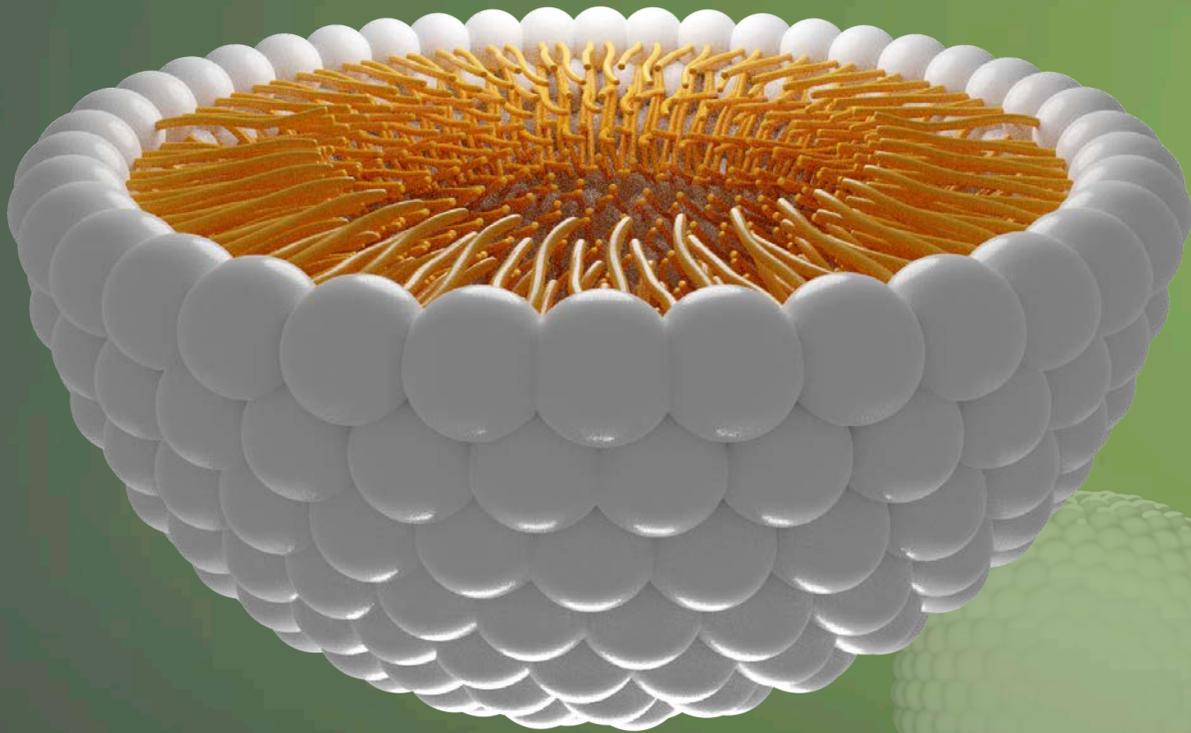




Pharmacology and Toxicology Characterization of Nanomedicines



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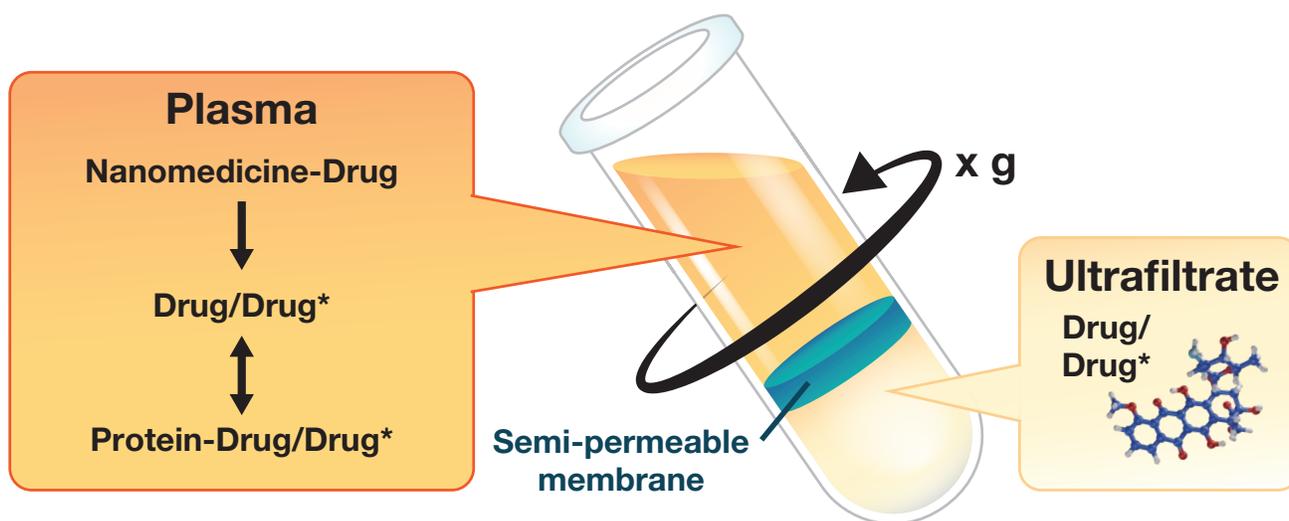


