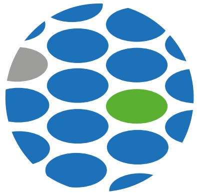
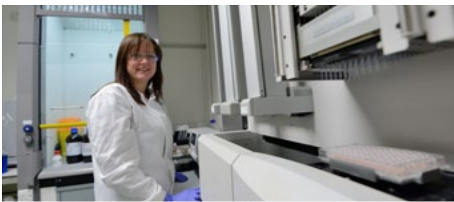
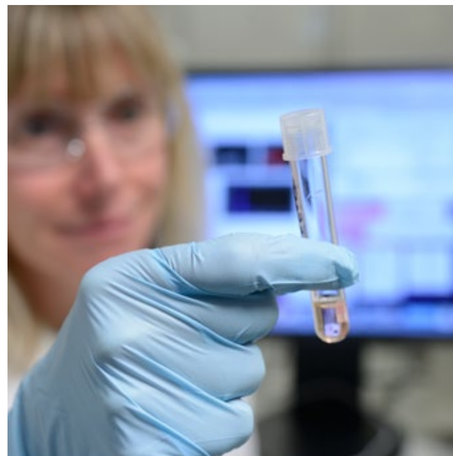


Bioassay Builder

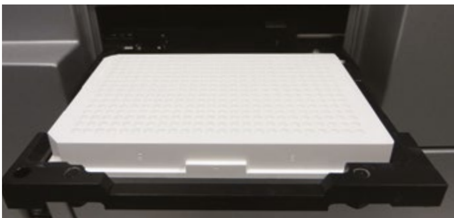
Assay development, screening and compound characterisation services

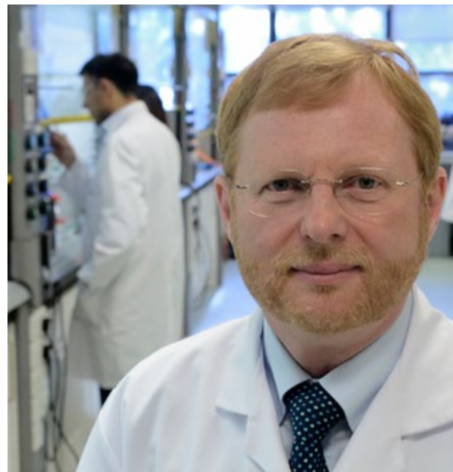


Our *BioassayBuilder* service is customised to your particular requirements

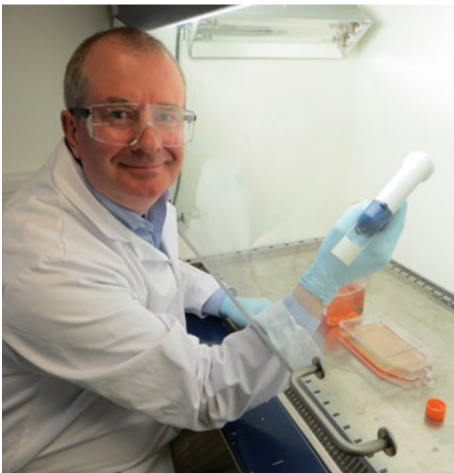


Our bioassay scientists believe that every datapoint is important





From protein expression and assay development to hit identification and optimisation



Our focus is providing you high-quality results with interpretation

domainex
the faster route to drug discovery

www.domainex.co.uk

Introduction

BioassayBuilder is a platform of bioassay services offered by Domainex, from protein expression to characterisation of pre-clinical candidates.

Domainex has assembled an experienced team of molecular, assay and cell biologists to support your drug discovery projects.

We offer a comprehensive suite of services, as follows:

- protein expression
- assay development
- hit identification
    - fragment screening via *FragmentBuilder*
    - virtual screening via *LeadBuilder*
- compound mechanism of action (MOA) analysis
- phenotypic profiling
- ADME testing
- ID of potential PD markers

Our skilled scientists will work with you to provide a high-quality service in a timely fashion. We provide detailed reports of results with interpretation and suggestions for further work.

