



Preclinical Services Catalog 2019



Bienta is a trademark for preclinical biology services of Enamine, the world's largest supplier of small molecules for drug discovery research. Bienta is operating as a Contract Research Organization providing biomolecular screening (HTS) services as well as comprehensive bioanalytical support for drug discovery projects, including in vitro ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) testing as well as pharmacokinetics (PK), toxicity studies and animal pathology models.

Our standard services are described below. Test conditions are amendable upon request and custom assays development option is available to meet your needs.



Discover your molecule with us!

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04

In Vitro ADMET tests

Shake-Flask Solubility Assay

Background:

Determining compound solubility is an essential tool for early stages of the drug discovery process, as well as for lead optimization. Low solubility can lead to unpredictable and unreliable results during in vitro testing, thereby increasing the development costs. Solubility issues at the later stages of the drug discovery may lead to poor bioavailability, underestimated toxicity and other obstacles, lowering the chances of a given drug candidate for success. Solubility can be measured either as a kinetic or thermodynamic value. Typically, for early-stage drug discovery the kinetic solubility method is used, as it is fast and well suited for HTS format. In this case, solid compounds are first dissolved in DMSO and then linear serial dilutions of each compound are added to an aqueous

buffer and observed for precipitate formation when the compound is not completely soluble. Precipitate appearance can be evaluated by light scattering (laser nephelometry method). For better precision, the solution can be subjected to high-speed centrifugation or filtration using special solubility filter plates and then the compound concentration is measured in the saturated solution directly by UV or LC-MS using separately built calibration curves. Thermodynamic solubility is important for lead optimization and drug formulation stages. It is usually determined for pure compounds: crystalline powders, amorphous substances and liquids. In this modification of solubility assay long (24 hours or more) incubations are required. Measurements are usually performed by the shake-flask method with UV-Vis or LC-MS detection.

Service Details:

This commonly used shake flask protocol is based on the use of Millipore Multiscreen solubility filter plates or centrifugation followed by UV-Vis quantitation of dissolved compounds. Microplate reader SpectraMax Plus (Molecular Devices) is used in our lab for UV-Vis measurements. LC-MS/MS quantitation (API3000 mass-detector, AB Sciex) can also be done for poorly UV-absorbing compounds, mixtures, and compounds prone to degradation. Kinetic solubility measurements are performed starting from DMSO stock solutions of the test articles; powders are used for thermodynamic solubility measurements.

Typical assay conditions are as follows:

- Kinetic solubility measurements: 2 h shaking time at 25°C in an aqueous buffer;
- Thermodynamic solubility measurements: 4 h and 24 h shaking time at 25°C.

The assay is run in duplicates. One or two reference compounds are included in each test batch. Assay/protocol customization is available upon request.

Deliverable:

Full study report is provided, including solubilities and calibration curves for test and reference compounds.

Sample Submission: Sample requirement is at least 50 µL of 20 mM stock compound solutions in DMSO for kinetic solubility measurements, and 2 x 4 µmoles of dry compound for thermodynamic solubility measurements. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.

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Solubility Assay by Laser Nephelometry

Background:

This is a high-throughput and affordable option for rapid evaluation of kinetic solubility for a large number of compounds in any aqueous buffer. Linear serial dilutions of each compound to be analyzed are prepared from DMSO stock solutions in an aqueous buffer of the specified composition and pH, and the approximate precipitation points for each compound are determined from the light scatter graphs (working solubility range is from 50-100 µM up to 1-2 mM). This assay is typically performed in duplicates.

For a rough estimate of aqueous solubility ("solubility not lower than..."), this method can be simplified: instead of linear serial dilutions, light scattering can be measured at one or two concentrations of compounds (high-throughput solubility threshold method). Those compounds yielding light scattering signal values significantly higher than that of the highly soluble compound (2'-deoxy-5-fluorouridine) are considered insoluble at tested concentrations. As a control, a reference compound poorly soluble at the same concentrations is used.

Service Details:

For the light scattering measurements we use BMG Labtech Nephelostar laser microplate nephelometer, which operates with 96-well plates. We typically perform high-throughput solubility threshold assay in singletons at up to 2 mM compound concentrations with Ondansetron as a poorly soluble reference and deoxyfluorouridine as a well soluble one. This method can be modified upon request.

Deliverable:

Compounds solubility normalized to deoxyfluorouridine (100% soluble) and Ondansetron (0% solubility) is calculated. Selected ranges of Relative Solubility (>0.8, 0.6-0.8 and <0.6) allow dividing compounds in clearly distinguishable groups (good solubility, intermediate solubility and poor solubility) at the chosen solubility threshold. Full study report is provided.

Sample Submission: Typically, a minimal accurately weighable quantity of dry compound (~1 mg or 2 µmol) or 50 µL of 20 mM stock DMSO solution) is required for this assay. However, the exact required amount would depend on the solubility range of interest.



LogD / LogP

Background:

A certain balance of lipophilicity and hydrophilicity is required for a successful drug candidate. Water solubility is essential for drug to be dissolved in plasma and other aqueous biological fluids, whereas lipophilicity is indispensable for penetrating through biological membranes and is of high importance for the compounds aimed for oral dosing. A widely used model to access physicochemical properties of the compound is LogP (partition coefficient). Lipophilicity is typically accessed as the distribution of the tested compound between two solvents - typically non-aqueous organic (n-octanol) and aqueous (pH-buffered water), and then LogP is expressed as a log₁₀ of the concentration ratio between two phases.

LogP is widely used in cheminformatics and is a component of the Lipinski's "rule of five", that is a golden standard to evaluate a drug-likeness of a compound. According to this rule, the successful drug candidate should possess LogP value not greater than 5.

LogD is a distribution coefficient widely used to measure the lipophilicity of ionizable compounds, where the partition is a function of the pH. For non-ionizable compounds LogP = LogD throughout pH range, whereas for ionizable compounds LogD takes into account the partition of both ionized and non-ionized forms. LogD is more convenient for practical measurements, as it takes into account solution pH, which is important for the analysis of the drug candidate properties in various biologic media with different pH values.

Service Details:

We use the miniaturized shake-flask method for LogD/LogP determination. The standard protocol is as follows: 10 μ L aliquot of 10 mM DMSO compound stock is dissolved in the previously mutually saturated* mixture containing 990 μ L of PBS buffer (pH 7.4) and 100 μ L of octanol followed by mixing in a rotator for 1 hour at 30 rpm. Incubations are done in duplicates. The concentrations of the compounds in each phase are measured by LC-MS (API3000, AB Sciex). Other pH buffers and customized assay setups are available upon request. Reliable measurable range is approximately -1 to 4.5.

*Reaching an equilibrium between aqueous and octanol phases may take much longer time than partition of a tested compound between the same phases. For this reason, mutual saturation of aqueous and octanol phases is important for stable and reliable LogD/LogP measurements.

Note:

Pricing for this service refers to logD determination at one selected pH. For non-charged compounds (at the pH used for logD measurements) logD equals logP, and there is no extra charge for logP measurement. For compounds with known pKa values, price for logP determination will be equal to the price of logD determination; the same applies for the cases where in silico calculated pKa values are used. Pricing for the experimental pKa measurements is subject to discussion and depends on the properties of the tested compounds.

Deliverable:

Calculations of the partition ratios (peak area of the analyte in octanol phase to the peak area of the analyte in PBS buffer). Full study report is provided.



Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 50 μ L of 10-20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.

Determining pK_a

Background:

The tendency of a compound to donate a proton is expressed as its acid ionization constant (dissociation constant), or K_a, and is usually represented as pK_a (pK_a = -log K_a). There is a documented correlation

between pK_a of a drug and its solubility that is widely used for the prediction of drug behavior in vivo. pK_a value may have a profound effect on pharmacological activity, bioavailability and toxicity of a drug, and therefore is a valuable readout in the selection process.

Service Details:

We determine pK_a of a test article by potentiometric titration, whereby the compound is dissolved in slightly acidified water and slowly titrated with sodium hydroxide while measuring the equilibrium pH of the solution. To allow full dissolution of the tested compounds, the titration is typically done in a binary methanol-water mixture (1:9, v/v). In some cases methanol content would have to be increased or replaced with another organic solvent (e.g. acetonitrile). pK_a values are calculated from the resulting pH versus buffering capacity graphs. Practical working range of this protocol is approximately from 2 to 12 pH units.

Deliverable:

Final report comprising information on the analysis, methodology, raw data, and interpretation of the results is provided.

Sample Submission:

Approximately 40 μ mol (20 mg) of dry compound is necessary for this test.



Plasma Protein Binding Assay (Equilibrium Dialysis)

Background:

The vast majority of small molecule drugs are reversibly bound to blood plasma proteins (albumin, lipoproteins, α 1-acid-glycoprotein) soon after the administration. The bound drug molecules fraction is generally considered not available for interaction with their biological targets. Determining the

extent of drug-protein binding is among key stages in drug development as it influences compound efficacy, dosing, clearance rate, and potential for drug interactions. Rapid Equilibrium Dialysis (RED) is the "golden standard" method to determine the percentage of the plasma protein binding (%PPB) for a drug candidate.