PHARMACOLOGY AND TOXICOLOGY CHARACTERIZATION OF NANOMEDICINES AT THE NCL

A thorough understanding of nanomedicine pharmacology and toxicology is essential to identify liabilities and optimize drug formulation. For example, without optimal platform stability, drug can be released too quickly or too slowly, resulting in off-target toxicities or poor efficacy. Similarly, toxicity can be dose limiting resulting in the inability to reach therapeutic drug concentrations.

Pharmacology and toxicology properties are frequently not identified until in vivo preclinical studies are performed later in the development process. There are, however, several predictive in vitro assays that can be extremely informative for development of nanomedicines, such as drug release in physiologically relevant matrices, cytotoxicity in cell lines relevant to a specific indication, and assays to evaluate mechanisms of toxicity. NCL provides 12 in vitro assays for nanomaterial pharmacology and toxicology evaluation on NCL's website (<https://ncl.cancer.gov/resources/assay-cascade-protocols>).

In addition to these in vitro studies, the NCL also provides in vivo pharmacokinetic and toxicity studies of nanomaterials submitted to the NCL's Assay Cascade characterization service. In vivo studies are conducted in collaboration with the Frederick National Laboratory's Laboratory Animal Sciences Program, <https://ncifrederick.cancer.gov/Lasp/Default.aspx>, which includes the Molecular Histopathology Laboratory and Small Animal Imaging Program, among others.. All in vivo studies are individually tailored for each tested nanoparticle and are collaboratively agreed upon with the developer before testing begins; in vivo studies are designed to mimic the intended clinical dose (adjusted for species), dosing regimen, and route of administration.



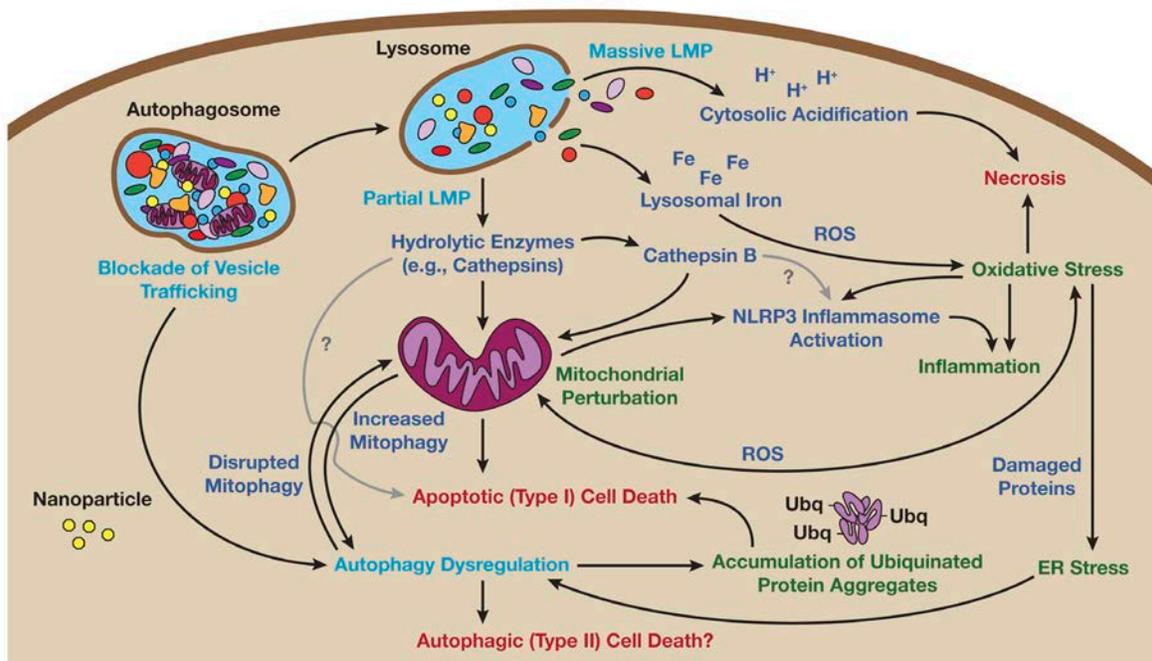
Stable Isotope Tracer Ultrafiltration Assay (SITUA). NCL developed a novel drug fractionation method to evaluate drug release in physiological matrix. (Reproduced with permission from *ACS Pharmacol Transl Sci*, **2020**, 3(3), 547–558.)

