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JUNE 21, 2023

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ORGANIZERS' WELCOME

Welcome to the 2023 NE-ADME Conference.

Our organizers have gathered another excellent group of speakers for the annual NE-ADME conference. The program is arranged to incorporate extensive audience participation and discussion. We encourage attendees to take full advantage of the opportunity to engage in discussion in order to receive the maximum benefit from the NE-ADME experience. Thank you for your participation.

ORGANIZING COMMITTEE

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Chris Rowbottom, Moderna
Conference Chair Elect:
Mitesh Patel, Novartis

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Ruchia Duggal, Merck
Li Di, Pfizer
Maria Fitzgerald, Ipsen
Vinayak Hosagrahara, Nimbus Therapeutics
Pei Li, Vertex
Steven Louie, Moderna
Mukesh Lulla, Biogen
Mitesh Patel, Novartis
Ron Xu, ORIC Pharmaceuticals
Jun Zhang, BMS

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NE-ADME 2023 CONFERENCE AGENDA

WEDNESDAY, JUNE 21

7:30 - 8:30 AM **Registration**

8:30 - 8:40 AM **Conference Opening**
Christopher Rowbottom, Moderna

SESSION I: ADME of Protein-Based Therapeutics
Moderators: Jun Zhang, BMS & Li Di, Pfizer

8:40 - 8:45 AM **Session Introduction**

8:45 - 9:10 AM ***Perspectives on ADME Studies of Therapeutic Proteins***
Hongbin Yu, Boehringer Ingelheim

9:10 - 9:35 AM ***Perspectives on DMPK Support for ADCs***
Seema Chauhan Kumar, Pioneering Medicines

9:35 - 10:00 AM ***Translational Modelling for a first in class MUC1xEGFR (M1231) Bispecific Antibody Drug Conjugate***
Anup Zutshi, EMD Serono

Plenary Speaker Introduction
Mitesh Patel, Novartis

10:00 - 10:40 AM **PLENARY:** ***Quantitative Proteomics-informed Modeling for Prediction of Drug Clearance and Drug Interactions***
Bhagwat Prasad, Washington State University

10:40 - 11:00 AM Break

SESSION II: Current Protein Degraders Targets
Moderators: Steven Louie, Moderna & Mukesh Lulla, Biogen

11:00 - 11:05 AM **Session Introduction**
Steven Louie, Moderna & Mukesh Lulla, Biogen

11:05 - 11:30 AM **VENDOR PRESENTATION:**
Recommendations for Successful in vitro Evaluation of Pharmacokinetic Drug Interaction Potential of ADCs
Maciej Czerwinski, BioIVT

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11:30 - 11:55 AM ***In vitro Binding Assays to Assess Exposure of Bifunctional Degradation Activating Compounds (BiDACs) in the Brain***
Ritu Singh, C4 Therapeutics

The Boston Society

11:55 - 12:20 PM ***Targeted Protein Degrader Applications for Gene Therapy and Small Molecule Drug Discovery***
Seth Carbonneau, Novartis

12:20 - 1:35 PM Lunch

1:35 - 1:45 PM **VENDOR PRESENTATION:**
Multiplexing Oligonucleotide Analysis with Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS)
Abdul Basit, CRL/Solvo

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SESSION III: Analytical Strategies for New Modalities
Moderators: Dallas Bednarczyk, Novartis & Maria Fitzgerald, Ipsen

1:45 - 1:50 PM **Session Introduction**
Dallas Bednarczyk, Novartis & Maria Fitzgerald, Ipsen

1:50 - 2:15 PM ***Use of High-Resolution Mass Spectrometry to Characterize Uptake and Metabolism of GalNAc- and Lipid-Conjugated siRNAs in Cultured Primary Human Hepatocytes In Vitro***
Michael Hayashi, Dicerna

2:15 - 2:40 PM ***NanoClick Assay: A High Throughput, Target-agnostic Permeability Assay that Combines NanoBRET Technology with Intracellular Click Chemistry***
Andrea Peier, Merck

2:40 - 3:05 PM ***Bioanalysis for Radio Ligand Therapies (RLTs) with LCMSMS and LC ICP MS***
Dan Wall, Novartis

3:05 - 3:25 PM Break

SESSION IV: Drug Discovery in Novel Modalities
Moderators: Ruchia Duggal, Merck & Chris Rowbottom, Moderna

3:25 - 3:30 PM **Session Introduction**
Moderators: Ruchia Duggal, Merck & Chris Rowbottom, Moderna

3:30 - 3:55 PM ***Model-informed Drug Development (MIDD) in the mRNA-based Therapeutics and Vaccines***
Stephen Greene, Moderna

3:55 - 4:20 PM **Antibacterial Efficacy of the Macrocyclic Depsipeptide Teixobactin in the Neutropenic Mouse PK-PD Model of Thigh Infection**
Peng Hsiao, Schrödinger Inc

4:20 - 4:45 PM **Poster Presentations**
Moderators: Pei Li, Vertex & Vinayak Hosagrahara, Nimbus

4:45 - 4:50 PM **Closing Remarks**

4:50 - 5:50 PM **Reception**

ABSTRACTS

SESSION I

Perspectives on ADME of Therapeutic Proteins

Hongbin Yu, Boehringer Ingelheim

Therapeutic proteins have become an integral part of therapeutics for a variety of diseases. They are subjected to different ADME processes due to their distinct physicochemical characteristics as compared to small molecule drugs (e.g. large size, lack of cellular permeability). The IQ ADME of Therapeutic Protein Working Group was formed in 2019 to share practices on studying absorption, distribution, metabolism and excretion across different companies. Findings for this working group were published in three papers in the Journal of Drug Metabolism and Disposition in 2022. This presentation will highlight recommendations by the Working Group on how to conduct ADME studies for therapeutic proteins.

Perspectives on DMPK Support for ADCs

Seema Kumar, Pioneering Medicines

Antibody-drug conjugate (ADC) combines the high specificity and long half-life of monoclonal antibodies with the high potency of small-molecule drugs. ADC aims to selectively deliver cytotoxic drugs to targeted tumor tissues, thereby limiting systemic exposure and increasing the therapeutic index of small-molecule drugs. Due to its complex multi-component structure (combining the large and small molecule drug components) and inherently heterogenous nature, ADC poses unique challenges in the characterization and understanding of its drug disposition. The talk will highlight some of these challenges.

Translational Modelling for a first in class MUC1xEGFR (M1231) Bispecific Antibody Drug Conjugate

Anup Zutshi, EMD Serono

M1231 is a first-in-class, bispecific MUC1xEGFR ADC that delivers a cytotoxic hemiasterlin-related payload to tumor cells expressing MUC1 and EGFR causing cell death through disruption of microtubule dynamics. A multi-scaled QSP model was developed to quantitatively analyze and expand the interactions of M1231 from the cellular level, to PDX tumor models, and to enable clinical translation of antitumor activity. The model predicted a tumor stasis dose (TSD) of 2.40 mg/kg and a tumor regression dose (TRD) of 4.28 mg/kg. Data obtained from the Phase I clinical trial (NCT04695847) seems to confirm these estimates.

