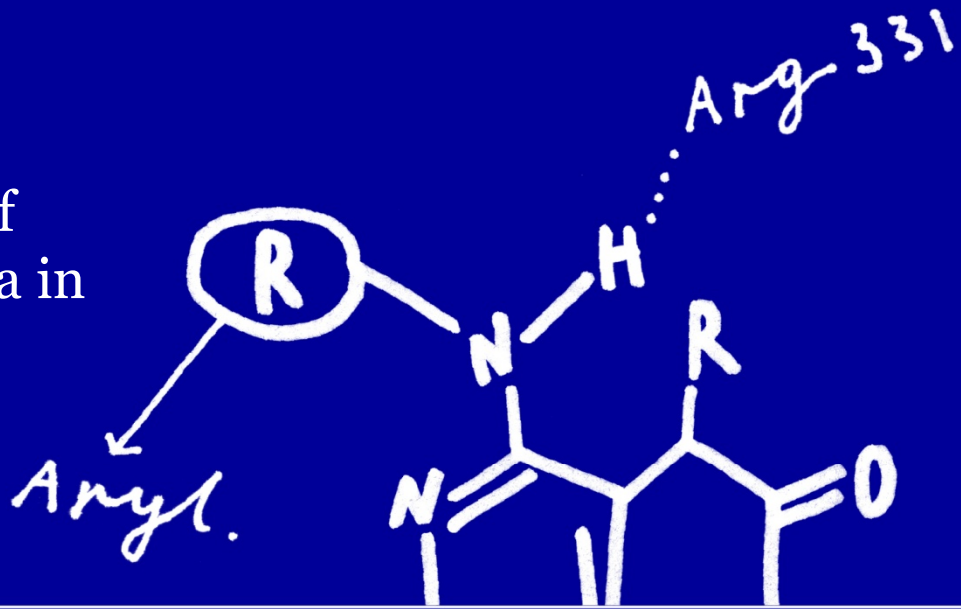
evotec

'RESEARCH NEVER STOPS'
Building innovative drug discovery alliances

Biophysical techniques for measuring kinetic and thermodynamics of binding

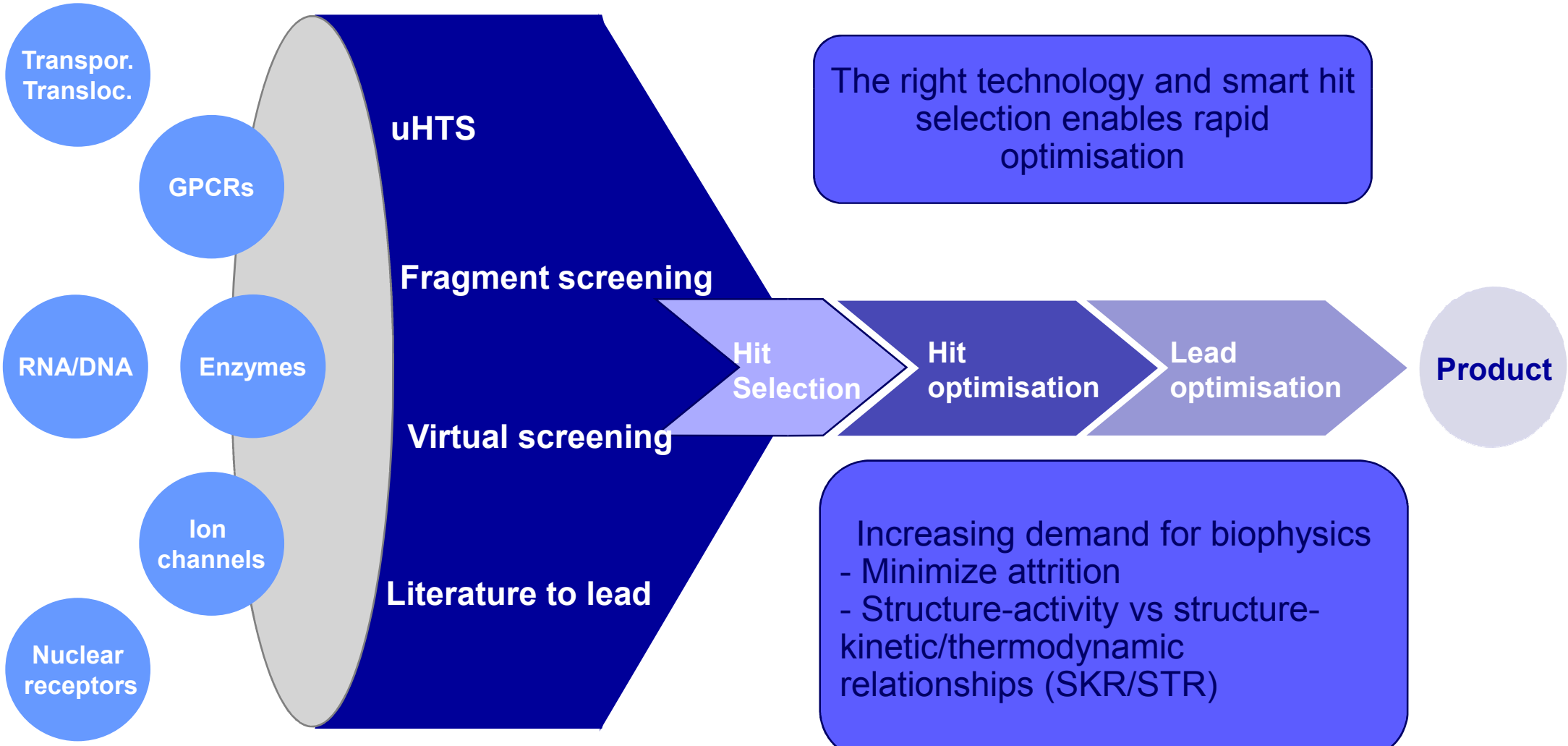
Enhancing Drug Quality: The benefits of kinetic and thermodynamic binding data in discovery

SCI HQ, London, UK
6 November 2012,



Selecting the right technology and approach for each individual target

Hit identification platform



Why Biophysics

Increase the quality of hits and leads in drug discovery

- Better understanding the mechanism of binding and activity
- Advance hits earlier and more efficiently
  - Identify and discard unwanted and promiscuous compounds early
  - Confirm and characterize weak hits, difficult hit series and singletons
  - Enable proper follow up for all hits
  - Prioritization of hits for expensive follow up (NMR, Xray)
  - Bona fide hits for MedChem optimization

Better selection of hits for:
Disease biology, Structural biology,CADD and MedChem