IN VITRO TOXICOLOGY

The NCL has about 10 different in vitro assays available to assess mechanisms of toxicity, including general cytotoxicity, apoptosis, oxidative stress and autophagy.

The cell viability and membrane integrity assays use the traditional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) reagents. We typically will conduct these assays in porcine renal proximal tubule (LLC-PK1) cells, human hepatocarcinoma (Hep G2) cells, as well as cell lines specific to a developer's indication. The LLC-PK1 and Hep G2 cell lines are good at detecting toxicity stemming from residual surfactants or other excipients in the formulation, and the NCL has a lot of historical data using these two cell lines.

In addition to characterizing the cytotoxic dose-response relationship of nanomedicines, the NCL offers in vitro assays to identify the underlying mechanisms of toxicity. While the combination of MTT- and LDH-assays, and time course studies, may point to primarily membrane or cellular mechanisms of action, the mechanistic assays can further elucidate mechanisms underlying toxicity. These mechanistic assays are designed to identify apoptotic, oxidative and lysosomal/autophagy dependent mechanisms. These primary areas of focus are the result of over a decade and half of research into the primary pathways involved in nanomedicine toxicity.



Toxic mechanisms of autophagy and lysosomal dysfunction. (Reproduced with permission from *Part Fibre Toxicol*, 2012, 9(1), 20.)

In Vitro Toxicity Assays

Cytotoxicity

- MTT & LDH
- Caspase 3, 3/7 activation

Oxidative Stress

- Glutathione
- Lipid peroxidation
- Reactive oxygen species

Autophagy

- Autophagic dysfunction
- MAP LC3I to LC3II conversion

Relevant NCL Publications

Sharma et al, *Methods in Molecular Biology*, 2018, Vol. 1628, p. 135-147. PMID: 29039099
Adisheshaiah et al, *Methods in Molecular Biology*, 2018, Vol. 1628, p. 211-219. PMID: 29039105
Stern et al, *Part Fibre Toxicol*, 2012, 9(1), 20. PMID: 22697169

IN VIVO TOXICOLOGY

NCL performs non-GLP animal studies in rodents to determine ADME (absorption, distribution, metabolism and excretion) and toxicity profiles. These toxicology studies can be used to provide preliminary identification of target organs of acute and repeat-dose toxicity, and may aid in the selection of starting doses for preclinical GLP and Phase I human trials. Although NCL's studies are non-GLP in nature, according to recent ICH guidelines, a non-GLP single-dose acute toxicity study may be utilized directly in an IND/IDE filing with the US FDA, in conjunction with a GLP repeat-dose acute toxicity study. Similarly, ADME studies are not required to be GLP, and NCL drug metabolism and pharmacokinetic studies are routinely included in IND and NDA filings.

In vivo ADME-toxicity studies are tailored for each nanoparticle, in collaboration with the developer, and, where feasible and appropriate, follow the intended clinical dosing regimen, including dose, schedule, and route of administration. Studies include a complete panel of hematology and clinical chemistry parameters as outlined below. Further, necropsy includes a comprehensive histopathological analysis of more than 40 tissues conducted by a board-certified veterinary pathologist.