PROTEIN EXPRESSION

- Cloning
- Combinatorial Domain Hunting (CDH) for complex multi domain proteins
- Protein expression in insect and E.coli cells
- Protein purification
- Protein characterisation and stability studies

Case Study 1: TBK1/IKKε Screening Cascade

TANK-binding kinase 1 (TBK1) and IkappaB kinase epsilon (IKKε) have been validated as novel drug targets in inflammatory diseases such as psoriasis, rheumatoid arthritis and COPD. Kinase activity assays were established for both TBK1 and IKKε in 384 well format whereby reactions were performed at ATP K_m and initial rate velocity. Structural and kinase activity data was generated to support medicinal chemistry with the result that low nanomolar compounds were identified (Figure 1). During further lead characterisation we demonstrated inhibition of the secreted cytokine IP10 in monocyte cell line THP1 following stimulation of the cells with lipopolysaccharide (LPS) (Figure 2). Mechanistic analysis demonstrated IP10 promoter IRF3 phosphorylation was inhibited by a TBK1 inhibitor in a concentration-dependent manner (Figure 3). Whole blood taken from healthy human donors was stimulated with LPS and IP10 secretion measured using AlphaLISA. At an established fixed concentration of LPS, TBK1 IC_{50} values were resolved and demonstrated sub-micromolar potency (Figures 4a &b).

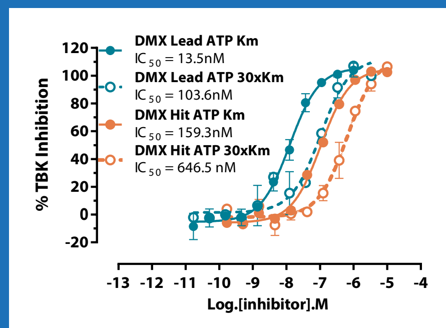


Figure 1: Biochemical profiling of TBK1 inhibitors and assessment of ATP sensitivity. TBK1/IKKε inhibitors were titrated and incubated with TBK1 purified recombinant protein. Enzymatic reaction was initiated by the addition of peptide substrate at either K_m or 30 times K_m ATP levels. Assay was stopped after 2hrs and IC_{50} values generated using four parameter fit.

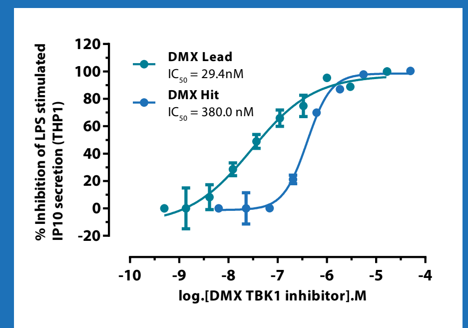


Figure 2: Inhibition profile of TBK1 inhibitors against LPS stimulated IP10 secretion. THP1 cells were pre-incubated for 1hr with a serial dilution of DMX compounds. Cells were subsequently stimulated with 0.1µg/ml LPS for 24hrs and the supernatant collected for IP10 assessment by AlphaLISA. Data was normalised to no stimulation (100%) and stimulation only (0%).

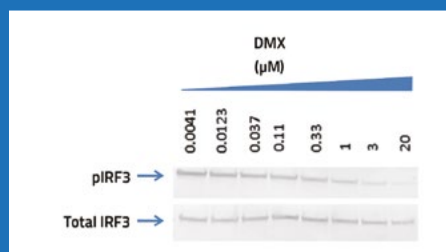


Figure 3: Effect of TBK1 inhibitor DMX on LPS stimulated IRF phosphorylation. THP1 monocytes were differentiated into macrophages by addition of PMA. IRF3 phosphorylation was stimulated after the addition of LPS. Levels of total and phosphorylated IRF3 were detected using Western blotting techniques. Figure shows total IRF3 is present in all samples and inhibition of phosphorylated IRF3 relative to total IRF3 with increasing concentrations of DMX following LPS stimulation.

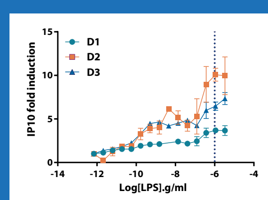


Figure 4a: LPS dose response effect on stimulation of IP10 in human whole blood from three human donors (D1, D2 and D3)

Whole blood was sourced from three healthy donors and treated with a serial dilution of LPS for 24hrs. IP10 levels were measured using AlphaLISA techniques and the fold induction compared to unstimulated was calculated. 1µg/ml LPS was selected for further experiments.

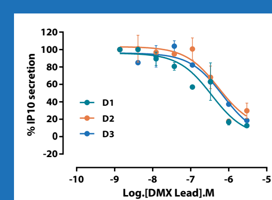


Figure 4b: Inhibition profile of DMX compound on LPS-stimulated IP10 secretion in human whole blood

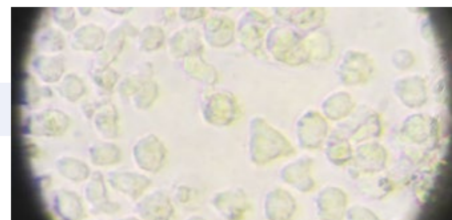
Whole blood was pre-incubated for 1hr with a serial dilution of DMX compound. It was subsequently stimulated with 1µg/ml LPS for 24hrs and the supernatant collected for AlphaLISA IP10 assessment. Data was normalised to no stimulation (100%) and stimulation only (0%).