Service Details:

To determine the capabilities of a drug to bind plasma proteins, we spike test compounds at a single concentration (typically 1 μ M or 2 μ M) into plasma followed by dialysis against the buffer until equilibrium is reached. The assay is performed in a 96-well dialysis unit HTD96b dialyser (HTDialysis). Each individual well consists of 2 chambers separated by a vertically aligned dialysis membrane of certain pore size (MWCO 12-14 kDa). Non-diluted plasma spiked with the compound of interest is added into one chamber, whereas the dialysis buffer is added into the other one. Unbound compound diffuses from the plasma chamber to the buffer chamber until equilibrium is reached. Concentrations of the compound in plasma and buffer are then determined

by LC-MS, and the percentage of plasma protein bound compound is calculated. All incubations are performed in duplicates. Whole plasma or an individual plasma protein (albumin, AGP) can be used in this assay. Plasma from different species is available, including human, mouse, dog and rat. Reusable dialysis apparatus is usually employed, however disposable units (RED, Thermo Scientific) are available upon request.

Deliverable:

The percentage of plasma protein-bound compound is calculated based on the LC-MS measurements of the compound concentrations in plasma and buffer solutions, full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 50 μ L of 10-20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.

Tissue Binding Assay (Equilibrium Dialysis)

Background:

Fraction unbound (f_u) of tissue is an essential parameter for the calculation of *in vivo* free drug

concentrations in the tissues (free drug concentration = total drug concentration $\times f_u$).

Service Details:

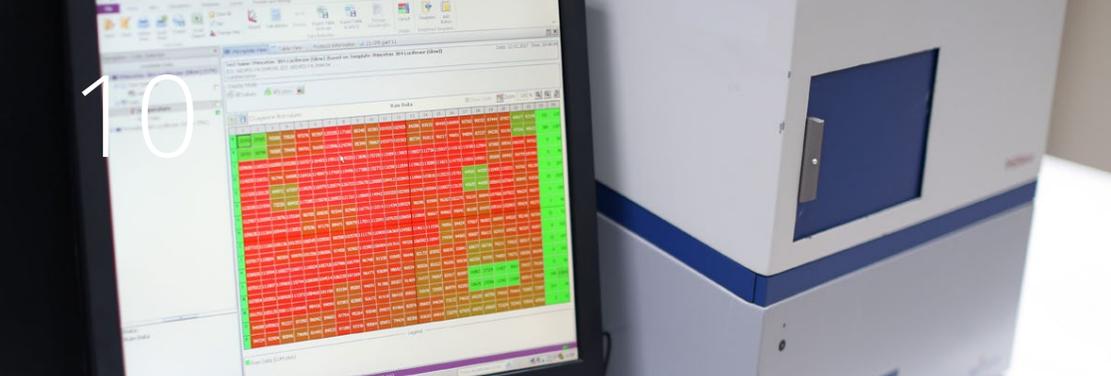
We determine binding capabilities of drug candidates to tissues by spiking test compounds into tissue homogenate in DPBS (1:4, w/v) and dialyzing against buffer until equilibrium is achieved (*Riccardi K. et al., Drug Metabolism and Disposition April 2018, 46 (4) 415-421*). The assay is performed in a multiple-use 96-well dialysis unit HTD96b dialyser (HTDialysis). Each individual well unit consists of 2 chambers separated by a vertically aligned dialysis membrane of predetermined pore size (MWCO 12-14 kDa). Tissue homogenate spiked with the compound of interest is added to one chamber and the dialysis buffer to the other chamber. Free compound diffuses from the

tissue chamber to the buffer chamber until equilibrium is reached. Concentrations of the compounds in homogenate and buffer are determined by LC-MS, and the fraction unbound is calculated. All incubations are performed in duplicates. This binding test can be performed for various tissues, including brain, liver, kidney, or lungs.

Deliverable:

Fraction unbound (f_u) and recovery are calculated based on the LC/MS measurements of the compound concentrations in homogenate and buffer solutions.

Sample Submission: A minimal weighted amount of dry compound (~1 mg) or 100 μ L of 10 mM stock DMSO solution is required for this assay. We do not need to know structures of the molecules for ADME testing. However, we ask our customers to provide brutto formulas, if at all possible, for all studies involving MS detection.



Microsomal Binding Assay (Equilibrium Dialysis)

Background:

Nonspecific microsomal binding in the *in vitro* metabolic assays can lead to an underestimation of the microsomal clearance because only the unbound

substrate is free to interact with drug metabolizing enzymes in microsomes. Determination of the unbound intrinsic clearance ($Cl_{in,u}$) is essential for the accurate comparison of compounds.

Service Details:

We determine binding capabilities of drug candidates to mouse, rat or human microsomes by spiking test compounds at concentration of 2 μ M into microsomes in phosphate buffer (0.42 mg of liver microsomal protein per ml) and dialyzing against buffer until equilibrium is achieved. The assay is performed in a multiple-use 96-well dialysis unit HTD96b dialyser (HTDialysis). Each individual well unit consists of 2 chambers separated by a vertically aligned dialysis membrane of predetermined pore size (MWCO 12-14 kDa). Free compound diffuses

from the tissue chamber to the buffer chamber until equilibrium is reached. Concentrations of the compounds in the each chamber are determined by LC-MS, and the fraction unbound is calculated. All incubations are performed in duplicates.

Deliverable:

The extent of binding is reported as a fraction unbound (f_u) value.

Sample Submission: A minimal weighted amount of dry compound (~1 mg) or 100 μ L of 10 mM stock DMSO solution is required for this assay. We do not need to know structures of the molecules for ADME testing. However, we ask our customers to provide brutto formulas, if at all possible, for all studies involving MS detection.

Plasma Stability Assay (human, rat, mouse)

Background:

Determination of stability of the potential drugs in plasma is indispensable in early stages of the drug discovery process, as it is crucial for pharmacokinetic readouts and has direct impact on *in vivo* efficacy. Certain classes of drug molecules, such as those containing ester or amide-linked groups are prone to

enzymatic hydrolysis by plasma esterases, amidases or proteases. On the other hand, enzymatic activation of some prodrugs that takes place in plasma is essential for their function. Hence, plasma enzymes can significantly alter the bioavailability of the active compounds, and therefore determination of the compound stability in plasma has both pharmacokinetic and clinical significance.

Service Details:

Incubations are carried out in 96-well polypropylene plates in 5 aliquots of 70 μ L each (one for each time point). Test compounds (1 μ M, final solvent concentration 1%) and reference compounds Verapamil and Propantheline are incubated at 37°C. Five time points over 120 minutes are analyzed (0, 20, 40, 60 and 120 min). All tests are performed in duplicates. The samples are analyzed by HPLC-MS (API3000, AB Sciex).

Deliverable:

The percentage of parent compound remaining in plasma after incubation is plotted versus incubation time; plasma half-life ($T_{1/2}$) is calculated from the obtained curve. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 50 μ L of 10-20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.

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Chemical Stability Assay

Background:

Stability in aqueous solutions is a fundamental requirement to a successful drug candidate. Degradation may be caused by a variety of mechanisms: hydrolysis, oxidation, light-catalyzed degradation and others. In early stages of drug discovery, screening for stability in buffer solutions at acidic, neutral and basic pH is desirable for eliminating potentially troublesome candidates.

Chemical stability analyses are often performed by HPLC with UV-Vis detection at three wavelengths. Yet,

in some cases degradation products may be poorly separated from parent compounds, causing inaccuracy in analysis. For this reason we recommend to perform this analysis by LC-MS. The assay is performed in a reusable 96-well Teflon plate (Millipore) to avoid possible artifacts caused by adsorption of certain compounds to polypropylene surfaces. Upon request, polypropylene plates can be used in the same assay setup for the non-specific binding assessment, which can be conveniently combined with the chemical stability assay, as PTFE (Teflon®) and polypropylene have different binding characteristics.

Service Details:

Glycine buffer (pH 8 – 11), PBS (pH 7 – 8) and acetate buffer (pH 4-6) are used in this assay to cover main pH ranges used in ADME. Stock solutions at a concentration of 10 mM of the test compounds are prepared in DMSO and stored at -20°C. Working concentration of test compounds is 1-5 µM solution in DMSO and buffers. The compound solutions are incubated in experimental buffers at 37°C for specified time intervals. The sample aliquots are taken at 6 time points: 0, 60, 120, 180, 240 and 300 min. To stabilize the samples prior to HPLC-MS analysis, they are stored at -25°C in 66% methanol/33% buffer, covered with adhesive sealing film. All samples are analyzed by HPLC-MS in a single batch within 8 hours after collection.

The HPLC-MS measurements are performed using Shimadzu VP HPLC system coupled with tandem mass spectrometer API3000 (AB Sciex). Data acquisition and analysis are performed using Analyst software (AB Sciex).

In this assay, propantheline is used as a quality control. This compound is stable at acidic pH, slightly unstable at pH 7.4 and unstable at pH 9.4.

Deliverable:

Stability is calculated as % test compound remaining relative to the T=0 peak area. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 µmol) or 50 µL of 10-20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.

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Hepatic Microsomal Stability (human, rat or mouse)

Background:

Liver is a primary site of drug metabolism, and drug metabolic transformations may have significant impact on its efficacy and safety. For this reason, drug candidates are screened early in the discovery process for metabolic stability. Microsomes from human or animal liver are useful models to quickly and inexpensively predict hepatic clearance in vitro for the corresponding species. Stability experiments

can be done either with hepatic microsomal fraction to investigate only Phase I metabolism or with the S9 fraction, which consists of both hepatic microsomes and cytosol. The advantage of using S9 fraction is that it contains both Phase I and Phase II enzymes and can be used to investigate Phase II metabolic pathways in vitro, when supplemented with the corresponding cofactors such as UDPGA (for glucuronidation) and PAPS (for sulphation).