Tissues Evaluated by Histopathology

- adrenal
- brain
- cecum
- colon
- duodenum
- epididymis
- esophagus
- eye
- femoral marrow
- femur
- gall bladder
- Harderian gland
- heart
- ileum
- jejunum
- kidney
- liver
- lung
- mammary gland
- mandibular lymph node
- mesenteric lymph node
- nasal sections
- ovary
- pancreas
- parathyroid
- pituitary
- prostate
- rectum
- salivary gland
- seminal vesicle
- skin/subcutis
- spinal cord
- spleen
- stomach
- testis
- thymus
- thyroid
- tongue
- trachea
- urinary bladder
- uterus
- vertebra
- any other tissues with gross findings at necropsy

Hematology Parameters

- differential leukocyte count (BA, EO, LY, MO, and NE)
- erythrocyte count (RBC)
- hematocrit (HCT)
- hemoglobin (Hb)
- mean corpuscular hemoglobin (MCH)
- mean corpuscular hemoglobin concentration (MCHC)
- mean corpuscular volume (MCV)
- mean platelet volume (MPV)
- platelet count (PLT)
- red blood cell distribution width (RDW)
- total leukocyte count (WBC)

Clinical Chemistry Parameters

- alanine aminotransferase (ALT)
- albumin (A)
- albumin/globulin ratio (A/G)
- alkaline phosphatase (ALP)
- amylase
- blood urea nitrogen (BUN)
- creatinine
- calcium
- globulin (G)
- glucose
- phosphate
- potassium
- sodium
- total bilirubin
- total protein count (WBC)

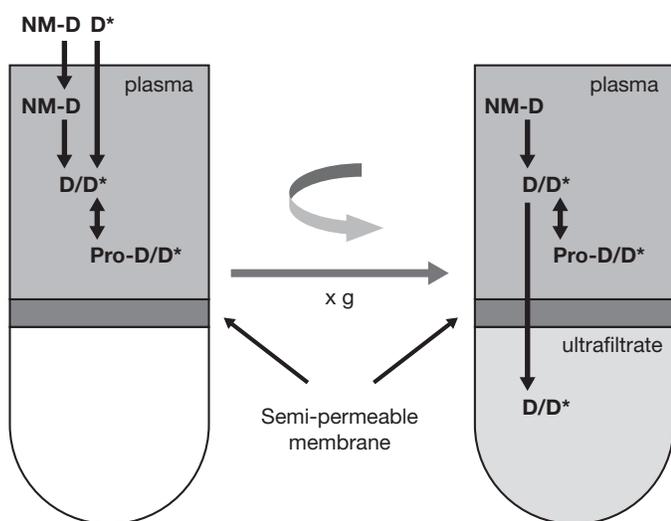
The Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals (Health Research Extension Act of 1985, Public Law 99-158, 1986). Animal care is provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996; National Academy Press, Washington, D.C.). All animal protocols are approved by the FNLCR institutional Animal Care and Use Committee.

IN VITRO PHARMACOLOGY

NCL has two methods to assess the in vitro drug release properties of a formulation. The first is a stable isotope tracer ultrafiltration assay (SITUA) used to quantitate drug release from the formulation in a biological matrix, i.e., plasma. This method is highly sensitive and requires no specialized labeling of the formulation. The primary requirement for the assay is a stable isotopically labeled version of the drug, which is often commercially available for many common chemotherapeutics, or can be custom synthesized at modest expense (NCL typically incurs this cost). A simple centrifugal ultrafiltration step coupled with highly sensitive mass spectrometry allows for the quantitation of nanoparticle-bound drug, free, unbound drug, and protein-bound drug fractions. NCL has tested more than 50 different formulations using this assay and has found good correlation to in vivo pharmacokinetic behavior. The references highlighted below offer more details on the SITUA method.

The second in vitro drug release assay available on NCL's website is a blood partitioning assay. The drawback to this assay, in comparison to the SITUA, is that it appears to be more sensitive to the types of formulations being tested, whereas the SITUA is more broadly applicable. The protocol as presented on the website also requires a dual radioactive labelled drug and nanoparticle platform. Although, it can be adapted to non-radioactive materials provided suitable analytical techniques are available.