PLENARY

Quantitative Proteomics-Informed Modeling for Prediction of Drug Clearance and Drug Interactions

Bhagwat Prasad, Washington State University

Drug metabolizing enzymes and transporters are critical determinants of drug absorption, metabolism, distribution, and elimination (ADME) and influence drug-drug interactions (DDIs) and toxicity. While significant progress has been made to utilize in vitro models to predict drug ADME using physiologically-based pharmacokinetic (PBPK) models, these models require comprehensive physiological data on inter-individual variability. In particular, PBPK models require quantitative information on the levels and activity of individual pathways involved in drug disposition across different tissues and populations (healthy vs. diseased or children vs. adults). A significant lack of quantitative knowledge regarding non-Cytochrome P450 (non-CYP) enzymes and transporters in human is the major limitation towards building PBPK models in the drug development which often results inaccurate in vitro to in vivo extrapolation (IVIVE) and poor prediction of interindividual variability of drug metabolism. Although non-CYP enzymes are expressed in multiple human tissues, differential tissue expression and interindividual variability in the expression of these

enzymes are not well studied. Uncharacterized sub-cellular localization of some non-CYP enzymes is another knowledge gap with respect to the development of quantitatively viable in vitro and in silico models. Similarly, animal to human scaling of non-CYP metabolism is not accurate because of the unknown inter-species differences. To address these issues, we utilize state-of-the-art quantitative proteomics in conjunction with metabolomics and genomics approaches to characterize abundances and activity of drug metabolizing enzymes and transporters in human tissues and biofluids. These data are then integrated into PBPK models to predict variability in drug clearance and DDIs, particularly in underrepresented populations such as children in whom clinical studies are not routinely performed.

SESSION II

Recommendations for Successful in vitro Evaluation of Pharmacokinetic Drug Interaction Potential of ADCs

Maciej Czerwinski, BioIVT

Numerous chemotherapies utilizing antibody drug conjugates (ADC) are being developed. Although the regulatory guidance for evaluation of drug interaction potential of ADCs is not well established, their toxic payloads can be examined following the recommendations for small molecule drugs. This presentation will discuss how to design studies to investigate ADC contribution to drug interaction potential.

In vitro Binding Assays to Assess Exposure of Bifunctional Degradation Activating Compounds (BiDACs) in the Brain

Ritu Singh, C4 Therapeutics

An important step in the development of drugs targeted for central nervous system (CNS) activity involves the accurate determination of the extent of drug exposure to the brain. The unbound brain-to-plasma partition coefficient or Kp,uu,brain is assessed routinely during the drug discovery process. It has been widely accepted as a key parameter related to calculating brain exposure and is based on the concept that the unbound or free concentration of a drug at the site of action drives the pharmacological response. The fraction unbound to brain homogenate for small molecules is typically determined using the rapid equilibrium dialysis (RED) method. However, this method is unsuitable for high molecular weight BiDAC degraders that often require prolonged equilibration time and may result in errors due to instability and significant non-specific binding. An alternative method is an ultracentrifugation-based binding assay, which has a lower propensity for non-specific binding but is a low throughput method. Both the RED and ultracentrifugation methods require animal derived brain homogenate for the assessment of unbound fraction. We have recently evaluated the TRANSIL Brain Absorption assay, a high-throughput matrix-free method that measures the affinity of drugs to porcine brain membranes to estimate their binding to brain tissue. The fraction unbound in the brain was determined for a few selected compounds using the ultracentrifugation and TRANSIL Brain Absorption methods and the calculated Kp,uu,brain values derived using the two methods were compared. The values derived from the fraction unbound using the TRANSIL Brain Absorption method were relatively higher than those calculated from the ultracentrifugation method. Correlating the unbound brain exposure to pharmacodynamic (PD) effect in vivo for select compounds showed that the PD effect was better explained by brain exposure based on the unbound fraction determined by the TRANSIL Brain Absorption method. An added advantage of this method is the ability to run the assay in a cassette format by co-incubating up to 4 compounds, which significantly improves the throughput as well as the cost of the assay. These studies have shown that the TRANSIL Brain Absorption method is a simple and efficient approach to determine fraction unbound of BiDAC degraders in the brain.

Targeted Protein Degrader Applications for Gene Therapy and Small Molecule Drug Discovery

Seth Carbonneau, Novartis

Targeted protein degradation (TPD) has been a fast-growing field for the last 10 years since the discovery of the mechanism of action of thalidomide because it has opened

a door to drugging targets that were once considered undruggable, presented opportunities to overcome resistance, and ways to selectively target paralogs. A wide array of chemical degraders have been discovered with modalities ranging from E3 ligase engagement, lysosomal engagement, and even direct proteasomal engagement for countless target and disease applications. Here we will share three drug discovery programs that represent different applications of TPD—Helios molecular glues for Immuno-Oncology, IMiD-regulatable CARTs as an example of gene therapy applications, and BRM-selective heterobifunctional degraders for non-small-cell lung cancer. Through the vignettes, we will provide an overview of each modality and share key aspects and considerations for drug discovery of each.

VENDOR PRESENTATION

Multiplexing Oligonucleotide Analysis with Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS)

Abdul Basit, Charles River Laboratories

Oligonucleotide-based therapeutics represent the changing face of drug discovery and development. Designed to prevent or modulate the translation of a specific gene, these therapies have the potential to tackle previously undruggable targets and could set the tone for the future of personalized medicine. As of January 2022, the FDA has approved 14 oligonucleotide drugs, but the number of therapeutics moving into and through the development pipeline is blossoming with 11 of these oligonucleotide drugs got approved in last 5 years.

With this new surge in oligonucleotide interest comes a new challenge for analytical scientist i.e. better chromatography while maintaining the MS response. Oligonucleotides are organic polymers in which nucleosides are linked by a phosphodiester into long chains up to 100s of bases in length. The combination of aromatic bases, polar sugars, and polyanionic phosphates can not only make LC separations of oligonucleotides very challenging; but also affect the sample extraction and ionization capabilities of the molecule.

We have optimized both sample extraction by solid phase and analysis using LC-HRMS for various oligonucleotides (siRNA/ASOs). LC-HRMS effectively distinguish and quantify not only the target analyte but also metabolites/impurities in the sample using right combination of scans in a single method. We coupled Full scan (FS) and Parallel reaction monitoring (PRM) mode together to enhance the throughput using single injection. PRM mode facilitates the quantification of targeted parent ions while FS open the doors for relative quantification of metabolites and impurities from the same injection. With this method we have achieved 5ng/mL LLOQ, 1000X dynamic linearity, and simultaneous quantification of metabolites (deamination, n-1, phosphodiester linkage) from the same sample.

SESSION III

Use of High-Resolution Mass Spectrometry to Characterize Uptake and Metabolism of GalNAc- and Lipid-Conjugated siRNAs in Cultured Primary Human Hepatocytes In Vitro

Michael Hayashi, Dicerna

Purpose: An in vitro assay for siRNA uptake and metabolism was developed in cultured primary human hepatocytes. The assay was developed to inform on uptake efficiencies, stability, and major metabolite formation on siRNAs conjugated to different moieties (i.e., GalNAc and lipids). Ultra-High Performance Liquid Chromatography High Resolution Mass Spectrometry (UPLC-HRMS) was used in conjunction with Novatia software to identify parent (full length product) and metabolites. The aim of this study was to identify uptake efficiency and metabolic soft spots for conjugated siRNAs in liver cells.

Methods:

- Cryopreserved, plateable, transporter-qualified primary human hepatocytes were plated in 12-well format (~0.9 x 106 cells/well).
- Allowed five days in culture to allow for maximal recovery

of ASGPR1 mRNA levels (measured by RT-qPCR). Media was changed every 48 hours.

• After five days in culture, incubated test articles in fresh medium. Same guide, different passenger (GalNAc-Conjugated siRNA, Lipid-Conjugated siRNA, and Unconjugated siRNA).

• Time points = 0, 0.5, 2, 6, 24, 48, and 72 hours.

• At the designated time points, supernatants were collected. Cells were washed 2x with 1X Williams Medium E. Cells were lysed with Phenomenex Clarity OTX Lysis-Loading Buffer and were collected.

• All collected specimens were processed by solid phase extraction (SPE) using the Phenomenex Clarity OTX SPE protocol.

• High resolution mass spectrometry (HRMS) analysis: Carried out on a Waters UHPLC system (H Class) coupled to a Waters HRMS (Xevo-G2-XS) in ESI negative mode. The separation was performed using a ACQUITY Premier Oligonucleotide C18 Column (130Å, 1.7 µm, 2.1 x 100 mm) at 70°C using mobile phase A (water) and B (methanol) both containing 1% HFIP (1,1,1,3,3,3-hexafluoroisopropanol), 0.5% DIPEA (N, N-diisopropylethylamine), and 1 µM EDTA (ethylenediaminetetraacetic acid).