Service Details:

Metabolic stability assays are typically performed using mouse, rat, or human microsomes or S9 fraction (microsomes from other species are available upon request). Test compounds are incubated with microsomes supplemented with cofactors at 37°C. Typical conditions are the compound concentration of 2 µM and 5 sampling time points over 40 min, in two independent replicates. At each time point, the reactions are terminated with acetonitrile. The samples

are centrifuged and the relative parent compound depletion is evaluated by LC-MS/MS. The incubation of two control drugs with microsomes and blank control reaction without co-factors are used as controls.

Deliverable:

Data include parent compound percent remaining, half-life ($t_{1/2}$), and intrinsic clearance (Cl_{int}) values. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 µmol) or 50 µL of 10-20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.



Single Time Point Microsomal Stability Pre-Screen

Background:

At early drug discovery stages it is critical to rapidly evaluate the metabolic stability of many new chemical entities. Single time point microsomal stability pre-screen assay, focused on stability ranking of large compound sets, allows increasing throughput and

reducing costs. Moderate incubation time used in this assay (e.g. 15 min for mouse liver microsomes) provides valid metabolic stability evaluations for both unstable and stable compounds, which are in good agreement with those derived from the standard multi time point experiments.

Service Details:

Single time point microsomal stability pre-screen is a 96-well plate automated assay performed on sets starting from 30 compounds. Test compounds are incubated with microsomes under the standard hepatic microsomal stability assay conditions. The parent compound loss is evaluated by LC-MS/MS measurements (API3000 detector, AB Sciex). This

service also includes incubation of two control drugs and a control without co-factors.

Deliverable:

Data include parent compound percent remaining. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μmol) or 50 μL of 10-20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, which should be discussed for each particular project. We do not need to know structures of the molecules for ADME testing. However, we ask our customers to provide brutto formulas for all studies involving MS detection.



Hepatocyte Stability (mouse, rat)

Background:

Drug metabolism and detoxification of about three quarters of all known drugs are primarily carried out in the liver. Hence, drug candidates have to be screened early in the discovery process for metabolic stability in order to rank them for further development and to

predict in vivo hepatic clearance values. Hepatocytes contain the full range of both Phase I and Phase II drug metabolizing enzymes, hepatic transporters, and cofactors. Hepatocytes can, therefore, serve as a very good in vitro model to determine in vitro clearance of test compounds as well as their metabolites formation, likely to reflect the in vivo metabolic processes.

Service Details:

Hepatocyte stability assay is performed using primary cryopreserved mouse or rat hepatocytes. Compounds at 3 μM concentration are incubated in the presence of hepatocytes at 37°C. The reactions are performed in two replicates per compound and terminated with acetonitrile at each of the 6 sampling time points: 0, 5, 10, 30, 60 and 120 min incubation. The samples are then centrifuged and the relative parent compound

depletion is determined by LC-MS/MS. The incubation of two positive control compounds with hepatocytes and a blank control reaction are used to verify assay validity.

Deliverable:

Data include parent compound percent remaining. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μmol) or 50 μL of 20 mM stock DMSO solution is required for this assay. For multiple ADME assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.



Cytochrome P450 Inhibition Assay (fluorogenic, panel of 5 CYP450)

Background:

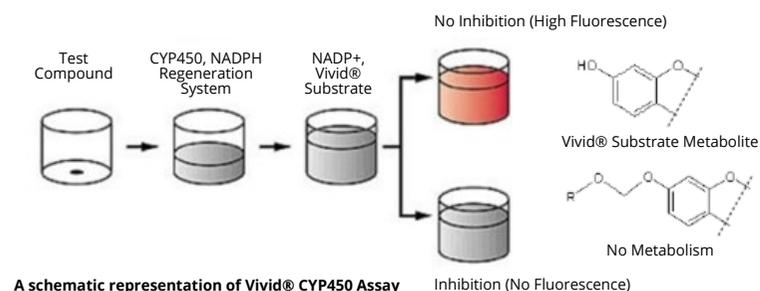
Cytochrome P450 (CYP) enzymes represent a heme-containing protein superfamily metabolizing a broad variety of xenobiotics, including drugs and toxic chemicals. 11 CYP families are expressed in a human liver and gastrointestinal tract (CYP1A2, CYP2A6, CYP2B6, CYP2C8/9/18/19, CYP2D6, CYP2E1, and CYP3A4/5), and 5 of them (CYPs 1A2, 2C9, 2C19, 2D6 and 3A4) are involved in about 95% of the known drug metabolism. Cytochrome P450s are of critical importance due to the two of the most significant problems in clinical pharmacology: metabolism-mediated drug-drug interactions (DDI) and individual variability in drug metabolism. Most drugs undergo deactivation by CYPs, either directly or by facilitated

excretion from the body. Some substances are bioactivated by CYPs to form pharmacologically active compounds. Also, many drugs may increase or decrease the activity of various CYPs due to the ability of binding to them. It is important to evaluate the potential inhibition of a new drug candidate for the most clinically relevant CYP450 enzymes. CYP450 inhibition may potentially lead to elevated in vivo plasma levels of a co-administered drug metabolized by the inhibited enzyme, and, consequently, to adverse drug reactions and toxicity. During the early stages of drug discovery process, routine assessment to identify the following major CYP enzymes for potential metabolism-mediated interactions is recommended: CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

Service Details:

The high-throughput fluorogenic CYP450 inhibition assay is fast and cost-effective method most frequently applied during drug discovery process. The potential for CYP450 inhibition is assessed by performing in vitro inhibition studies using specific fluorogenic CYP450 substrates with the corresponding individually expressed CYP450 enzymes and NADPH regeneration

system (Vivid® CYP450 Screening Kits). The fluorogenic probes are transformed by CYP450 to give fluorescent compounds (hydroxycoumarin or resorufin analogues). The fluorescent signal produced from reaction is directly proportional to the cytochrome P450 activity. In the cases when tested compounds interfere with the CYP450 enzyme-substrate reaction, the fluorescent signal decreases, which is detected using fluorometric multi-well plate reader.



Fluorogenic CYP450 substrates and reference inhibitors used in CYP450 inhibition assay

CYP450	Substrate	Reference Inhibitor
1A2	EOMCC	α -Naphthoflavone
3A4	BOMCC	Ketoconazole
2C9	OOMR	Sulphaphenazole
2C19	EOMCC	Ticlopidine
2D6	MOBFC	Quinidine

For the rough estimate, single point assays are typically performed for each compound at 10 μ M concentration or another concentration stipulated by the customer. Reference inhibitors specific for each CYP450 enzyme are used to assess CYP450 inhibition in the control experiments for every batch of tested compounds. Test concentrations of the reference compounds correspond to approximately 50x fold reported IC50 values for the corresponding cytochromes P450, which is expected to produce 80-100% inhibition in the properly performing assay. The negative control (baseline) does not contain CYP450 enzyme. If a noticeable inhibition is detected, the IC50 values for the tested compounds can be

determined upon request. For this purpose, 8-point, 3-fold serial dilution dose-response inhibition curves of the compounds are built, starting at 100 μ M (or another) compound concentration. Based on this data, IC50 values for a compound for each of the CYP450 enzymes are calculated using GraphPad Prism software. All test points are performed in quadruplicates.

Considering that interference can occur from the test compound exhibiting intrinsic fluorescence or fluorescence quenching, which can lead to the false results, it is recommended to test them using alternative LC-MS/MS based assay.

Deliverable:

Either single point assay data for each compound at 10 μ M concentration or IC50 values based on 8-point, 3-fold serial dilution dose-response inhibition curves (upon request). Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 100 μ L of 10 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, which should be discussed for each particular project.



LC-MS/MS based Cytochrome P450 Inhibition Assay (Panel of 5 or 7 CYP450)

Background:

Cytochrome P450 (CYP) enzymes represent a heme containing protein superfamily metabolizing a broad variety of xenobiotics, including drugs and toxic chemicals. 11 CYP families are expressed in a human liver and gastrointestinal tract (CYP1A2, CYP2A6, CYP2B6, CYP2C8/9/18/19, CYP2D6, CYP2E1, and CYP3A4/5). Five major isoforms (CYPs 1A2, 3A4, 2C9, 2C19, and 2D6) are involved in about 95% of the known drug metabolism. Recent studies showed the increasing role of CYP2C8 and CYP2B6 in metabolism of numerous drugs and important endogenous compounds. Cytochrome P450s are of critical importance due to the two of the

most significant problems in clinical pharmacology: metabolism-mediated drug-drug interactions (DDI) and individual variability in drug metabolism. It is important to evaluate the potential inhibition of a new drug candidate for the most clinically relevant CYP450 enzymes. CYP450 inhibition may potentially lead to elevated in vivo plasma levels of a co-administered drug metabolized by the inhibited enzyme, and, consequently, to adverse drug reactions and toxicity. Assessment of the following CYP450 enzymes inhibition by a new drug candidate is recommended by FDA¹ and EMA^{2,3}: CYP3A4, CYP2C9, CYP2C19, CYP2D6, CYP1A2, CYP2C8 and CYP2B6.