In conjunction with this work, the NCL develops and optimizes bioanalytical techniques suitable for analysis of each unique formulation, including novel fractionation techniques, LC-MS methods, and more. As needed, NCL will also assist developers in transferring these methods to appropriate CROs where GLP capabilities are needed.



Depiction of the stable isotope tracer ultrafiltration assay (SITUA). Stable isotopically labeled tracer drug (D^*) is spiked into plasma containing the nanomedicine (NM-D). D^* is assumed to behave identically to normoisotopic drug (D) with regard to both formulation and protein binding ($\text{Pro-D}/D^*$). After reaching binding equilibrium, the sample is transferred to an ultrafiltration device and the filtrate is separated by centrifugation. The ultrafilterable fraction, which contains unbound drug fraction, is then used to calculate protein bound, unencapsulated, and encapsulated drug fractions. (Reproduced with permission from Skoczen et al, *J Control Release*, **2015**, 220(Pt A), 169–174.)

Relevant NCL Publications

Skoczen & Stern, *Methods in Molecular Biology*, **2018**, Vol. 1628, p. 223-239. PMID: 29039106

Stern et al, *Drug Metab Dispos*, **2016**, 44(12), 1934-1939. PMID: 27670412

Skoczen et al, *J Control Release*, **2015**, 220(Pt A), 169-174. PMID: 26596375

Ambardekar & Stern, in Non-Biological Complex Drugs, *The Science and the Regulatory Landscape*, **2015**, 261-287. ISBN: 978-3-319-16240-9

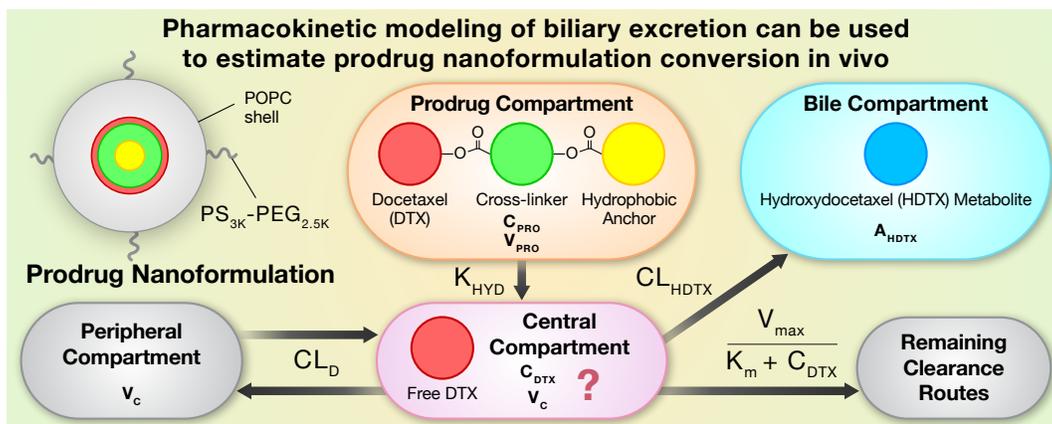
IN VIVO PHARMACOLOGY

NCL ADME and pharmacokinetic (PK) studies track the various components of a nanoparticle formulation in blood and tissues and aid determination of tissue and systemic exposure, the routes and rates of clearance, and systemic half-life. In comparison to toxicity studies, PK studies are generally quicker and cheaper to conduct. Because of this, PK studies can often be used to quickly assess the viability of the formulation before delving into more costly or time expansive studies, identifying formulations that need optimization or those that may not be viable.

NCL conducts single and repeat dose PK studies, tissue distribution studies and bioequivalence comparisons. Through the NIH Pharmacy, the NCL has access to most commercial chemotherapeutics in use today and can include these comparator arms in all studies. NCL designs each study to mimic the intended clinical treatment cycle as best as possible, including treatment dose, schedule and route of administration, and all study designs are collaboratively agreed upon before commencing any study. Pharmacokinetic parameters are calculated using the most current versions of traditional pharmacokinetic software packages such as Phoenix WinNonlin. NCL also offers the capability to perform customized pharmacokinetic modeling.