Results: HRMS data was processed using untargeted analysis via Novatia ProMass Deconvolution Software, and targeted analysis via extracted ion chromatography (Waters MassLynx software). siRNA full length products were detected in cell lysates beginning at 0.5 h (2-fold over baseline). Full length product detection demonstrated time-dependent increases over the course of the 72-hour incubation. Major metabolites observed for the GalNAc-conjugated siRNA were sense metabolite 1, sense metabolite 2, antisense metabolite 1, and antisense metabolite 2. Major metabolites observed for the lipid-conjugated siRNA were sense metabolite 1 and antisense metabolite 1. The unconjugated siRNA did not demonstrate appreciable uptake or metabolism.

Conclusions: In comparison to the in vivo data, the in vitro cultured primary human hepatocytes can demonstrate uptake efficiency and metabolic soft spots for certain classes of siRNA.

NanoClick Assay: A High Throughput, Target-agnostic Permeability Assay that Combines NanoBRET Technology with Intracellular Click Chemistry

Andrea Peier, Merck

Macrocyclic peptides open new opportunities to target intracellular protein-protein interactions (PPIs) that are often considered non-druggable by traditional small molecules. Specifically, peptides have the potential to bind to highly expansive binding surfaces (orthosteric blocking) of such PPIs and/or other unique allosteric binding sites. However, their clinical development may be limited by their ability to efficiently penetrate into cells to modulate their cognate PPI targets. The ability to have a predictive, high-throughput assay to assess cell permeability is a critical tool to support peptide drug discovery programs.

We developed a high throughput, quantitative, target-agnostic cell permeability assay that essentially measures the cumulative cytosolic exposure of a peptide in a concentration-dependent manner. The assay has been named NanoClick as it combines in-cell Click chemistry and monitoring of a NanoBRET signal in cells. The assay is based on cellular expression of the NanoLuc-HaloTag system and relies on the Click reaction of azide-containing peptides with DiBac-chloroalkane (CA) anchored to the HaloTag. Subsequent introduction of an azido-dye followed by the NanoLuc substrate allows the detection of a BRET signal that is reduced by the presence of Click-reactive peptides in the cytosol. The readout can be expressed as a permeability ratio of EC50s when compared to the response of a low permeability control.

We validated the assay using known cell penetrating peptides and were further able to demonstrate correlations to cellular activity using a p53/MDM2 model system. The assay has been applied across multiple programs and has been used to guide and establish structure-permeability relationships in the optimization of macrocyclic peptides for cellular potency across intracellular PPI target programs.

Bioanalysis for Radio Ligand Therapies (RLTs) with LCMSMS and LC ICP MS

Dan Wall, Novartis

AIM: With recent improvements to LC-ICP-MS technology, discovery in vivo pharmacokinetics (PK) and mass balance studies can now be conducted with cold metal radioligand therapeutics (RLTs), enabling a far simpler experimental workflow, than with their radioactive counterparts. These in vivo studies require sensitive and selective qualified bioanalytical methods for the parent RLT, any related metabolites and total metal in biological samples. **Results:** In this work we showed a novel application of LC-ICP-MS, that enabled us to perform reversed phase, high performance liquid chromatography for the cold Lu RLT peptides and related metal tagged species. This LC-ICP-MS approach opened the possibility to simultaneously detect different forms of a selected Lu RLT peptide, where the chromatographic separation provided a profile for the parent and different metal loaded metabolites and catabolites of the dosed Lu RLT peptide in biological matrices. The LC-ICP-MS configuration was then also adapted to a no-column FIA-ICP-MS format, used to determine total metal in biological samples. We show that when LC-MS/MS is used for parent Lu RLT quantitation, in combination with the parent and metabolite profiling made possible by LC-ICP-MS, and total metal from FIA-ICP-MS, the PK, and mass balance for the dosed RLT can be determined. **Conclusion:** The qualified methods provided sensitive and selective bioanalysis of Lu RLT peptides in biological samples, and we integrated the LC-ICP-MS, FIA-ICP-MS and LC-MS/MS approaches to enable a logical gated approach that can be used in a discovery setting, to efficiently answer questions of Lu RLT peptide PK and mass balance.

SESSION IV

Model-informed Drug Development (MIDD) in the mRNA-based Therapeutics and Vaccines

Stephen Greene, Moderna

Model-informed drug and vaccine Development (MIDD/MIVD) for mRNA-based therapies and vaccines present opportunities for both unique and traditional MIDD/MIVD approaches. Case examples of how these traditional and unique approaches were applied to answer specific development questions will be explored. Examples of how these unique approaches were also used to support regulatory interactions will also be discussed.

Antibacterial Efficacy of the Macrocyclic Depsipeptide Teixobactin in the Neutropenic Mouse PK-PD Model of Thigh Infection

Peng Hsiao, Ferring Research Institute Inc.

Teixobactin, a macrocyclic depsipeptide discovered in the Gram-negative bacterium Eleftheria terrae, represents a novel class of peptidoglycan synthesis inhibitors that are highly potent against a broad range of Gram-positive microbes, including methicillin-sensitive Staphylococcus aureus (MSSA) and vancomycin-resistant Enterococci (VRE), with no observable resistance. Minimum inhibitory concentrations (MIC) of teixobactin against MSSA and VRE were determined to be 0.138 and 0.453 µg/mL, respectively. The pharmacodynamics of teixobactin against MSSA and VRE were then investigated using the neutropenic mouse thigh infection model. Mice infected with 1.2 × 106 CFU/thigh at the initiation of therapy were administered 1, 5, or 20 mg/kg teixobactin bolus intravenously. Teixobactin was effective at inhibiting both MSSA and VRE in a dose-dependent manner. The maximal bacterial reduction counts in thigh tissue at 24 hr were –4.0 log10 and –2.8 log10 CFU/thigh for MSSA and VRE, respectively. Further, a refined fractionized dose study against MSSA was conducted at a dose range of 1-30 mg/kg (in 1, 2, or 4 equally fractionated doses) over 24 hr. Of all the pharmacokinetic parameters evaluated (Cmax/MIC, AUC/MIC, and fT> MIC), the efficacy was best linked with the percentage of a 24 hr dosing interval at which the free teixobactin plasma concentration exceeded 4X the MIC (fT> 4XMIC; R2 = 0.92).

SPEAKER BIOGRAPHIES

ABDUL BASIT, PHD, Charles River Laboratoties Dr. Basit is Associate Director at Charles River's Worcester Discovery Bioanalysis group in Massachusetts, where he leads the large molecule bioanalysis using state of art high resolution mass spectrometry. In this role he oversees characterization and quantification of oligonucleotides, peptide and protein therapeutics and endogenous metabolites using cutting edge technologies in omics science.

Dr. Basit is has been in the field of research and development for over 12 years, working at various research organization in a myriad of roles. Prior to joining Charles River in 2021, He has worked as Research Assistant Professor at Washington State University, Spokane, WA and senior fellow/technical director for quantitative proteomics and metabolomics at Department of Pharmaceutics, School of Pharmacy, University of Washington, Seattle, WA. He also has working experience with in vitro DMPK and bioanalysis at Sailifesciences and Vanta Bioscience, India.