ASSAY DEVELOPMENT

- Reagent sourcing/production
- Biochemical, biophysical and phenotypic assay formats
- Flexible experimental schedule covering:
  - titration studies to optimise assay parameters
  - kinetic studies
  - check solvent tolerance
  - confirm pharmacology (if applicable)
  - inter and intra plate variability
  - low to high-throughput dependant on requirements

HIT DISCOVERY

- Access *LeadBuilder* to identify virtual hits with desirable properties
- Test diverse, focused and fragment libraries
- Fragment-based drug discovery via *FragmentBuilder* with MicroScale Thermophoresis (MST) at its core
- Client-provided, sourced or Domainex-generated test compounds
- Access Pharmaprofiler, a high-performance screening library of commercial drugs
- Flexible assay format including:
  - single, multi or full concentration effects
  - generate up to 50,000 data points
  - rapid deconvolution of virtual hits
  - orthogonal fragment approaches
  - pre-agreed QC and cut-off rate criteria, e.g. minimum Z'



HIT/LEAD CHARACTERISATION

- *In vitro* profiling
  - potency and selectivity assessment
- Mechanism of action studies
- Kinetics
- Phenotypic responses (see Phenotypic Assay Portfolio)
- Pathway dissection
- Target engagement
- Biomarker identification and analysis

Key features & deliverables of the BioassayBuilder service

- Highly customised service to meet your needs
- Ability to offer a complete screening solution
- Careful stewardship with minimised consumption of your compounds
- Quality assurance: pre-set assay acceptance criteria
- Provision of high-quality data, proteins or assay systems in a timely manner
- Ownership of test results assigned to you
- Ability to troubleshoot, generate ideas and be flexible
- Can be efficiently linked to medicinal chemistry

Platforms

ADME

- Microsomal clearance
- Aqueous solubility
- Cyp inhibition
- LogD
- Further assays tested via strategic partners

EQUIPMENT	HTS plate readers with stacker	Real-time PCR/ thermal cyclers	FACS
	Automated liquid handling	HTS liquid dispensing	LC-MS
	Western Blotting	Cell culture	AKTA and protein purification
	Fluorescent Imaging Plate Reader	MicroScale Thermophoresis	
READOUTS	Fluorescence	Fluorescence polarization	HTRF
	Luminescence	AlphaScreen/ AlphaLISA	Differential fluorimetry scanning
	Absorbance	q-PCR	
TARGETS	Protein:protein interaction	Enzymes	Epigenetic targets
	GPCRs	Biomarkers	Reporter and pathway analysis
	Phenotypic	Target engagement	ADME

Case Study 2: Lysine Methyltransferases

Lysine methyltransferases (KMTs) are involved in epigenetic gene regulation by covalent modification of histones. Domainex has addressed key technical challenges associated with KMTs, including generating a number of crystal structures, assays and a KMT-focused library. For instance, an EZH2 screening assay was developed using a peptide substrate and a more physiologically relevant substrate H3/H4 tetramer (Figure 1a&b). The biochemical assay was then utilised to screen a KMT library identified using *LeadBuilder* virtual screening focusing on both the SAM and peptide binding sites (Figure 2). KMT specific chemotypes were resolved for EZH2, SMYD2, SMYD3, G9a and NSD2. Binding conformations of chemotypes were illustrated against G9a using MicroScale Thermophoresis (MST) technology, and binding dependency of SAM evaluated (Figure 3a&b). Finally, EZH2 is known to methylate histone H3K27(me3) and we have demonstrated cellular efficacy of tool compounds via both FACS and Western Blotting techniques (Figure 4).

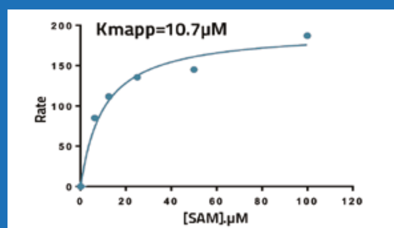


Figure 1a: Development of an EZH2 AlphaScreen assay. Purified recombinant EZH2 complex was tested for its ability to methylate histone based substrates and resolve SAM ATP K_{mapp}. Rate of reaction was elucidated from a serial dilution of SAM in the presence of fixed concentrations of biotinylated peptide and EZH2 complex. Methylation was detected using alpha donor streptavidin and alpha acceptor anti- H3K27me3 beads.