PK studies are often complemented with analysis using orthogonal techniques such as ICP-MS analysis for metallic-containing nanomaterials, ELISA, and scintillation counting for radioactive materials, among others. Often, drug fractionation methods, such as the SITUA method described above, are utilized to characterize nanomedicine-encapsulated, unbound and protein bound drug fractions. Note, in rare cases, the NCL can work with ^3H and ^{14}C radioactive materials when other testing options are not available but prefers to develop other bioanalytical techniques in lieu of radioactivity. No other radioisotopes are permitted per our current license.

In addition to PK studies, NCL conducts limited efficacy studies primarily to confirm sponsor findings or add value to existing models. NCL does not typically have resources to conduct broad cancer model sensitivity studies. NCL efficacy studies include clinically relevant route and schedule, and appropriate drug and nanoformulation controls.



Metabolite modeling of a macromolecular prodrug. (Reproduced with permission from *J Control Release*, **2013**, 172(2), 558–67.)

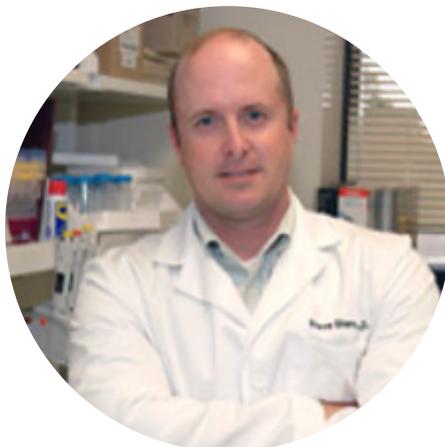
Pharmacokinetic Parameters

- area under the time concentration curve to time infinity (AUC_{inf})
- area under the time concentration curve to time last (AUC_{last})
- area under the first moment curve (AUMC)
- concentration at time zero (C₀)
- maximum concentration (C_{max})
- clearance (CL)
- terminal half-life (t_{1/2})
- apparent volume of distribution (V_d)
- volume of distribution steady state (V_{ss})
- Mean residence time (MRT)

Relevant NCL Publications

Skoczen et al, *ACS Pharmacol Transl Sci*, **2020**, 3(3), 547-558. PMID: 32566919
Price et al, *Science Advances*, **2020**, 6(29), eaay9249. PMID: 32832614
Stern et al, *J Control Release*, **2013**, 172(2), 558-567. PMID: 23664969

MEET THE EXPERT



Dr. Stephan T. Stern, PhD, DABT

Director of Research & Development,
Head of Pharmacology, Toxicology & Formulation

About Dr. Stern

Dr. Stern is a Diplomate of the American Board of Toxicology and has led the NCL's Pharmacology & Toxicology program since its founding in 2004. He has been instrumental in the design and implementation of pharmacology and toxicology studies used to characterize nanoparticle disposition and assess nanoparticle biocompatibility. Steve is widely regarded as an expert in the pharmacological and toxicological properties of nanomedicines. He has been an invited speaker at many national and international nanotechnology meetings, serves as an expert reviewer to the NIH Source Evaluation Group and international nanomedicine funding programs, and provides recommendations to regulatory bodies on appropriate testing of nanotech formulations. His areas of expertise include biochemical toxicology of the liver and kidney, analytical methodology, and drug metabolism/pharmacokinetics.

Prior to joining the NCL, Steve completed a post-doctoral fellowship at the University of North Carolina - Chapel Hill in the Division of Drug Delivery and Disposition, and Curriculum in Toxicology. In this position, his research focused on examining the role of intestinal metabolism in modulating the gastrointestinal toxicity of chemotherapeutic agents. He received his B.S. degree in biochemistry from the University of Rochester and his Ph.D. in toxicology from the University of Connecticut at Storrs.