Dr. Basit received his Bachelor of Pharmacy degree from Pune University, India and master's degree in pharmaceutical science from National Institute of Pharmaceutical Education and Research, India. He did PhD in Drug Discovery from Istituto Italiano Di Technologia (Università degli Studi di Genova), Italy. Dr. Basit has authored and co-authored > 40 peer-reviewed articles, >30 conference abstracts, one book chapter and Co-inventor of two U.S. Patents.
https://www.linkedin.com/in/abdul-basit-3934b815/

SETH CARBONNEAU, Novartis Mr. Carbonneau is a Senior Principal Investigator at Novartis Institutes for Biomedical Research where he focuses on taking chemical biology approach to drug discovery. In recent years, he has led several targeted-protein degradation drug discovery programs primarily for oncology targets. He is a graduate of College of the Atlantic in Bar Harbor, ME and has worked with Dr. James Coffman at the Mount Dessert Island Biological Laboratory and with Dr. Thomas Look at the Dana Farber Cancer Institute.

MACIEJ CZERWINSKI, PHD, XenoTech Dr. Maciej Czerwinski currently serves as a Director in the Scientific Consulting department at XenoTech. He received his Ph.D. from the University of Maryland at Baltimore Medical School in the Department of Pathology. Dr. Czerwinksi conducted his doctoral thesis research in the Laboratory of Dr. Frank Gonzalez at National Cancer Institute in Bethesda, followed by a post-doc at the Department of Pharmaceutics, University of Washington in Seattle. He has been with XenoTech since 1999 and guides the development of products and services for the in vitro analysis of drug safety. Dr. Czerwinski designed a patented method to analyze the in vitro cytokine-mediated drug-drug interactions between biologics and small molecule drugs. and he is also the inventor of XenoTech's CryostaX single-freeze pooled hepatocytes.

STEPHEN GREENE, PHD, Moderna Dr. Stephen Greene is an Associate Scientific Director of Clinical Pharmacology and Pharmacometrics at Moderna Therapeutics. Dr. Greene's current professional emphasis is on model-informed development of mRNA vaccines and therapeutics across multiple therapeutic areas including infectious diseases, oncology, and rare diseases. Stephen completed his Bachelor of Arts in mathematics from the University of Utah in 2010 and his doctorate of pharmacy (PharmD) from Purdue University in Indiana in 2016. Stephen also completed a 2 year post-doctoral fellowship at the University of North Carolina and Nuventra Pharma Sciences with a particular emphasis on PK/PD modeling in 2018. Stephen has experience in clinical pharmacology and pharmacometrics applied across pre-clinical and clinical phases of development and post-marketing commitments in multiple therapeutic areas including infectious diseases, CNS, rare diseases, and oncology.

MICHAEL HAYASHI, Dicerna Pharmaceuticals Michael Hayashi is currently a Scientist II at Dicerna Pharmaceuticals, a Novo Nordisk Company. He has 28+ years of experience in the pharmaceutical industry. His major focus has been the development and application of in vitroADMET assays, specifically to look at large and small molecule stability, metabolite formation, and gene regulation. Michael has also focused on mass spectrometry (LC/MS/MS and HRMS) to conduct targeted and untargeted bioanalysis in his research. He has provided study conduct and reporting for multiple therapeutic areas from screening to regulatory filings.

PENG HSIAO, MS, PHD, Schrödinger, Inc. Dr. Hsiao received both his MSc and PhD from the University of Washington, department of Pharmaceutics. Following graduation, Peng worked briefly as a Senior Fellow in the University of Washington School of Medicine, department of Radiology, conducting clinical research in collaboration with the Fred Hutchinson Cancer Research Center, using radio-labeled monoclonal antibodies.

For the past 10 years, Dr. Hsiao has worked in industry exploring diverse chemical modalities and therapeutic areas. He gained experience working at Seagen, focusing on the mechanism of action and ocular toxicity of antibody drug conjugates for oncology. His prior H3 Biomedicine entailed an agnostic chemical construct approach to cancer therapy, i.e., small molecules, PROTAC, and biologics. Peng also worked on establishing PK/PD models of antimicrobial peptides for C. diff infection, as well as full ADME/DMPK profiling, PD/PD with human dose projection for small molecules for the treatment of infertility at Ferring Research Institute, and IBD/CD at Boehringer Ingelheim.

Dr. Hsiao's research interests include the regulation and function of transporters in the physiological restrictive sites (e.g., P-gp at the BBB), PK/PD modeling of hepatic toxicities and clinical predictions in humans, based on in vitro assays and in vivo animal models, and ADME/PK/PD modeling of drugs at both the physiological and cellular levels.

SEEMA KUMAR, PHD, Pioneering Medicines Seema Kumar is a Senior Director at Pioneering Medicines, a business unit of Flagship Pioneering. In her role, Dr. Kumar leads DMPK and Bioanalytical support across biotherapeutic modalities. Prior to her current role, Dr. Kumar served as a Director and senior DMPK lead at EMD Serono Inc. (a business of Merck KGaA, Germany), where she led Clinical Bioanalytical Sciences group that provided drug-disposition support including Bioanalysis, Immunogenicity, and DMPK support for clinical stage NBE programs. Before that, Dr. Kumar led the Bioanalytical group at Pfizer. Prior to joining Pfizer, Dr. Kumar served in roles of increasing responsibility as Director of Quality Control and Director of CLIA-certified Clinical Bioanalytical Lab at XBiotech USA Inc.

Dr. Kumar holds a Ph.D. from Johns Hopkins University and has published several publications in peer-reviewed journals and contributed to several book chapters. Dr. Kumar has given numerous talks at national and international scientific conferences and meetings and is an active member of industry consortia (IQ, AAPS, etc.).

ANDREA PEIER, PHD, Merck Dr. Peier is currently a Senior Director of Cell Sciences in the Screening & Compound Profiling Department at Merck. She received her Ph.D. in Molecular & Human Genetics at Baylor College of Medicine and completed a post-doctoral fellowship under Ardem Patapoutian at the Genomics Institute of the Novartis Research Foundation where she cloned and characterized novel TRP channels involved in thermoregulation and pain. During her 17- year tenure at Merck, she's held multiple positions in Disease Area biology and Pharmacology leveraging cellular models to profile small molecules and improve translatability to preclinical in vivo models. In her current role, her group uses cellular models to accelerate Target ID, Validation, and Hit-to-Lead Efforts, specifically focusing on the development of cellular assays to study peptide permeability, target engagement, protein degradation, and disease biology to advance drug discovery.

BHAGWAT PRASAD, PHD, Washington State University Dr. Bhagwat Prasad is an Associate Professor in the Department of Pharmaceutical Sciences, Washington State University (WSU), Spokane, WA. He leads several federally- and industry-funded research programs on characterization of interindividual variability in drug disposition (drug transport and metabolism) and serves as a director of the proteomics-based research in non-cytochrome P450 enzymes (PRINCE). Dr. Prasad has published >110 peer-reviewed articles and >100 conference abstracts and delivered over 85 invited talks at various national and international conferences. Dr. Prasad is the recipient of 2018 ISSX North American New Investigator Award. Dr. Prasad also received the Early Career Faculty Showcase award at the 2018 ASPET meeting. Dr. Prasad also served as Secretary of the Drug Metabolism and Disposition Division of ASPET. Dr. Prasad is a member of the editorial boards of Drug Metabolism and Disposition and Clinical Pharmacology and Therapeutics. Dr. Prasad obtained his MS in 2006 and Ph.D. in 2010 in Pharmaceutical Analysis from NIPER, Mohali, India, and he was a postdoc and assistant professor at the department of Pharmaceutics, University of Washington, Seattle, WA from 2011-2014 and 2014-2019, respectively.

RITU SINGH, PHD, C4 Therapeutics Dr. Singh is an Associate Director in the Drug Metabolism and Pharmacokinetics group at C4 Therapeutics in Watertown, MA. She earned her PhD in Pharmaceutical Sciences from Rutgers University. Prior to joining C4 in 2022, Ritu worked at the Broad Institute, Corning Life Sciences (Gentest), Synta Pharmaceuticals and Merck. Her interests and expertise include ADME optimization of small molecules and targeted protein degraders, drug metabolism, metabolite identification, bio-analysis, and drug-drug interactions.