Service Details:

The potential inhibition of 7 major cytochromes CYP1A2, CYP3A4, CYP2C9, CYP2C8, CYP2C19, CYP2D6, and CYP2B6 is assessed using LC-MS/MS based assay, in which biotransformations of the CYP450 specific substrates are used as markers to quantify the enzymatic activity. The FDA recommended CYP-selective substrates are used in the assay, which represent well characterized currently or previously marketed drugs. Quantification of a decrease in the formation of a

metabolite in the presence of an inhibitor compared to the vehicle control is used to determine the CYP450 inhibition. Due to high specificity of the substrates to CYP450 isoforms not only individually expressed human cytochromes can be used in the assay but also human liver microsomes comprising a full set of CYP450 enzymes. The human liver microsomes assay is more comparable to the in vivo processes occurred in the liver and it is considered as "gold standard" for in vitro drug-drug interaction evaluation. This testing system is accepted for regulatory in vitro DDI studies.

Isoform-specific CYP450 substrates, metabolites and inhibitors

CYP450	Substrate	Metabolite	Reference Inhibitor
1A2	Phenacetin	Acetaminophen	Furafylline
3A4	Testosterone	6 β -Hydroxytestosterone	Ketoconazole
2C9	Diclofenac	4'-Hydroxydiclofenac	Sulphaphenazole
2C19	S-Mephenytoin	S-4-Hydroxymephenytoin	Tranylcypromine
2D6	Dextromethorphan	Dextrorphan	Quinidine
2B6	Efavirenz	8-Hydroxyefavirenz	Ticlopidine
2C8	Amodiaquine	Desethylamodiaquine	Montelukast

Advantages of LC-MS/MS method over fluorogenic assay:

- LC-MS/MS analysis method is sensitive and specific, therefore human liver microsomes can be used. The fluorescent probes are not isoform-specific and should be used only with individual recombinant cytochromes;
- In the fluorogenic assay, interference can occur from the test compound exhibiting fluorescence or fluorescence quenching and lead to false results.

The service is available both in panel of 5, comprising the most clinically important CYPs (1A2, 3A4, 2C9, 2C19, and 2D6), and more expanded panel of 7 (1A2, 3A4, 2C9, 2C19, 2D6, 2B6, and 2C8) formats. Both individual recombinant cytochromes and human liver microsomes can be used. For the rough estimate, single point assays are typically performed for each compound at 10 μ M concentration or another concentration stipulated by the customer. All test points are performed in duplicates. If a noticeable inhibition is detected, the IC50 values (test compound concentration which produces 50% inhibition) can be determined upon request. For this purpose, dose-

response inhibition curves (8 points, 3-fold serial dilution) of the test compound and reference inhibitor are built starting at 100 μ M concentration. The IC50 values are calculated using Microsoft Excel and GraphPad Prism software. Reference inhibitors specific for each CYP enzyme are used to assess inhibition in the control experiments for every batch of tested compounds. Test concentrations of the reference compounds correspond to approximately 5x fold of IC50 values for the corresponding cytochromes P450, which is expected to produce 80-100% inhibition in the properly performing assay.

Deliverable:

Either single point assay data for each compound at 10 μ M concentration or IC50 values for the tested compounds based on 8-point, 3-fold serial dilution dose-response inhibition curves (upon request). Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 100 μ L of 10-20 mM stock DMSO solution is required for this assay.

¹ US FDA Draft Guidance for Industry – In Vitro Metabolism- and Transporter-Mediated Drug-Drug Interaction Studies. Oct 2017. <https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM581965.pdf>

² European Medicines Agency (EMA) Guideline on the Investigation of Drug Interactions. June 2012 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf

³ Concept paper on a revision of the Guideline on the investigation of drug interactions, March 2017 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2017/04/WC500225363.pdf



Cytochrome P450 Time Dependent Inhibition (IC₅₀ shift)

Background:

Time dependent inhibition (TDI) gains an increasingly greater attention as a predictor of the drug-drug interaction potential of clinical candidates. IC₅₀ shift assay is a current standard approach for preliminary assessment of TDI. In addition to competitive inhibition of CYP450, some compounds display time dependent inhibition. CYP450 mediated transformation of these compounds results in metabolites which act as reversible inhibitors or modify chemically enzyme (e.g. via covalent bond formation). Their inhibitory potency increases with incubation time. TDI is manifested by difference in

IC₅₀ values measured under two different conditions: pre-incubation of a test article with an enzyme and a cofactor NADPH, which produces metabolites, and pre-incubation without NADPH. TDI can be divided into a number of mechanistic categories including irreversible (covalent) modification of the enzyme (mechanism based inactivation), quasi-irreversible (metabolite-intermediate complex), reversible (metabolite more potent inhibitor than parent), etc. TDI inhibition is of particular importance because it may result in a long-lasting inhibition since usually the enzyme re-synthesis is required to recover the CYP450 activity.

Service Details:

Time dependent inhibition of 5 major cytochromes CYP1A2, 2C9, 2C19, 2D6, and 3A4 is studied using IC₅₀ shift approach. In this assay, the IC₅₀ value (concentration which produces 50% inhibition) of a test compound is determined under two different experimental conditions:

- 1) 30 min pre-incubation of test article with enzyme without NADPH,
- 2) 30 min pre-incubation of test article with enzyme and NADPH.

Following the pre-incubation step, the specific substrates are added to the incubation mixture. The experiment is performed using non-dilution method (Kozakai et al, Drug Metab. Pharmacokinet. 2014, 198-207).

If the compound is a time dependent inhibitor, an increase in potency will occur between the measurements

done at 30 min pre-incubation "minus NADPH" (reversible inhibition component) vs. 30 min pre-incubation "plus NADPH" (time dependent inhibition component). The ratio of these two values yields the IC₅₀ shift:

$$IC_{50} \text{ shift} = IC_{50} \text{ "minus NADPH"} / IC_{50} \text{ "plus NADPH"}$$

The ratio higher than the cut-off value (from 1.5 to 2-fold) indicates a time dependent inhibition.

Accordingly to FDA recommendations, the time dependent inhibition experiments are performed using LC-MS/MS based assay, in which biotransformations of the CYP450 specific substrates are used as markers to quantify the enzymatic activity. The extensively-characterized pooled human liver microsomes (50 or 200 donors) are used in the assay to provide consistent and reproducible results. Quantification of a decrease in the formation of a metabolite in the presence of an inhibitor is used to determine the IC₅₀.

Isoform-specific CYP450 substrates, metabolites and time dependent inhibitors

CYP450	Substrate	Metabolite	Reference time dependent inhibitor
CYP1A2	Phenacetin	Acetaminophen	Furafylline
CYP2C9	Diclofenac	4'-Hydroxydiclofenac	Tienilic acid
CYP2C19	S-Mephenytoin	S-4-Hydroxymephenytoin	Fluoxetine
CYP2D6	Dextromethorphan	Dextrorphan	Paroxetine
CYP3A4	Testosterone	6β-Hydroxytestosterone	Verapamil

Deliverable:

Dose-response inhibition curves (8 points, 3-fold serial dilution) of the test compound and the reference inhibitor starting from 100μM concentration. All test points are performed in duplicates. The IC₅₀ values are calculated using Microsoft Excel and GraphPad Prism software. Data include tables with inhibition % for each compound concentration and SD values, two dose-response inhibition curves (plus/minus NADPH), and IC₅₀ shift value.

Sample Submission: A minimal accurately weighable quantity of dry compound (~2-3 mg or 5 μmol) or 100 μL of 40 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, which should be discussed for each particular project.



Cytochrome CYP450 reaction phenotyping

Background:

Cytochrome P450 (CYP) enzymes represent a heme containing protein superfamily metabolizing a broad variety of xenobiotics, including drugs and toxic chemicals. Eleven CYP families are expressed in human liver and gastrointestinal tract (CYP1A2, CYP2A6, CYP2B6, CYP2C8/9/18/19, CYP2D6, CYP2E1, and CYP3A4/5), and 5 of them (CYPs 1A2, 2C9, 2C19, 2D6 and 3A4) are involved in about 95% of the known drug metabolism. Cytochrome P450s are of critical importance due to the two of the most significant

issues in clinical pharmacology: metabolism-mediated drug-drug interactions and individual variability in drug metabolism (CYP450 gene polymorphism).

CYP450 reaction phenotyping involves the identification of enzymes responsible for metabolism of the test article, which is useful for prediction of drug-drug interactions (DDI) and is recommended by the FDA. In case when a new drug candidate is metabolized by more than one isozyme, blocking one metabolic pathway by coadministered inhibiting drug engender metabolic switching to an alternative uninhibited enzyme.

Service Details:

In this assay two metabolically active test systems are used: individual human recombinant cytochromes (CYPs 1A2, 2C9, 2C19, 2D6 and 3A4) and human liver microsomes. The test compound is incubated with each CYP450 isoform and the metabolising ability of the enzyme is estimated by disappearance rate of parent drug using HPLC-MS/MS analysis. Reference cytochrome-specific substrates are used as controls. In the microsomal assay the isoform-specific CYP450 inhibitors are used, and the increase of the half-life ($t_{1/2}$) in the presence of the inhibitor indicates which enzyme is responsible for the metabolism of the compound.

Isoform-specific substrates and inhibitors for in vitro CYP450 reaction phenotyping

CYP450	Substrate (reference)	Inhibitor	Inhibitor concentration, μM
1A2	Phenacetin	α -Naphthoflavone	1
3A4	Testosterone	Ketoconazole	1 and 10
2C9	Diclofenac	Sulphaphenazole	10
2C19	Omeprazole	Ticlopidine	20
2D6	Dextromethorphan	Quinidine	1

Sample Submission:

A minimal accurately weighable quantity of dry compound (~1 mg or 2 μmol) or 50 μL of 20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We do not need to know structures of the molecules for ADME testing. However, brutto formulas have to be provided for all studies involving MS detection.