Additional Resources

- Select protocols for assays described here can be downloaded for free on the NCL's website:
<https://ncl.cancer.gov/resources/assay-cascade-protocols>
- Additional publications on these topics are available here:
<https://ncl.cancer.gov/resources/ncl-scientific-bibliography>
- Information on in vitro and in vivo pharmacokinetic services available for purchase through the TSA mechanism is detailed here:
<https://ncl.cancer.gov/working-ncl/technical-services>

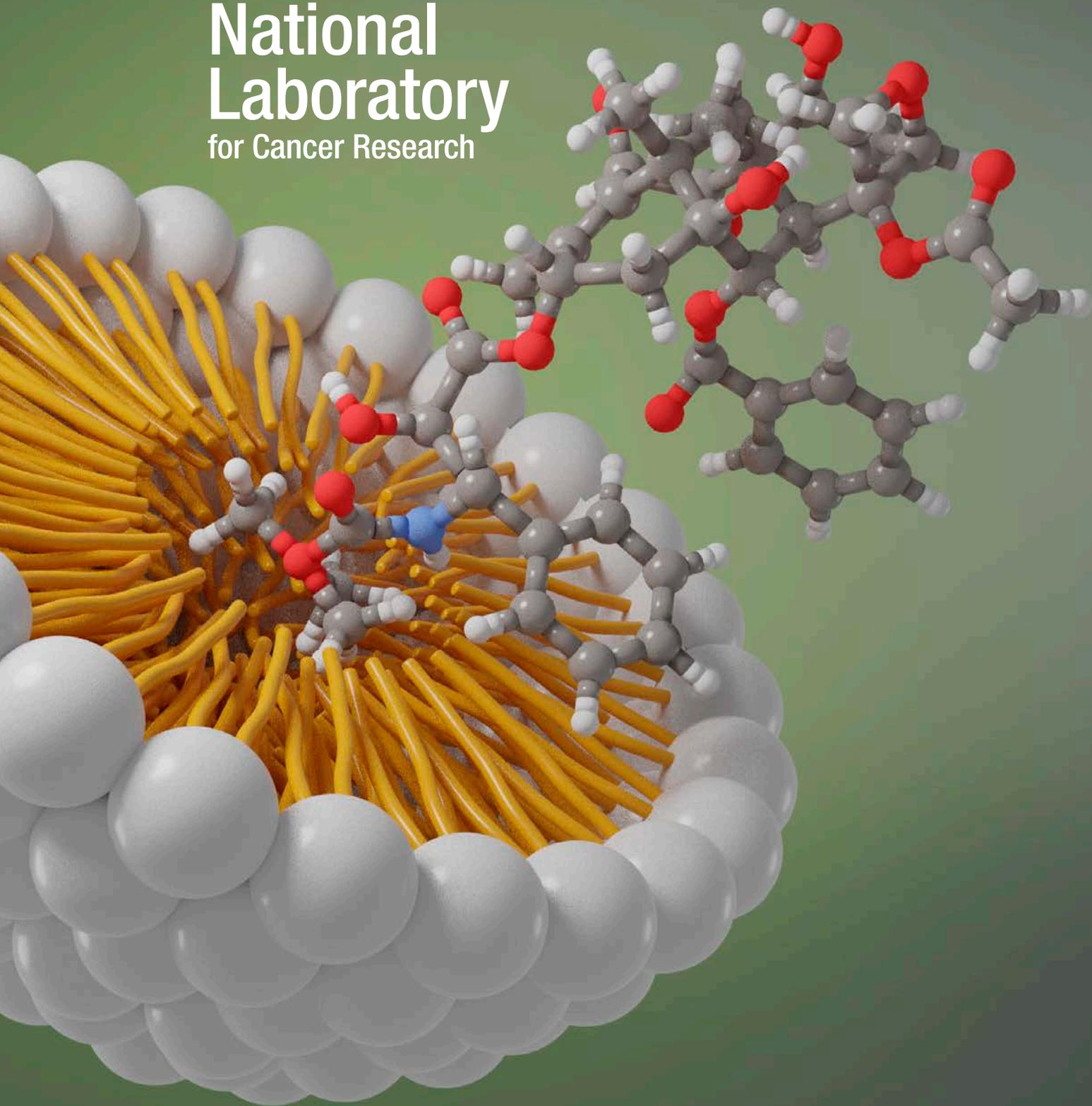
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For questions or further discussion on any of the topics highlighted here, please feel free to reach out to Dr. Stern via email.

Frederick National Laboratory

for Cancer Research



The NCL is part of the Frederick National Laboratory for Cancer Research (FNLCR). FNLCR is operated by Leidos Biomedical Research, Inc., for the National Cancer Institute, under contract 75N91019D00024.

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