DAN WALL, Novartis Mr. Wall has been working in Pharma research for over 18 years. He has many years of experience as a team leader and bench scientist. His role now is as team leader. His primary skill set is based on custom sample preparation and LC-MS/MS, however he is also versed in Ligand Binding Assay bioanalytics, as well as hybrid immunocapture / LC-MS/MS. He is leading a dynamic and highly experienced and innovative group of low MW discovery bioanalytical scientists working in Cambridge, MA, USA and Basel Switzerland. They are building cutting edge advanced bioanalytical methods, so to tackle the wide diversity of new molecular entities and complex modalities that come to us. They also utilize LCMS methods to analyze PK and ADA for protein-based biologics. Every day they work with project teams to answer business critical questions about the compounds that are being discovered. They are part of a larger PK Sciences Bioanalytical Labs group, that also has extensive expertise in Biologics and Gene Therapy.

HONGBIN YU, PHD, Boehringer Ingelheim Dr. Yu obtained his Ph.D. in Chemistry from the University of Missouri and conducted his postdoctoral work at MIT. He joined the DMPK Department of Boehringer Ingelheim since 2006 and has worked on ADME and PK of small molecules and therapeutic proteins. Currently he is the Oncology Therapeutic Area Lead for Global Development at BI. He is an active member of the IQ Consortium and BioSafe and has led/co-led multiple scientific working groups.

ANUP ZUTSHI, PHD, EMD Serono Dr. Zutshi is a Sr. Scientific Director and Global Head of Translational Quantitative Pharmacology at EMD Serono laboratories (a division of Merck KGaA, Darmstadt, Germany). Since joining the organization in June 2015, Dr. Zutshi has built a high performing team of scientists to support projects from early discovery to Phase II Proof of Concept studies and beyond. The team supports projects in Immunology, Immuno-Oncology and Oncology across research sites within Merck.

Dr. Zutshi obtained a B.S. and M.S. in Pharmacy from India and a Ph.D. in Pharmaceutical Sciences from the University of Florida, Gainesville, FL. He has worked in contract research for 9 years and in pharmaceutical research for 22 years. Dr. Zutshi is interested in understanding the mechanisms of new chemical/biological entities as drugs and their impact on physiological

systems of healthy as well as diseased individuals. Dr. Zutshi's work involves studying large biotherapeutic molecules as well as small xenobiotic molecules. His research leads directly to predicting first in human dose, dose escalation strategies, dose stopping criterion, predicted changes to biomarkers and disease end points. This helps to design the optimum clinical trials to establish proof-of-concept.

POSTER ABSTRACTS

In Vitro Assessment of Physicochemical Properties of Biologics for the Early Identification of Pharmacokinetic Liabilities in a Developability Workflow

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PURPOSE

Historically, the assessment of pharmacokinetics (PK) of an Fc-asset biotherapeutic has primarily relied on in vivo characterization. Recently, it has emerged in the literature that certain physicochemical properties of biotherapeutics can be assessed in vitro with strong correlative potential to in vivo behavior [1]. Consequently, in vitro screening approaches, comparable to that of Lipinski's rule of 5 for small molecules, are emerging as an evolving field of science for biotherapeutics. Applying this along a protein engineering developability workflow, it follows that physicochemical attributes relating to target binding domain optimization & framework selection can have negative PK implications, which can be identified using early in vitro assessments in lieu of conducting in vivo PK studies [2-4]. Evaluation of disparate in vitro measured properties, in relation to measured PK parameters, highlight that the most correlative physicochemical properties tend to fall into categorical associations including charge based or non-specific interactions, self-association or aggregation, and biological interaction with the FcRn receptor [1,5]. In this work a series of in vitro assays addressing different categorical physicochemical properties was implemented into the developability workflow to aid in the earlier identification of PK liabilities of biologic modalities. Accelerated identification of PK liabilities would provide numerous benefits to drug development efforts, reducing the time and effort expended on sub-optimal molecular material, thereby reducing the cost, expense, and animal expenditure of non-essential in vivo studies.

METHODS

Following a comprehensive literature review and an internal validation process, four in vitro assays measuring varied categorical physicochemical properties were identified as potentially impactful predictors of PK liability: A baculovirus particle (BVP) binding assay assessing for non-specific binding, a cross interaction chromatography assay (CIC) assessing for self-interaction/association, and an FcRn column assay and FcRn cellular recycling/transcytosis assay assessing for FcRn interaction. These assays were selected based upon a multifaceted scoring motif, selecting for correlative potential to PK, appropriate throughput to be able to inform against the numbers of molecules encountered in early screening campaigns, and the ability to be easily implemented into current developability workflow schema. These assays were internally developed and implemented, then utilized to evaluate a diverse set of 43 monoclonal antibodies (mAbs) selected from an early discovery campaign which exhibited a known range of developability properties that would be representative of an early-stage protein production campaign. The individual assays were validated to ensure differentiation of in vitro output scores could be achieved, and then used to evaluate and rank order the mAbs in terms of developability risk criteria. Finally, each individual mAb was administered to hFcRn Tg32 mice for PK characterization, and the resulting clearance (CL) was used to establish the in-vitro-to-in vivo-correlation (IVIVC) of the assays and help shape the screening cascade for implementation of these in vitro assays into the existing developability protein engineering workflow.

RESULTS

Each individual assay resulted in a positive correlation to in vivo CL, with a range in ρ of ≥ 0.56-0.72 between all assays as follows: CIC assay: ρ = 0.72, BVP assay: ρ = 0.67, FcRn Column assay: ρ = 0.61, FcRn recycling: ρ = 0.56. CL values for the test set varied greatly, ranging from 0.093 mL/h/Kg to 2.21 mL/h/kg, ensuring that a broad scope of high, medium, and low clearance molecules for correlation were inclusive in the evaluation dataset for the derivation of the IVIVC relationship of each individual assay. Thresholds for acceptance values were established in each in vitro assay to minimize potential for false positive scorings, while maximizing predictability of true positive and negative scoring. All mAbs with CL values > 0.7 mL/h/kg were successfully identified as PK liabilities in at least one of the in vitro assays (n = 9). Furthermore, clinically marketed mAbs (n = 5; Eculizumab, Ramucirumab, Rituximab, Fremanezumab, and Obinutuzumab) were included as a test set of "well behaved" control compounds, and were identified in all assays as classifying with below threshold scores. Collectively, these results demonstrate the potential for this suite of assays to be utilized in a developability workflow as an accurate early-stage predictor of potential PK liability in lieu of conducting an in vivo PK evaluation.

CONCLUSIONS

The positive correlations observed between the in vitro assays and in vivo CL values support the concept of utilizing categorical in vitro assay data to augment and direct the implementation of screening approaches in protein engineering screening cascades. Utilization of this strategic approach will allow for early identification of potential PK liabilities, guiding the lead selection and optimization strategies, as well as informing the early-stage protein engineering efforts to ensure that the PK properties of a targeted drug product profile are achievable.

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Understanding metabolism of novel lipids for mRNA vaccines and therapeutics using LC/MS/MS

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BACKGROUND

Novel lipids Lipid 8 and polyethylene glycol-2000-dimyristoyl-glycerol (PEG-DMG) form an integral part of the lipid nanoparticles (LNP) that coat and protect mRNA medicines. Lipid 8 is an ionizable lipid that facilitates cytosolic transport and helps protect mRNA from extracellular nucleases. PEG-DMG increases the in vivo stability of LNP by decreasing particle aggregation, opsonization, and phagocytosis, prolonging circulation half-life.

PURPOSE

Based on FDA guidance for nonclinical safety testing of drug metabolites, metabolites of Lipid 8 and PEG-DMG in conventional animal models need to be identified and profiled. Any metabolites unique to humans (i.e., not found, or present at markedly lower levels in conventional animal models) also need to be identified.