In Vitro Metabolite Profiling and Identification

Background:

The enzymatic biotransformation of drugs in living systems strongly affects their biological activity, sometimes resulting in metabolites with decreased

bioavailability and enhanced toxicity. The knowledge of specific sites of metabolic transformation is useful for guiding synthetic optimization of the lead compounds or drug candidates to overcome the stability and toxicity issues.

Service Details:

To elucidate the main routes of hepatic metabolism, incubations of new chemical entities are carried out with liver microsomes (mouse, rat or human), that provide a major range of the metabolizing enzymes (phase I metabolism). Additionally, S9 fractions from mouse, rat and/or human liver are used for a broader coverage of possible biotransformations (both phases I and II of metabolism). Some compounds are unstable in blood plasma; therefore plasma stability test is usually included in the service. A typical protocol includes incubation of the test compound (at concentration of 2 μM) with one chosen metabolically active test-system (liver microsomes, liver S9 or plasma) in the presence (or absence, in the case of plasma) of cofactors at 37°C, sampling at 2 time points (0 and 60 minutes), reaction quenching with acetonitrile and centrifugation. The analysis of the incubation sample and comparison to the liquid (quenched at time 0 min) is performed using liquid chromatography/tandem mass spectrometry (LC-MS/MS, API3000, AB Sciex).

Our approach to drug metabolite identification integrates both software- and knowledge-based predictions of metabolic pathways allowing for list-dependent search of metabolites (1), as well as standard tandem mass spectrometry protocols (2) including MS/MS spectra of parent compound and metabolites, precursor ion and neutral loss scans to ensure the

detection of unexpected metabolites formed by less common metabolic reactions.

Deliverable:

Data include ion chromatogram of parent compound and metabolites, table containing metabolites references and molecular formulas where possible, names of biotransformations, masses, m/z, mass differences from the parent, and retention times. The structure identification data comprise MS/MS spectra and details of the product ion fragments for parent compound and metabolites, as well as structural assignment based on the key fragments observed. Finally, we provide a detailed report containing proposed metabolites structures and expected metabolic pathways.

Sample Submission:

A minimal accurately weighable quantity of dry compound (~1 mg or 2 μmol) or 50 μL of 20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project. We need to know structure of the molecules for metabolite profiling studies.



Parallel Artificial Membrane Permeability Assay (PAMPA)

Background:

Parallel Artificial Membrane Permeability Assay (PAMPA) is used to determine passive diffusion across an artificial lipid membrane made from lipid-like organic compound or a lipid from natural source and supported by porous filter. Passive diffusion is an important factor in determining absorption of orally administered compounds in the gastrointestinal tract (GIT), penetration of the blood-brain barrier (BBB), as well as general transport across cell membranes. PAMPA provides a simplified approach to permeability by addressing just a single transport mechanism. This avoids the complexities of active transport as well as metabolism and enables ranking of the compounds

on a single permeability factor. Depending upon the particular lipid and the buffers used, PAMPA assay could be predictive of gastrointestinal tract absorption (PAMPA-GIT), blood-brain barrier permeability (PAMPA-BBB) or transdermal penetration (Skin-PAMPA). Typically, PAMPA experiments are carried out in the early drug discovery phase to select leads with promising oral bioavailability/brain penetration potential by cost-efficiently ranking candidates within large compound sets. By combining the data generated in PAMPA with more labor-intensive (as well as more predictive) cell-based permeability assays, quick structural modifications of discovery compounds to improve their in vivo characteristics, can be efficiently guided.

Service Details:

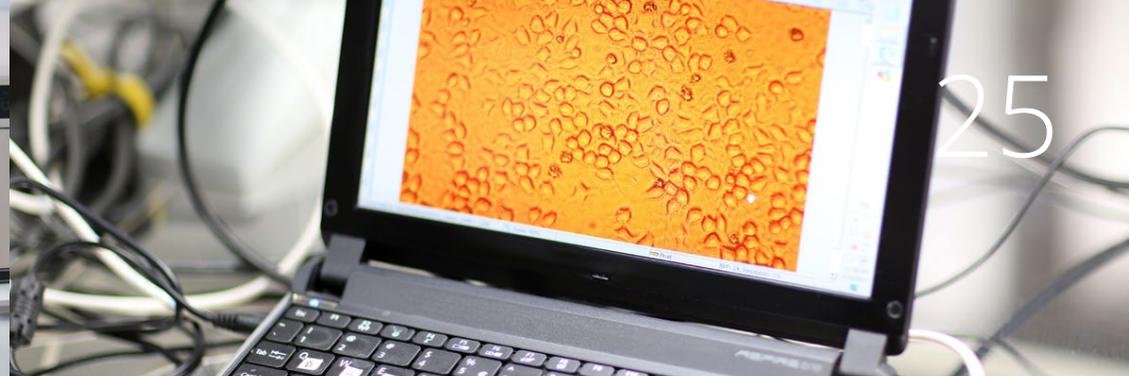
"PAMPA sandwich" is assembled from two disposable multi-well plates. One plate contains a porous filter disk at the bottom of each well (a donor plate), while the other one is an acceptor plate. The potential permeability contact between the two plates occurs at the filter, which is coated with a solution of lipid material in inert organic solvent to prepare the artificial membrane. Aqueous buffer containing 0.5% DMSO is added to each well of the acceptor plate. 50 μ M test compound solutions (containing 0.5% DMSO) are added to the wells of the donor plate. The donor plate is inserted into the acceptor plate and the plates are then incubated at room temperature, in a humid environment, for 1, 2 or 4 hours. Analytical standards are prepared from

test compound solution. Test compound permeability is assessed in two independent replicates. Two compounds of known permeability are run as controls on each plate. The donor and acceptor samples for the test and control compounds are quantified by UV or LC-MS/MS using a 3-point calibration curves with the appropriate dilutions of the samples. The experimental analyte recovery is calculated using both donor and acceptor compartment concentrations.

Deliverable:

The apparent permeability coefficient for each compound (P_{app}) is calculated. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 50 μ L of 20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient. Brutto formulas are required for all studies involving MS detection.



Caco-2 Permeability Assay

Background:

Solubility and intestinal permeability are the most important factors determining oral absorption of drugs. Caco-2 (human colon adenocarcinoma cells) assay is a widely used model in drug discovery for evaluation of compound permeability properties. When grown to confluence and allowed to differentiate the cells form a monolayer resembling luminal epithelium of human intestine by structure and properties. Caco-2 cells have a variety of active transporters, which are relevant to the absorption process in the gastro-intestinal tract. Therefore, in contrast to the PAMPA (parallel artificial membrane permeability) assay, Caco-2 method is more suitable for the prediction of in vivo drug efflux. Importantly, this assay has a good correlation with in vivo studies of absorption. For Caco-2 permeability assay, cells are grown on semi-permeable supports inside inserts in multi-well plates. The

system is composed in the way that a semi-permeable support separates apical and basolateral compartments, as differentiated Caco-2 monolayer is asymmetrical. Therefore, this system enables measurements of drug transport in both directions (apical to basolateral or basolateral to apical / A-B or B-A), across the cell monolayer. After certain incubation time, the solutions of tested compounds and samples from appropriate compartments are taken and analyzed by LC-MS/MS. Based on compound concentrations measured, apparent permeability coefficient (P_{app}), reflecting the ability of a compound to penetrate cell monolayer, is calculated. Assessing transport across the monolayer in both directions (A-B and B-A) enables determination of an efflux ratio, which is an indicator as to whether a compound undergoes active efflux. A P-glycoprotein (P-gp) inhibitor, typically verapamil, can also be included to identify whether active transport is mediated by this efflux pump.

Service Details:

Caco-2 (human colorectal adenocarcinoma line) was purchased from ATCC (cat.#HTB-37) and cultured according to the supplier's recommendations. The Caco-2 assay is carried out in 24-well insert plates (Millipore). Prior to use, the integrity of Caco-2 monolayers is verified by transepithelial electrical resistance (TEER) measurements. The assay is performed by spiking a compound (in duplicates, at 10 μ M) into the apical and/or basolateral compartments of the trans-well insert, and monitoring the appearance of this compound on the basolateral and/or apical side at a predetermined time point. Incubation time for tested compounds is 1.5 h; buffer pH is 7.4 (or 6.5) in the donor and 7.4 in the acceptor compartments.

High and low permeability controls are run with every experimental batch to verify assay validity. Due to the long set-up time for the assay, the lead time could be up to 3-4 weeks. Similar permeability assay using MDCK (Madin-Darby canine kidney) cell line is also available upon request.

Deliverable:

Based on compound concentrations measured by LC-MS/MS, apparent permeability coefficient (P_{app}), reflecting the ability of a compound to penetrate cell monolayer, is calculated. Efflux ratios and P-gp substrate liability are determined if applicable. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 50 μ L of 20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient. Brutto formulas are required for all studies involving MS detection.