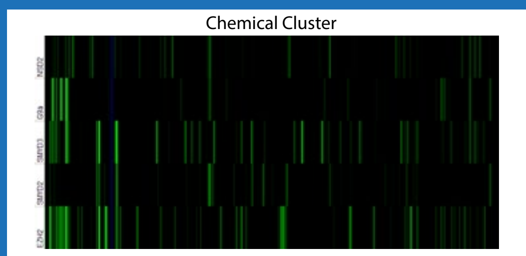


Figure 2: KMT small molecule inhibition profile. A KMT library was identified through a *LeadBuilder* virtual screen. From this 1130 hit compounds were tested at 100µM for EZH2 inhibitory activity. In addition, selectivity assays were screened including SMYD3, SMYD2, NSD2 and G9a (green denotes >75% inhibition). Both selective and non-selective inhibitors were identified, along with other profiles.

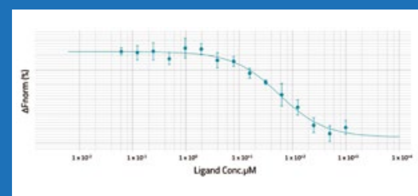


Figure 3a: Binding of Domainex proprietary G9a inhibitor in the presence of SAM. Purified recombinant G9a was fluorescently labelled via lysine linkage. Low nano molar concentration of G9a was incubated with a titration of SAM and loaded onto the Nanotemper pico Monolith instrument. Using MST technology, changes in mobility of G9a were recorded and Kd resolved for SAM binding at 55µM.

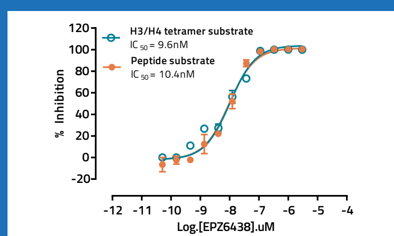


Figure 1b: Effect of EPZ6438 on EZH2 enzyme activity. EPZ6438, a known EZH2 inhibitor, was titrated and pre-incubated with EZH2 complex plus SAM. Enzymatic reaction was initiated by the addition of 100nM biotinylated substrate. Assay was performed at 10 times SAM Km. Assay was stopped and IC_{50} values generated using four parameter fit.

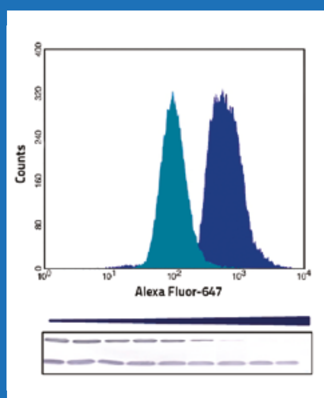


Figure 4: Quantification of H3K27me3 in SU-DHL-6 cell lines. H3K27(me3) levels were measured in the SU-DHL-6 cell line either by FACS or Western blotting using an antibody to the methylation mark. Secondary antibodies were either labelled with Alexa Fluor-657 or AP respectively. The standard compound, EPZ6438 was tested at 20µM using FACS or titrated using Western blotting techniques. Decrease in methylation was observed in the presence of the EZH2 inhibitor.

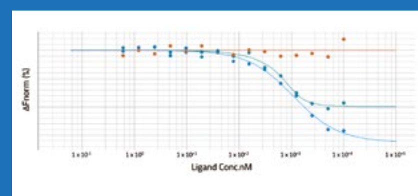


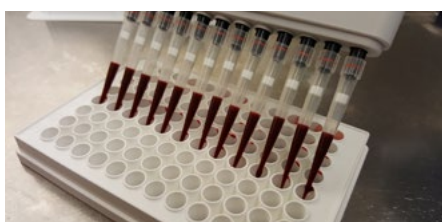
Figure 3b: Effect of DMX compound on G9a mobility in the presence and absence of SAM. A titration of Domainex proprietary compound was incubated with fluorescently labelled G9a in the presence of; 10 times K_d SAM (blue line), K_d SAM (green line) and no SAM (orange lines). Compound binding is dependent on SAM binding.