METHODS

In vitro studies of Lipid 8 were performed by dosing rat, non-human primate (NHP), and human hepatocytes with Lipid 8 LNP; then identifying the resulting metabolites by de novo structural characterization using a combination of retention time, accurate mass, and MS/MS fragmentation. The in vivo metabolism of Lipid 8 was further confirmed by analyzing rat plasma, urine, and bile following IV infusion of live animals with Lipid 8 LNP. LC-HRMS signal was used for semi-quantitative profiling.

In vitro studies of PEG-DMG were performed by incubating rat, NHP and human sera with PEG-DMG; then identifying the resulting metabolites by matching retention time, accurate mass, and MS/MS fragmentation with synthesized standards.

The metabolite identification (MetID) workflow utilized an Agilent 1290 UHPLC, an Agilent 6550 Q-TOF, and Mass-MetaSite software to 1) evaluate the presence of expected metabolites from biotransformation via phase I and phase II metabolic pathways; and 2) identify unknown metabolites via LC-HRMS differential analysis and MS/MS structural elucidation.

RESULTS

MetID of Lipid 8 revealed twelve total metabolites, with five metabolites detected in hepatocyte cultures, eight detected in plasma, four detected in urine, and all twelve detected in bile. No human-specific metabolites were detected. Generally, urine contained more hydrophilic, lower MW metabolites (M1-M4) whereas plasma and bile contained more hydrophobic, higher MW metabolites. Lipid 8 was the predominant species in circulation over the sampling period of 24 hours. Lipid 8 concentrations fell to <10% and <1% of the maximum level in plasma by 6 h and 24 h post dose, respectively.

MetID of PEG-DMG revealed two metabolites: polyethylene glycol- monomyristoyl-glycerol (PEG-MMG) and polyethylene glycol (PEG-OH). No human-specific metabolites were detected. In vitro, PEG-DMG decreased by 81% over the course of 24 h; PEG-OH increased by 418-fold over the course of 24 h; and PEG-MMG increased by 270% during the initial 2 h but then declined by 91% (relative to initial levels at time point 0) from 2 to 24 h.

CONCLUSIONS

Both the in vitro and in vivo studies of Lipid 8 show that metabolites are formed primarily by ester hydrolysis and beta oxidation. As a result, Lipid 8 is largely cleared via the renal and hepatic routes of administration. The in vitro studies of PEG-DMG show that metabolites are formed primarily by ester hydrolysis, presumably enzymatic, with much higher rates of degradation observed in rodent sera (which contains comparatively higher abundances of esterases). In vivo studies to understand PEG-lipid metabolism in rats vs NHPs are ongoing. This research will improve our understanding of the metabolism of novel LNP components used in nucleic acid vaccines and therapeutics and provide information about the specific biotransformations involved in clearance of these novel lipids.

Lysosomal trapping measurement in multiple cell types using a High Content Imaging (HCI) system

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For correct in vivo predictions, it is essential to obtain relevant in vitro pharmacokinetic data and to determine correct intracellular free drug concentrations. Lysosomal trapping has been described to affect intracellular free concentrations of basic lipophilic drugs, which in turn may result in modified bioavailability, clearance and DDI of these compounds, and could cause phospholipidosis or drug resistance [1]. To evaluate lysosomal trapping of drugs, different methods are available, e.g., detection of lysosomal dye accumulation, or determination of intracellular concentration of a drug in the presence of lysosome inhibitors [2]. Our goal was to set up an assay for the prediction of lysosomal trapping in MDCKII and Caco-2 cell lines as well as human hepatocytes using High Content Imaging (HCI) readouts. HEK293 cells were used to compare the performance of lysosomal trapping detection with High Content Imaging versus a traditional Microplate Reader. Lysotracker Red dye was added to the cells for indirect detection of lysosomal trapping, where lysosomotropic compounds will extrude the dye from the lysosomes, resulting in reduction of fluorescent signal intensities. This change serves as the basis for IC50 calculations. We chose to test chloroquine, propranolol, imipramine and verapamil as compounds previously reported to undergo lysosomal trapping as well as rosuvastatin as a compound without lysosomotropism [1,2]. Ammonium-chloride was used as control for total inhibition of lysosomal function [2]. Ketoconazole, which is known for causing phospholipidosis and could potentially be lysosomotropic based on its physicochemical properties, was also investigated [3]. IC50 values of imipramine were similar in MDCKII, Caco-2 and human hepatocytes, and were also comparable to the available literature data obtained using immortalized cell lines [1]. The IC50 values of chloroquine differed more between the immortalized cell types and hepatocytes but were similar between the different hepatocyte lots and were also comparable with the literature data based on rat hepatocytes [2]. To our knowledge, lysosomal trapping IC50 values for these pharmaceuticals in Caco-2 and primary human hepatocyte cells are first reported here. We were also able to detect lysosomal trapping of ketoconazole in single donor human hepatocytes and Caco-2 cells. In addition to the HCI readouts, lysosomal trapping was also investigated using direct LC/MS quantification of the test compounds in the presence and absence of ammonium-chloride. In conclusion, the new HCI-based assay is a sensitive, reproducible, and fast method to evaluate lysosomal trapping for multiple cell types and compounds.

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ADAPTATION OF IN VITRO METHODS TO DETERMINE UNBOUND CELL TO MEDIUM PARTITION COEFFICIENT, $K_{p,uu}$

Virag Bujdoso-Szekely, Katalin Jemnitz, Johanna Pacsuta and Zsuzsanna Gaborik

Hepatic overall clearance is the outcome of the complex interplay between ADME processes (sinusoidal uptake, metabolism, canalicular secretion, sinusoidal efflux) [1]. Extended Clearance Model (ECM) serves as a reference to classify drugs based on their in vitro determined clearance mechanisms [1]. This model groups compounds into four classes by which the rate determining step of their elimination can be predicted. For transport proteins and metabolic enzymes only unbound drugs are available, the concentration of which determines efficacy, toxicity, pharmacokinetics, and pharmacodynamics. ECM not only serves as a classification system but also provides indirect estimates of unbound intrahepatic drug concentration i.e., unbound liver-to-blood partition coefficient ($K_{p,uu}$) [2]. If transport is the rate determining step in drug disposition, concentrations in plasma and liver might be asymmetric ($K_{p,uu} \neq 1$) that determines the rate of intracellular processes. $K_{p,uu}$ can be calculated based on K_p: hepatocellular drug accumulation and hepatic unbound fraction (f_u). The latter parameter can be measured by 3 approaches (homogenization/temperature/logD7.4 method), of which the temperature method resulted in best approach compared to calculation based on the ECM [2]. Our aim was to adapt and validate the method for f_u and $K_{p,uu}$ determination for compounds with different plasma-binding from distinct classes of ECM groups using radioactive and bioanalytical methods. Using the temperature method [2] active/passive uptake were determined at steady state for 30/60/90 minutes, at 37°C/4°C using plated human hepatocytes. Rosuvastatin, pitavastatin, atorvastatin, fluvastatin, pravastatin, verapamil and ketoconazole were used as substrates. f_u values were determined at 4°C. K_p was evaluated based on the media and intracellular concentrations, and $K_{p,uu}$ was calculated as follows: $K_{p,uu} = K_p \times f_u$. The order of f_u values is FLV ≈ PTV < ATV < RSV < PRV which correlate well to literature data, together with our $K_{p,uu}$ parameters. Results of radiolabelled versus unlabelled substrate measurements are also corresponding. Interestingly, in the literature these values vary in a broad scale possibly due to different hepatocyte lots. However, our data fits into this range of parameters. $K_{p,uu}$ and unbound intracellular concentration are parameters of growing interest in drug discovery, especially for class 3 and 4 drugs, which are cleared mostly by active and passive uptake and efflux processes [1]. Thus, intracellular unbound concentration is probably unequal to the one applied extracellularly in in vitro experiments. To generate reliable in vitro data for in vivo hepatic clearance prediction, $K_{p,uu}$ should be taken into account.