MDR1-MDCKII Permeability Assay

Background:

Solubility, intestinal and CNS permeability are the most important factors determining oral absorption of drugs. MDR1-MDCKII is one of common in vitro models for a permeability assay and for identification of P-gp substrates and inhibitors. MDR1-MDCKII is a Madin-Darby canine kidney cell line transfected by human MDR1 gene, which encodes the efflux protein P-gp. The advantages of using this cell line are faster proliferation and formation of monolayer with tight junction which reduces the time required to conduct the in vitro transport studies.

MDR1-MDCKII cells derive from MDCKII line (MDCKII is a subclone derived from the heterogeneous parent line MDCK) transfected with the human ABCB1 gene encoding the well characterized transmembrane drug efflux pump, P-glycoprotein (P-gp). Designed this way, MDR1-MDCKII became of a particular value to identify compounds with intestinal or blood-brain barrier permeability, or select for P-gp substrates or inhibitors. For MDR1-MDCKII permeability assay, cells are grown on semi-permeable supports inside inserts in multi-well plates. The system is composed in the way that a semi-permeable support separates apical and basolateral compartments, as

differentiated MDR1-MDCKII monolayer is asymmetrical. Therefore, this system enables measurements of drug transport in both directions (apical to basolateral or basolateral to apical/A-B and B-A), across the cell monolayer. After the selected incubation time point, the solutions of tested compounds and samples from appropriate compartments are collected and analyzed using LC-MS/MS. PAPP, the permeability coefficient which reflects the ability of a compound to penetrate cell monolayer, is calculated based on the compound concentrations measured. Transport assessment across the monolayer in both directions (A-B and B-A) enables to determine an efflux ratio which indicates whether a compound undergoes active efflux. A P-gp inhibitor, verapamil or cyclosporine A, can also be included into the assay settings to identify whether active transport is mediated by P-gp efflux pump.

Both the MDR1-MDCKII and Caco-2 models produce comparable results with non-P-gp substrates and compounds with low and medium permeability. However, MDR1-MDCKII model is generally considered to be a better tool to predict and classify compounds that are likely to pass through the blood-brain barrier, while Caco-2 model is the standard for predicting intestinal absorption.

Service Details:

MDR1-MDCKII cell line (Sigma-Aldrich, cat.#MTOX1303) is cultured according to the supplier's recommendations. The MDR1-MDCKII assay is carried out in 24-well insert plates (Millipore). Prior to use, the integrity of MDR1-MDCKII monolayer is verified by transepithelial electrical resistance (TEER) measurements. The assay is performed by spiking a compound (in duplicates, at 10 μ M) into the apical and/or basolateral compartments of the trans-well insert, and monitoring the appearance of this compound on the basolateral and/or apical side at a predetermined time point. Incubation time for tested compounds is 1.5 h; buffer pH is 7.4 (or 6.5 optionally) in the donor and 7.4 in the acceptor compartments. High and low

permeability controls are run with every experimental batch to verify assay validity. Similar permeability assay using Caco-2 (human colorectal adenocarcinoma line) cell line is also available upon request.

Deliverable:

Based on compound concentrations measured by LC-MS/MS, apparent permeability coefficient (P_{app}), reflecting the ability of a compound to penetrate cell monolayer, is calculated. Efflux ratios and P-gp substrate liability are determined if applicable. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (2 μ M) or 50 μ L of 20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient. Brutto formulas are required for all studies involving MS detection.



FluxOR™ hERG Assay

Background:

hERG (human Ether-a-go-Related Gene, KCNH2) is a gene encoding alpha-subunit of a potassium channel. Properly formed and functional potassium channels are essential for normal electrical activity in the heart. Interference with the potassium channel due to the drug inhibition can result in acquired long QT syndrome, life-threatening conditions, and even cardiac death.

In the past few years, several drugs were withdrawn from the market due to safety issues linked to hERG inhibition. Because the hERG potassium ion channel can accept molecules of many different chemotypes, a broader number of molecules can block its function. This is why a functional screening to identify and remove potential hERG inhibitors is a pivotal step in early stages of drug development.

Service Details:

For high-throughput screening of potassium ion channel and transporter activities we use the FluxOR™ II Green Potassium Ion Channel Assay (Thermo Fisher Cat# F20016) and HEK293-hERG cells (human embryonic kidney cell line stably expressing hERG). The test can be carried out in Poly-D-Lysine-coated 384-well microplates. For the assay, the culture medium is removed and the cells are incubated with the Loading Buffer, followed by incubation in the Assay Buffer and the FluxOR™ II Background Suppressor. The cells are then treated with drug or controls for 30 min. DMSO, used as a negative control, and reference compounds Dofetilide and Haloperidol are run with every experimental batch

to verify validity of the test. Then, the Stimulation buffer containing Thallium is added to each well and the intracellular fluorescence is measured at the spectrum for extinction at 460-490 nm and emission at 520-540 nm in a kinetic assay using FLIPR Tetra cellular screening system (Molecular Devices). The assay conditions, the cell density, as well as the concentrations of the reference compounds were optimized in-house for the best performance of the assay.

Deliverable:

The results are expressed as % of inhibition, compared to the positive control. Full study report is provided.

Sample Submission: A minimal accurately weighable quantity of dry compound (~1 mg or 2 μ mol) or 40 μ L of 10-20 mM stock DMSO solution is required for this assay. For multiple assays, lesser amount of compound per assay may be sufficient, depending on the particular project.



Bacterial reverse mutation test (Ames test)

Background:

The bacterial reverse mutation test (also called Ames test) is used to detect point mutations, which involves substitution, insertion or deletion of one or a few DNA base pairs. The test is performed according to the OECD Guidelines for Testing of Chemicals, Test No. 471 (adopted in 1997) and employs auxotrophic strains of *Salmonella typhimurium* and *Escherichia coli*. Point mutations were made in the histidine (*S.typhimurium*) or the tryptophan (*E.coli*) operon, thus blocking bacterial biosynthesis of the corresponding amino

acids. A chemical's mutagenic potential is evaluated by detecting the appearance of the reverse mutants of the auxotrophic strains, making them prototrophs, able to grow in corresponding deficient media. This test is commonly used as a quick screen to estimate the mutagenic potential of new chemicals and drug candidates.

Modified Ames test can also be performed upon request. It includes addition of chemically induced rat liver S9 fraction to simulate the effect of metabolism, since certain compounds, like benzopyrene, become mutagenic only after their metabolic conversion.

Service Details:

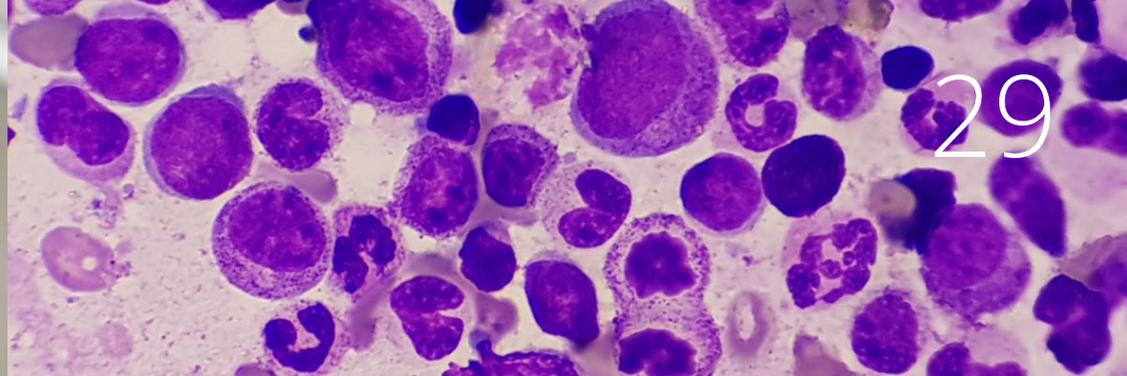
Six bacterial strains (two of them as a mixture) are used according to the OECD Guidelines for Testing of Chemicals: *S.typhimurium* TA98, TA100, TA1535, TA1537, and *E.coli* wp2[pKM101] + wp2 uvrA mixed 1:2. Chemicals are tested according to the traditional Ames test using standard incorporation protocol (Revised methods for the Salmonella mutagenicity test. Maron D.M., Ames B.N.//Mutat. Res. 1983, 113 (3-4):173-215). Three concentrations of the test agent as well as a positive and negative controls are tested using 6 tester strains (two are mixed). The colonies are counted and the results are expressed as the number of revertant colonies per plate. Compounds with known mutagenic activity

are used for positive control for each tester strain: TA98 - 2-nitrofluorene (0.4 µg/ml); TA100 - 4-nitroquinoline N-oxide (0.04 µg/ml); TA1535 - NaN₃ (0.2 µg/ml); TA1537 - 9-aminoacridine (3 µg/ml); *E.coli* strains - 4-nitroquinoline N-oxide (0.04 µg/ml). Formetabolicactivationtesting(S9),2-aminoanthracene (0.4 µg/ml) is used for all strains. DMSO is used as a negative control.

Deliverable:

Final report comprising information on the analysis, methodology, raw data, and interpretation of the results is provided.

Sample Submission: Approximately 80 µmol of dry compound (or equivalent in stock solution) is necessary for this test. Exact amount depends on the selected testing range and/or solubility of the test article.



Micronucleus test

Background:

Micronuclei are formed during metaphase/anaphase transition of mitosis and represent broken fragments of daughter chromosomes outside the nucleus. Micronucleus test is an important step in toxicology screening for new drug candidates. Analysis of micronuclei formation can be used as a tool for chromosomal DNA damage detection as it is a key marker of genotoxicity of a compound. Evaluation of xenobiotic metabolite genotoxicity is achieved by using rat liver S9 fraction.