Phenotypic Assay Portfolio

NAME	DESCRIPTION	FORMAT	STIMULI	READ-OUT	UNIT	COMMENTS
Monocyte cytokine release	IC_{50} or % inhibition of test compounds to reduce cytokine release in THP1 and RAW246.7 cell lines	96 or 384 well	LPS, PolyIC, IMIQ R848, PMA	AlphaScreen (Perkin Elmer), ELISA, FACS, qPCR	pg/ml or S/N or % inhibition	ILß, IL6, IL8, IP10, MCP1, TNFα
PBMC* cytokine release (healthy donor)	IC_{50} or % inhibition of test compounds to reduce cytokine release	96 or 384 well	LPS, PolyIC, IMIQ R848, PMA	AlphaScreen (Perkin Elmer), ELISA, FACS, qPCR	pg/ml or S/N or % inhibition	ILß, IL6, IL8, IP10, MCP1, TNFα
Whole blood cytokine release (healthy donor)	IC_{50} or % inhibition of test compounds to reduce cytokine release	96 or 384 well	LPS, PolyIC, IMIQ R848, PMA	AlphaScreen (Perkin Elmer), ELISA, FACS, qPCR	pg/ml or S/N or % inhibition	ILß, IL6, IL8, IP10, MCP1, TNFα
Cell viability	IC_{50} or % inhibition of test compounds to reduce cell viability over 1-6 days	96 or 384 well	On request	Cell Titer Glo – luminescence (Promega)	% viability	2D and 3D cell growth available
Cell toxicity	IC_{50} or % inhibition of test compounds to induce cell death up to 72hrs	96 or 384 well	On request	Cell Tox green – Fl (Promega)	% cell death	Can be multiplexed with cell viability
Apoptosis	IC_{50} or % inhibition of test compounds to induce Caspase 3 6-24hrs	96 or 384 well	On request	Caspase 3 luminescence (Promega)	% caspase activity	Can be multiplexed with cell viability
Cell cycle	Sub-G1, G1/S phase/G2 assessment	6 well	On request	Propidium iodide	% Sub-G1, G1/S phase/G2	Can be multiplexed with cell markers

In Vitro ADME Assay Portfolio

NAME	DESCRIPTION	FORMAT	READ-OUT	UNIT	COMMENTS
Microsomal stability	Liver microsome stability assessment in a variety of species (please enquire)	96 well, LC-MS	% of parent remaining, $Cl_{int,app}$	(uL/min/mg prot), half life	
Cyp inhibition	Measures enzyme-drug interactions and inhibition of cytochrome p450 enzymatic activity	96 well, fluorescence	Fluorogenic substrate cleavage/ turnover, kinetics	% inhibition and IC_{50}	CYP2C9 CYP2C19 CYP2D6 CYP3A4 CYP1A4
Aqueous solubility	Thermodynamic solubility, typically PBS pH7.4. Other buffers available on request	Micro shaken flask, peak quantification by DAD, confirmation by MS	2&24hrs time points	µg/ml and µM	
	Kinetic solubility	96 well	Absorbance	µM	
LogD	Octanol/PBS pH7.4 partition	Micro shaken flask, peak quantification by DAD, confirmation by MS	90 minutes		
Hepatocyte stability	Cryopreserved hepatocyte stability assay which measures clearance and metobolite formation	96 well, LC-MS	% of parent remaining, $Cl_{int,app}$	(uL/min/mg cells), half life	
Caco-2	Measure of human intestinal permability to investigate drug efflux	96 well, LC-MS	Recovery	p^{app}, Efflux ratio	



About Domainex

Domainex is a fully integrated drug discovery service company based near Cambridge, UK serving pharmaceutical, biotechnology, academic and patient foundations globally. Domainex's drug discovery service business was established in 2001 and since that time has continued to expand to serve a wider range of clients across the world including UCB, FORMA Therapeutics, St George's University, The Institute of Cancer Research and Auspherix. Our expertise and commitment to providing high quality services has resulted in a strong success record in drug discovery, delivering an average of one candidate drug every year for the past six years.

How Can Domainex Help Your Drug Discovery Project?

Domainex's highly experienced molecular biologists, assay biologists, medicinal, computational and analytical chemists can be leveraged through our CRO services. Domainex provides highly efficient and well considered scientific solutions to enable successful drug discovery programmes against a wide range of drug targets. Whether your project is at an early stage of drug discovery or has already identified chemical matter, our processes have been shown to result in a 30% time-saving compared to industry standards and use less resource, allowing prudent management of your own budget.

Contacts

If you would like to know more about Domainex's discovery services, or speak to us regarding your own drug discovery needs, please contact us at: enquiries@domainex.co.uk

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Publications

Reich *et al.*, "Identification of soluble protein domains adaptable to Combinatorial Domain Hunting: An effective approach for high-throughput applications" *Protein Sci.* (2006) 15: 2356-2365

Maclagan *et al.*, "A combinatorial method to enable detailed investigation of protein–protein interactions" *Future Med. Chem.* (2011) 3: 271–282

Meier *et al.*, "Engineering human MEK-1 for structural studies: A case study of combinatorial domain hunting" *Journal of Structural Biology.* (2012) 117: 329–334