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A Unique In Vitro Assay to Investigate ABCB4 Transport Function

Csilla Temesszentandrási-Ambrus, Gábor Nagy, Annamária Bui, Zsuzsanna Gáborik

ABCB4 is almost exclusively expressed in the liver, where it plays an essential role in bile formation by transporting phospholipids into the bile. ABCB4 polymorphisms and deficiencies in humans are associated with a wide spectrum of hepatobiliary disorders, attesting to its crucial physiological function. Inhibition of ABCB4 by drugs may lead to cholestasis and drug-induced liver injury (DILI), although compared with other drug transporters, there are only a few identified substrates and inhibitors of ABCB4. Since ABCB4 shares up to 76% identity and 86% similarity in the amino acid sequence with ABCB1, also known to have common drug substrates and inhibitors, we aimed to develop an ABCB4 expressing Abcb1-knockout MDCKII cell line for transcellular transport assays. This in vitro system allows the screening of ABCB4-specific drug substrates and inhibitors independently of ABCB1 activity. Abcb1KO-MDCKII-ABCB4 cells constitute a reproducible, conclusive, and easy to use assay to study drug interactions with digoxin as a substrate. Screening a set of drugs with different DILI outcomes proved that this assay is applicable to test ABCB4 inhibitory potency. Our results are consistent with prior findings concerning hepatotoxicity causality and provide new insights for identifying drugs as potential ABCB4 inhibitors and substrates.

An Approach for Comparative Semi-Quantitative Estimation of the Efficiency of Trapping Reactive Metabolites in Human and Animal Liver Microsomes

Igor Mezine, Chris Bode, Sid Bhoopathy, Ismael Hidalgo

Purpose

To develop an approach for comparative evaluation of the efficiency of trapping reactive metabolites in human and animal liver microsomes as a tool to help validate preclinical tox species selection.

Method

The reactive metabolites (RM) of three model compounds— acetaminophen (APA), clozapine (CLZ), and nefazodone (NFZ)— were generated in human liver microsomes (HLM) as well as monkey (MkLM), rat (RLM) and mouse (MsLM) liver micosomes. RM were trapped via GSH conjugation with (+) or without (-) a competitive chemical trapping agent, N-acetyl cysteine (NAC). The corresponding stable isotope-labeled (SIL) GSH conjugates (GC) of each model compound were generated in HLM in the presence of SIL-GSH and were used as internal standards for the purpose of semi-quantification. The samples were analyzed by LC-HRAMS, and the levels of GC were established relative to those in the (-)NAC HLM incubation sample. The enzymatic trapping efficiency (ETE) in HLM, defined as the ratio of GC in (-)NAC/(+)NAC samples were highly RM-dependent, and it is suggested that poorly trapped RM (lower ETE) could be of higher toxicological concern.

Results

The enzymatic trapping efficiency (ETE) in HLM, defined as the ratio of GSH conjugates (GC) in (-)NAC/(+)NAC samples, were highly RM-dependent. Two-dimensional graphical representation of normalized (to human) ETE (NETE) vs. normalized (to human) relative yields (NRY) of GC for each RM assigns animal RM to one of four different zones (NRY/NETE) 1) Low/High, 2) Low/Low, 3) High/Low, and 4) High/High. It is suggested that achieving adequate exposure in tox species to a particular RM in zone 1 would require more attention, because RM in this category are likely to be lower in abundance and more readily trapped in animal species than in humans.

Conclusions

The developed semi-quantitative approach allows intra- and inter-species differentiation of reactive metabolites, both in terms of relative yield and relative trapping efficiency as compared to HLM.

DMPK perspectives to inform Lipid nanoparticle design and development

Liu Yun

Purpose

Appropriate cellular tropism is the most sought properties in the development of LNP. LNPs with targeted delivery profile could not only enhance the effectiveness but also reduce undesired side effect. To address this issue, several methods will be discussed to characterize the ADME/DMPK profile of LNP.

Methods

Current In vitro/in vivo characterization methods and its application to establish IVIVC will be discussed. Generally, fluorescently labeling strategies have been widely applied for LNP. In combination with IVIS imaging system, fluorescent microscope, and flow cytometry, cellular and subcellular ADME profile of LNPs will be revealed. In addition, high-throughput screening technologies, barcoded LNP will be introduced and discussed to bridge the cellular tropism of LNP across different species.

Results

High-throughput DNA barcoding system could simultaneously measure nucleic acid delivery mediated by dozens of different LNPs in a single mouse/NHP. Compared with fluorescently labeling strategies, hundreds and thousands of LNPs could be evaluated across different species in a time and cost efficient manner.

Conclusions

The LNP tropism could be evaluated in several ways. Fluorescently labeling strategies could reveal subcellular LNP distribution profile. Barcoded delivery system provides a high-throughput screening methods to accelerate the development of nanoparticle drug delivery systems.

Predictive Pharmacokinetic Platform Merging Human Tissue Chips and Translational Software

Murat Cirit, PhD, CEO & co-founder, Javelin Biotech

The estimation and optimization of drug properties to develop efficacious and safe therapies for humans is a critical step in preclinical discovery. Although efforts have been made to develop in vitro methods that deliver translational and predictive data, much testing still utilizes animal models, which create a large financial burden during lead optimization and can be inaccurate in predicting human outcomes. To address this need, we have developed an integrated platform that combines human tissue chips with translational software to predict clinical parameters of investigational drugs.

Human tissue chips, aka microphysiological systems (MPS), are traditionally microfluidic devices designed to recapitulate human physiology at the tissue level and enable long-term in vitro (co-)cultures. While current microfluidic-based tissue chips are primarily used in basic research, such technologies have limited utility in pharmacokinetic applications because the flow-through fluidic design, chip material, and small media & tissue volumes do not support drug quantification.

Javelin's single- and multi-tissue chips are designed to generate multi-scale (media- and tissue-based) data by incorporating media recirculation in a COC-based milli-fluidic chip with larger media volumes (>1ml) and tissue sizes (>200K cells per tissue) than other microfluidic chips.

Liver, kidney, and muscle tissues on single- and multi-tissue chips were evaluated both for pharmacokinetic and safety pharmacology context of use applications. Additionally, transcriptomics analysis of the liver tissue demonstrated higher transcriptional similarity scores of in vitro human liver tissues to human liver tissues than animal liver similarity scores to human liver. Then, a diverse set of compounds were tested on each tissue and tissue-specific pharmacokinetic parameters (such as hepatic and renal clearance and volume of distribution) were measured. Then, on-chip results were extrapolated to human scale with companion software using quantitative systems pharmacology (QSP)-based algorithms to quantify predictability of each CoU application. The predicted PK (CLint, CLr and VDss) parameters showed high correlation to clinical parameters.

These studies demonstrated that the Javelin Biotech platform can deliver accurate clinical predictions for several applications, and offer an alternative to, and hopefully a replacement of, laboratory animal studies for the purposes of understanding human pharmacokinetics in an intact mammal as a surrogate for human.

Evaluation of fraction unbound values over varying concentrations of CNS compounds in brain, blood, and plasma

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Biogen (Cambridge, MA)

Purpose

O-linked N-Acetylglucosamine (O-GlcNAc) is implicated in various diseases including cancers, diabetes, cardiac dysfunction, and neurodegenerative diseases1. Inhibition of OGA is proposed to delay the progression of tau-related diseases by slowing the accumulation of hyper-phosphorylated, insoluble tau filaments. Independent pharmacological proof-of-biology experiments have demonstrated that chronic OGA inhibition dramatically reduced tau pathology in brain and improved functional deficits in preclinical models of tauopathy. Indications of target mediated drug disposition (TMDD) were observed in PKPD studies. Herein, we present the in vitro binding data in several tissues where OGA is differentially expressed and show how target expression levels result in drug concentration dependent binding. These studies provide critical support in interpreting the results from PKPD studies.