The in vitro micronucleus assay is conducted in CHO-K1 cells treated with test articles. Compounds with known mutagenic activity are used as positive controls in the assay: 4 nitroquinoline-N-oxide - clastogen, active

without metabolic activation; cyclophosphamide - clastogen which requires metabolic activation; colchicine - compound with aneugenic activity. Dimethyl sulfoxide (DMSO) is used as a vehicle control in micronucleus assay both with and without metabolic activation. Cytochalasin B inhibits actin polymerization and blocks cytokinesis which results in formation of binucleated cells. This allows detection and analysis of micronuclei only in those cells that have completed mitosis.

For evaluation and analysis of the results, 2000 binucleated cells per each concentration of the tested compound are scored. Statistical analysis is performed using Chi-square test comparing the number of micronucleated cells in the experimental and negative control samples.

Service Details:

The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster. CHO-K1 cells (ATCC CCL-61) are cultured according to the supplier's recommendations. The cells are seeded in 6 cm diameter Petri dishes and treated with the tested or reference compounds either for 24h in the assay without metabolic activation or for 4h in the assay with metabolic activation. Cytochalasin B is added after 24h and the cells are incubated for additional 24h. After this, the cells are harvested, fixed and scored for micronuclei. The highest test concentration of the compounds should correspond to 10 mM if no solubility

or cytotoxicity limitations are observed. Three test concentrations with no more than a 3-fold difference between the concentrations are used. The experiments with the tested compounds and positive and negative controls are conducted in duplicate. The reference compounds with the known cytotoxicity are run with every experimental batch to verify assay validity.

Deliverable:

Based on the statistically significant increase of frequencies of binucleated cells with micronuclei (in the assay with or without metabolic activation) compound can be classified as genotoxic. Full study report is provided.

Sample Submission: Up to 280 µmol (~150 mg) of compound is required for this assay.



Animal Studies

Pharmacokinetic studies in mice or rats

Background and Service Details:

Pharmacokinetic (PK) studies can significantly vary in design depending on their goals and parameters of the tested compounds. We offer PK in common inbred (C57BL/6N, BALB/c) or outbred (CD-1) mouse strains or Wistar rats. A typical PK study in mice involves two drug delivery routes (e.g. PO and IV), 6 time points for each route (for example - 5, 15, 30, 60, 120, 240 min for IV and 15, 30, 60, 120, 240 and 360 min for PO) and 4 animals per each time point group/route, plus the common control plasma group ("Vehicle") - 50 animals in total. We will prepare blood plasma samples and measure compound concentrations by LC-MS/MS using AB Sciex API3000 mass spectrometers and Shimadzu VP HPLCs.

Cassette dosing, analyte measurements in different tissues, measurements of various pharmacodynamic parameters, development of optimized drug delivery formulations, as well as metabolite ID services are offered at an extra charge.

Deliverable:

A detailed study report including full description of study design, analytical method, measured test article concentrations, all common PK parameters and PK graphs. Raw experimental data are available upon request.

Sample Submission: Dry compound or compound in a pre-made animal dosing formulation. Amounts depend on the dosing levels. For a PK study in mice at 10 mg/kg PO and 10 mg/kg IV about 16 mg of compound is required. We do not need to know structures of the molecules. However, brutto formulas have to be provided for all studies involving MS detection.



Formulation Screen

Background:

Pharmacokinetics, in vivo pharmacology/efficacy and short-term toxicology are the typical animal studies crucial for prioritization of lead candidates in the early stages of drug discovery process. Selection of proper compound formulations meeting a variety

of requirements, related to intrinsic physico-chemical properties of the compound, routes of delivery, stability, toxic side-effects, interference with bioanalytical methods etc., can have a substantial effect on the outcome of such studies. This issue is aggravated by the commonplace occurrence of poorly soluble NCEs in drug discovery.

Service Details:

We will test solubilization of the test articles in 10-20 different formulation vehicles containing a variety of excipients commonly used in early preclinical formulations. Exact number of vehicles tested depends upon the properties of the compound and its behavior in the consecutive solubilization tests. Typically, 2 selected concentration levels can be tested through a dilution step. All formulations will be checked for full solubilization of the test article as well as the phase stability of each formulation over 24 hour period.

This is accomplished by the visual observation using white-light transilluminator. Follow-up studies, such as longer-term chemical stability of the test article in the selected formulation, can be done for an additional fee.

Deliverable:

Report containing compositions of the tested formulation vehicles, preparation of the formulations, solubilization and stability is provided.

Sample Submission: A minimal accurately weighted aliquot of dry compound (~1 mg) is required for each formulation recipe to be tested. We do not need to know the structures of the molecules for this test. However, some knowledge of the chemical nature of the tested compounds may facilitate the choice of most suitable formulation vehicles.



Acute toxicity study in rodents

Background:

Acute toxicity studies are designed to study adverse effects after administration of a single dose or multiple doses of a test substance given during a period not exceeding 24 hours. Acute toxicity studies in animals are necessary for early stage preclinical molecules to evaluate the perspectives of their further development. The information obtained from such studies is also useful in

choosing doses for efficacy studies, providing preliminary identification of the target organs for drug-specific toxicity, and, in some cases, revealing delayed toxicity. Acute toxicity studies in animals are often conducted using two routes of drug administration - the route intended for human therapy as well as intravenous administration. We can run acute toxicology studies of various designs in mice or rats. We can also provide follow-up studies, such as toxicokinetics or histopathology if requested.

Service Details:

A typical protocol includes investigation of 4 escalating doses in "up-and-down" manner after a single dose administration by one or more delivery routes, one of which is the intended route of administration in humans. After administration of the first chosen dose to a group of 6-10 mice or rats (equal number of males/females), animals are observed for 7 days and routinely checked for mortality, morbidity, changes in the body weight and other signs of toxicity. An additional group of animals is then dosed at a higher or lower dose, depending on the presence or absence of signs of toxicity or mortality. The final dosed group of animals is

observed for at least 14 consecutive days. One control vehicle-dosed group is included in addition to the four test article-dosed groups. Determination of the signs of toxicity includes general indicators such as mortality, morbidity, body weight, as well as terminal blood draws for hematological and clinical chemistry measurements. Gross necropsy is performed on all animals that die and on all survivors at the end of the study.

Deliverable:

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in a pre-made animal dosing formulation. Amounts depend on the dosing levels and the design of the study.



Acute toxicity - LD₅₀ study

Background:

Development of any new drug involves assessment of the benefit-risk balance between the effective therapeutic dose levels and potential side effects and toxicity levels, which may diminish or nullify the curative effects of new molecules. Therefore, importance of early evaluation of the toxicity of drug candidates cannot be underestimated. Several types of acute toxicity studies can be done to determine the Median Lethal Dose 50% (LD₅₀), Maximum Tolerated Dose (MTD) or No Observable Adverse Effect Level (NOAEL). The main objective of LD₅₀ study is establishing the drug dose which causes death of 50% of the treated animals under the defined conditions

of the test. This type of study is usually conducted to help select approximate doses for MTD/NOAEL studies and repeated-dose toxicity tests. The classical method for determining LD₅₀ may involve large numbers of animals and has high mortality ratio. Due to such limitations, the LD₅₀ test is often replaced by alternative toxicity tests or modified to reduce the number of animals involved. The most frequently used protocols designed with the intention to minimize the number of animals are the fixed dose procedure (FDP) method (OECD 420), the acute toxic category (ATC) method (OECD 436) and the up-and-down (UDP) method (OECD 425). Choice of a particular method depends largely on the expected toxicity of the tested drug and exact goals of the study.

Service Details:

We use a modified protocol to approximately estimate LD₅₀ levels by "up-and-down" or "staircase" method using a small number of animals. Typical experimental design involves 10 female BALB/c mice or Wistar rats, 5 single dose levels (2 animals per dose), and oral, intraperitoneal or intravenous drug delivery routes. The animals treated with the first selected dose are monitored for signs of toxicity and mortality at the first, second, fourth and sixth hour for toxicity signs. Mortality observed within 24 hours is recorded. Thereafter, depending on the level of tolerance of the first dose, subsequent doses (less than the initial dose, if not well tolerated or greater than the initial dose, if well

tolerated) are administered to the animals in groups of two to six, depending on the balance adjustment between minimizing the number of animals sacrificed and precision of LD₅₀ calculations. They are observed daily for additional 14 days for signs of delayed toxicity. The percentage mortality values are converted into LD50 values using the method of maximum likelihood. Standard service also includes the final gross necropsy study.

Deliverable:

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in pre-made dosing formulation. Amount depends on the toxicity of the test article.



Rodent Models

MTD/NOAEL studies

Background:

Development of any new drug involves assessing the benefit-risk balance between the effective therapeutic dose levels and potential side effects and toxicity levels, which may diminish or nullify the curative effects of new molecules. Importance of the early evaluation of toxic properties of drug candidates cannot, therefore, be underestimated. Several types of acute toxicity studies can be done to determine the Median Lethal Dose 50% (LD50), Maximum Tolerated Dose (MTD) or No Observable Adverse Effect Level (NOAEL). The MTD is defined as the dose of a drug that produces an acceptable level of toxicity or the highest dose of

a drug that does not cause unacceptable side effects. The NOAEL is the highest level of exposure to the drug at which there are no biologically significant increases in the severity or frequency of adverse effect between the treated animal group and the appropriate control group; some effects which are not considered adverse may be produced at this level. The main objective of the MTD/NOAEL studies in early preclinical stage is to identify optimal range for therapeutic doses to be used in animal efficacy models. These studies also help to identify specific target tissues/organs for toxicity, determine reversibility of toxicity, and identify dosage parameters for repeated-dose toxicity tests.

