparticle characterization data presented in the report are peer-reviewed by the NCL Scientific Oversight Committee (SOC) consisting of experts from the supporting organizations (i.e., NCI, NIST, and FDA). Once data are disclosed to, and reviewed by the sponsor, the report is eligible for public release in ninety days. The report is made public via the NCL website and portions of the report may be submitted to scientific journals for publication. Proprietary sponsor information is not included in the publicly-available report.

Q. How do you protect the sponsor's proprietary information, yet make data available to the public?

A. The exchange of confidential information and materials between the provider and NCL is protected by the terms of the Material Transfer Agreement (MTA) (http://ncl.cancer.gov/working_application-process_annex2.pdf). All confidential/proprietary information (that is not otherwise publicly available) disclosed to the NCL is strictly protected. Data generated by the NCL and its partners (i.e. NIST and FDA) may be made publicly available 90 days after the NCL releases the results to the sponsor. This delay is intended to allow the sponsor time to further secure its IP position prior to public disclosure.

Q. Will the NCL evaluate a company's nanoparticles against other nanoparticles? If so, do you publish the results?

A: The NCL may test multiple nanoparticles from the same family (e.g., dendrimers with different surface charges) to assess whether the various nanoparticles share similar biocompatibility or toxicity. The NCL conducts structure-activity relationship (SAR) studies aimed at understanding how the physicochemical attributes of nanoparticles (such as size, surface characteristics, architecture, composition, and polydispersity) influence biocompatibility. The goal of SAR studies is to examine trends by comparing various types of particles. If a comparison of various nanoparticles from different sponsors provides a significant contribution to the field of nanotechnology, and is of interest to the broad scientific community, such results would be published in collaboration with the contributing sponsor(s).

Q. What happens if the NCL determines nanoparticles are toxic or cannot reproduce a sponsor's results? Does the NCL publish unfavorable results?

A: The NCL works in close collaboration with sponsors to address any safety/efficacy issues or inconsistencies. Development of a sponsor's nanotech strategy is an iterative process and the NCL will attempt to offer suggestions as to how to further optimize a sponsor's nanoparticle. The NCL will then generally continue characterization on the improved "batch" of nanoparticles. If the scope of improvements to a nanoparticle's formulation is drastic, such as a change in the targeting modality, the NCL would require the sponsor to reapply for evaluation. The new material would need to demonstrate efficacy and would require the characterization to start from scratch.

The NCL is tasked with making its data available to the public – even "unfavorable" results are of value to the nanotech community. ■