Method

Brain homogenate (diluted 1 part brain:3 parts PBS buffer), fresh blood, and plasma from the rat and human were the matrices used in this assay. Concentrations of 10, 30, 100, 300, and 1000 nM of test articles A and B were spiked into the matrices and incubated for 6 h. Multiplexing LC system (LX4) connected to an API-5500 Triple Quad was used for the quantitation of this experiment. ESI MRM positive mode was MS mode selected.

Results

Compounds A and B exhibited concentration dependent binding in both the rat and human homogenates, however, there were no significant changes in concentration contingent binding in blood and plasma in either species. Compound B showed significantly more binding than compound A in both rat and human brain homogenate at 10nM. The binding in rat brain homogenate was more pronounced than human at lower concentrations indicating potentially higher expression of OGA in rat than human brain homogenate.

Figure 1. Fold increase of f_u when raising the concentration of test articles from 10nM to 1μM

Compound / Species	Brain Homogenate	Whole Blood	Plasma
1 / Rat	32	1.8	No significant change
1 / Human	12.6	No significant change	No significant change
2 / Rat	299	2.2	No significant change
2 / Human	45	1.8	No significant change

Conclusions

Concentration-dependent binding in rat and human brain where OGA is highly expressed was shown. This study corroborates the TMDD profile observed from PK/PD studies. This study was critical in deriving the PKPD parameters correctly which would otherwise result in underestimating the potencies and result in discarding promising new chemical entities. There was much more binding in the brain at lower concentrations than blood and plasma for both species, which indicates potentially more expression levels of OGA in brain than the other matrices.

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Characterization of Selective CYP46A1 Inhibitors and Activators Across Compound Library Using a High-Throughput Screening Platform

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Purpose

Cytochrome P450 46A1(CYP46A1) is predominantly expressed in brain and catalyzes the conversion of cholesterol to 24-hydroxy cholesterol as well as the rate of cholesterol production and turnover. Like other cholesterol metabolites, 24-hydroxy cholesterol has role in the central nervous system signaling. In-Vitro and In-Vivo studies have revealed that some sterols, steroids and marketed drugs can also bind to CYP46A1. Inhibition or activation of CYP46A1 enzyme can have a therapeutic potential. Here, we describe the development of rapid, sensitive and automated CYP46A1 assay in a 384-well plate format for the detection of potential inhibitors or activators using a compound library (2321 FDA approved drugs). Based on the literature, testosterone is found to be a substrate of the CYP46A1 enzyme. Therefore, testosterone is used as a probe substrate for reliability of initial screening of cassetted compound library due to short LC-MS/MS run time compared to cholesterol. Based on the cassetted analysis results individual compounds were tested with both testosterone and cholesterol as probe substrates. The present work also provides a better understanding as to which compounds should be tested as potential inhibitors or activators for CYP46A1.

Methods

Time and Protein Concentration

To determine the optimum protein concentration and incubation time for the CYP46A1 assay, a range of protein concentrations (0.1, 0.25 and 0.5 mg/mL) of CYP46A1 bactosomes were incubated across several incubation times (0, 5, 10, 30, 60, and 120 minutes). Testosterone or cholesterol were added as probe substrate to designated wells and the NADPH was added to initiate the reaction. The samples were filtered by centrifugation and analyzed using LC-MS/MS to monitor 16 beta hydroxy testosterone or 24 hydroxy cholesterol metabolite formation respectively.

Determination of K_m and V_{max}

Using the protein concentration and incubation time determined in the previous section, the formation of testosterone or cholesterol metabolites were measured with several concentrations of probe substrate covering the expected range of Km values.

Validation of the CYP46A1 assay in 384-well plate

The CYP46A1 testosterone inhibition/activation assay was performed in a high-throughput 384-well format using Bravo (liquid handler). CYP46A1 bactosomes (0.25 mg/mL) in potassium phosphate buffer containing magnesium chloride were pre-incubated for 5 minutes. Soticlestat (final concentrations of 0.5 and 5uM) was used as a positive control inhibitor and vehicle was used as a negative control. The samples were incubated for 30 minutes at 37°C The formation of 16 beta hydroxy testosterone compared to the vehicle control will be used to calculate % inhibition as well as performance of the assay.

Cassette Analysis

Test compounds (library containing 2,321 compounds) were pooled into 384-well plates (4 compounds/well) using cassette analysis for initial screening. The assay was performed as described above.

Individual Testing

Based on the results of the cassette analysis, 242 test compounds were defined as hits and selected for individual testing.

CYP46A1 Cholesterol Inhibition/Activation assay

The same 242 test compounds and control were then tested with cholesterol as probe substrate. The samples were incubated for 30 minutes at 37°C in a CO2 incubator. The formation of 24 hydroxy cholesterol compared to the vehicle control will be used to calculate % activity and % inhibition.

Results

The time and protein linearity results showed that 30 min incubation time and 0.25 mg/mL CYP46A1 bactosomes were optimum to run testosterone and cholesterol assay. The control inhibitor, soticlestat, in both testosterone and cholesterol assay showed inhibition of 99% and 97% respectively. The % CV was found to be less than 2.5% for the testosterone inhibition using soticlestat at both the concentrations tested across the 384 well plate. Cassette testing detected the activation or inhibition of CYP46A1 activity in 69 wells out of two 384 well plates. This resulted in 242 compounds to be individually tested for inhibition or activation of CYP46A1 using both testosterone and cholesterol. Cassetted analysis vs individual testing of compounds in the testosterone assay for inhibition or activation data showed less than 20% CV between the results. The individual testing resulted in 15 common inhibitors and 12 common activators of CYP46A1 for the testosterone and cholesterol assays.

Conclusions

The system described here not only establishes high throughput automated method using 384-well plates to predict the therapeutic inhibitors or activators of CYP46A1. The low % CV for the inhibition of testosterone using soticlestat reveals the reliability of the assay across the 384 well plate. The assay also shows testosterone could be used as the probe substrate for ease of analysis as compared to cholesterol. Cassetting was reliable to screen the compounds for the activation or inhibition of CYP46A1 activity. A possible limitation to this approach is an interaction between compounds leading to false positive or false negative results. To demonstrate the efficacy of this screen, we identified clotrimazole as an inhibitor of CYP46A1 which agrees with the previous literature. Our study results give insight into structurally diverse compounds that can inhibit or activate the CYP46A1 enzyme.

A Comprehensive LC-MS Based Review of Factors Influencing Protein Biotinylation

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Purpose

Biotinylation is one of the most common protein conjugations applied in ligand-binding assays, at which critical biotinylation conditions are required for quality control and CMC process. In some cases, inconsistent protein biotinylation occurred followed by the failure of the ligand binding assay. Therefore, urgent needs and raised interest are to understand the impacts of various Biotin labeling conditions (i.e., challenge ratio) on the efficacy and efficiency of protein biotinylation.

In this study, Relationship between biotin labeling efficiency and labeling conditions was then clearly illustrated, contributing to further optimization of protein conjugation with biotins, as well as other potential labeling reagents.

Methods

Mass spectrometry combined with liquid chromatography (LC-MS) is one of the powerful tools to elucidate protein-protein and ligand-protein interactions. In this study, LC-MS based intact MS and peptide mapping methods were successfully developed to detect a protein target and its biotinylation under different labeling conditions.

Results

Four biotinylation labeling conditions were investigated. Biotinylation efficacy and efficiency were interfered by the challenge ratio and the incubation time, while the starting material amount and light condition provided similar biotin levels. In addition, same top 3 Lys sites were labeled by biotin under different labeling conditions.

Conclusions

A combination of intact MS and peptide mapping analysis was successfully developed and applied to detect the protein target and its biotinylation. Total biotin levels and the specific labeling sites were verified and relatively quantified by the developed MS-based analysis. The factors influencing the labeling efficiency of protein biotinylation were then investigated at both protein and peptide level. Further optimization of protein biotinylation will be performed based on the results of the dataset.

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