Service Details:

In a simple exemplary study which can be run in preparation for an animal efficacy study, we dose three groups of 10 mice each (5 male/5 female) with 3 selected doses of the test article by one or more delivery routes, one of which is the intended route of administration in humans. A smaller vehicle dosing group (4-6 animals) is typically included. Animals are observed for mortality, signs of gross toxicity and behavioral changes at 30 min, 2, 4 and 6 hours after the each administration and thereafter daily for a period of 14 consecutive

days. Body weight is recorded prior to dosing, and on days 7 and 14. Gross necropsy is performed on all animals at the terminal sacrifice. Specific tests, such as hematological, urinary and clinical chemistry analysis, in combination with more definitive toxic or gross pathology endpoints, are also available upon request.

Deliverable:

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in pre-made dosing formulation. Amount depends on the toxicity of the test article and study design.

Bienta continuously increases the number of rodent models for use in basic research and nonclinical drug discovery and development. Many models can be quickly established on request. This section represents an exemplary selection of rodent diabetes and metabolic disease models.

Glucose Tolerance Test (GTT)

Background and Service Details:

Glucose tolerance test (GTT) measures the clearance rate of high glucose load from animal body. GTT can be used for rapid pharmacological screening of anti-diabetic drug candidates in rodents by assessing hypoglycemic activity of test substances following acute or chronic administration. This method is highly convenient for quick investigation of various routes and time schedules for compound and glucose administration (PO, IP, IV, SC or IM).

oral dosing with glucose solution (2 g/kg) 30 minutes later. Glucose levels in blood are monitored using strip glucometer before compound administration (-30 min), before glucose challenge (0 minute) and at 15, 30, 60, 90 and 120 minutes. Two control animal groups of the same size are treated with blank vehicle or Metformin.

Deliverable:

In a typical oral glucose tolerance test (OGTT) experiment, a single dose of test compound is administered to a group of five C57BL/6N mice by gavage, followed by

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in a pre-made dosing formulation. The amount required depends on the dosing levels. For example, to treat 6 mice at 10 mg/kg, single dose of about 2 mg of compound is needed.



Insulin Tolerance Test (ITT)

Background and Service Details:

Insulin tolerance test (ITT) is a simple method for evaluating sensitivity of insulin receptors in tissues by measuring blood glucose levels in circulation before and after bolus insulin injection. This technique can be used for rapid pharmacological screening of insulin-like drug candidates or insulin sensitivity modulators by assessing their hypoglycemic activity following acute or chronic administration.

In a typical ITT experiment bolus intraperitoneal injections of human insulin (0.75U/kg) in a group of five C57BL/6N mice are used to induce hypoglycemia. Test compounds can be administered either prior to or

instead of insulin injections. Glucose levels in blood are monitored using strip glucometer immediately before insulin administration (0 minute) and at 20, 40, 60 and 120 minutes. A control group of the same size is treated with blank vehicle or insulin, depending on the purpose of the study.

Deliverable:

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in a pre-made dosing formulation. The amount required depends on the dosing levels. For example, to treat 6 mice at 10 mg/kg, single dose of about 2 mg of compound is needed.



Streptozotocin-induced Type 1 Diabetes

Background:

Type 1 Diabetes (T1D) is a pathological condition characterized by the loss of insulin-producing β -cells of the pancreatic islets of Langerhans, resulting in a deficiency in insulin production and secretion. The advantages of non-genetic animal models of diabetes compared with the genetically determined ones are caused by their better translatability of the drug candidate testing results to the treatment of this polygenic disease in humans. T1D can be modeled by treating experimental animals (rats or mice) with Streptozotocin (STZ),

a compound that has preferential toxicity toward pancreatic β -cells. Selective partial destruction of β -cells leads to reduction in insulin production, decreased levels of circulating insulin, elevated blood glucose levels and, eventually, development of various diabetes-associated pathologies, such as diabetic nephropathy, cardiomyopathy, diabetic ulcers, etc. STZ-induced experimental model of T1D can be used for efficacy studies of anti-diabetic drugs, including agents acting on insulin-independent pathways and providing end organ protection from diabetes.

Service Details:

Depending on the goals of a particular study, a single high dose (~150 mg/kg) or consecutive multiple low doses (40-50 mg/kg/day for 5 days) of STZ are injected in C57BL mice or Wistar rats to induce the disease state. Induction of diabetes is monitored by routine tests – blood levels of glucose and glycosylated hemoglobin (HbA1c). Markers of diabetes' side effects can be monitored upon request. To develop a standard STZ model of diabetes to test a drug candidate, we suggest using 8-10 animals per each planned experimental group, 5-7 days of STZ treatment, and 4 weeks of post-

STZ treatment monitoring body weight, food/water intake, blood glucose and glycosylated hemoglobin levels to confirm that the mice are diabetic. If the study goal is to test an accompanying pathology, the time allowed for disease development may be increased up to 6-8 months.

Deliverable:

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in pre-made dosing formulation (amount required depends on the dosing levels and schedules). For example, for treating a group of 8 mice at 10 mg/kg, twice daily (b.i.d.) for 1 week, about 16 mg of the test compound is needed.



Streptozotocin/High Fat Diet-induced Type 2 Diabetes

Background:

Unlike type 1 diabetes, type 2 diabetes (T2D) is associated with resistance to insulin action. To develop the corresponding animal pathology model, High Fat Diet (HFD) is often used. HFD leads to the development of obesity, metabolic syndrome and decrease in insulin sensitivity in mice, in contrast to low fat diet (LFD). Combination of HFD and single Streptozotocin (STZ) injection leads to metabolic changes that are characteristic of Type 2 Diabetes, including peripheral

insulin resistance and pancreas β -cell impairment. HFD/STZ-induced model of experimental T2D can be used for efficacy testing of anti-diabetic and anti-obesity drugs. The induction of disease can be carried out in rats or mice. This model is suitable not only for the study of the effectiveness of the drugs stimulating the production/release of insulin from β -cells, but also drugs that reduce the production of glucose in the liver, improve the sensitivity of tissues to insulin, regulate the production of incretins, etc.

Service Details:

To induce T2D in C57BL/6J mice, the standard HFD (Research Diets, Inc.) in combination with a single high dose (95 mg/kg) of STZ is used. Development of the diabetic state is monitored by routine tests – food and water intake, body weight gain, glucose levels, glycosylated hemoglobin in the blood, glucose tolerance test (GTT), and insulin tolerance test (ITT). Various markers of diabetes' side effects can be monitored upon request. To develop the HFD/STZ model of Type 2 diabetes for testing a drug candidate, we suggest using 8-10 animals per each planned experimental

group, 12 weeks of HFD for obesity induction and post-HFD/STZ treatment monitoring to confirm that the mice are diabetic. If the study goal is to test an accompanying pathology, the time allowed for disease development may be increased to 6-8 months.

Deliverable:

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in pre-made dosing formulation (amount required depends on the dosing levels and schedules). For example, to treat a group of 8 mice at 10 mg/kg, twice daily (b.i.d.) for 1 week, about 16 mg of the test compound is needed.



Obesity-induced Type 2 Diabetes

Background:

Obesity is associated with the development of many diseases, with Type 2 Diabetes (T2D) the most common one among them. Obesity-induced experimental model of T2D is characterized by significant weight gain, increased fasting glucose, insulin resistance, and a number of metabolic disorders, such as abnormal lipid metabolism, cardiovascular complications, nephropathy, etc. Obesity-induced T2D model in CD-1 (ICR) or C57BL/6J mice demonstrates good reproducibility and reliability

of the obtained results. Due to better translatability of the drug candidate testing results to the treatment of this polygenic disease in humans, non-genetic animal models of diabetes have numerous advantages compared to the genetically determined models. This model can be used for pharmacological screening of anti-diabetic and anti-obesity drugs, as well as for the study of drugs intended for the treatment of side effects of diabetes and obesity – diabetic nephropathy, cardiomyopathy, etc.

Service Details:

For obesity induction, the standard High Fat Diet (Research Diets, Inc) with fructose supplements in ICR male mice is used. Development of the diabetic state is monitored by routine tests – food and water intake, body weight gain, blood glucose and glycosylated hemoglobin levels, glucose tolerance test (GTT), and insulin tolerance test (ITT). Various markers of diabetes' side effects can be monitored on request. To develop the obesity-induced model of diabetes for testing a drug candidate, we suggest using 8-10 animals per

each planned experimental group, 12 weeks of HFD for obesity induction and post-HFD monitoring to confirm that the mice are diabetic. If the study goal is testing an accompanying pathology, the time allowed for disease development may be increased to 6-8 months.

Deliverable:

A detailed study report including full description of study design and all experimental data.

Sample Submission: Dry compound or compound in pre-made dosing formulation (amount required depends on the dosing levels and schedules). For example, to treat a group of 8 mice at 10 mg/kg, twice daily (b.i.d) for 1 week, about 16 mg of test compound is needed.

To find out more about our services or inquire about a custom-developed assays and models, please feel free to contact us at info@bienta.net. Our well-experienced personnel will be happy to help.

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