- Most biophysical methods are complex, costly, resource intensive and do not provide HT
- Biophysical methods are complementary technologies and need to be well positioned in the workflow

evotec

Kinet/Therm

Biophysical Technology Toolbox

In use / under consideration at Evotec

TECHNOLOGY	TYPE OF INFORMATION
Biochemical binding assasy (FCS+plus, Reporter Displacement)	Yes/No Binding, Kd, Ki, kon/koff, ΔH, ΔS
Surface Plasmon Resonance (SPR) and related technologies (switchSENSE, MolSense, Biolayer Interfer.)	Yes/No Binding, stochiometry, Kd, Ki, kon/koff, (ΔH, ΔS)
Mass spectrometry (LC-MS) and HT MS (RapidFire), HX-MS, MS-based Proteomics	Yes/No Binding, Kd, Activity, Protein Dynamics, PTM, Pathways
Nuclear-magnetic resonance (NMR)	Yes/No Binding, Kd, allosteric sites
Isothermal Titration Calorimetry (ITC)	Stochiometry, ΔH, ΔS
Differential Scanning Fluorimetry (DSF)	Protein (de)stabilization, Tm
Dynamic Light Scattering (DLS)	Cpd Aggregation, Solubility
Fragment Molecular Orbital Calculations (FMO)	Electrostatic Interactions, ΔH

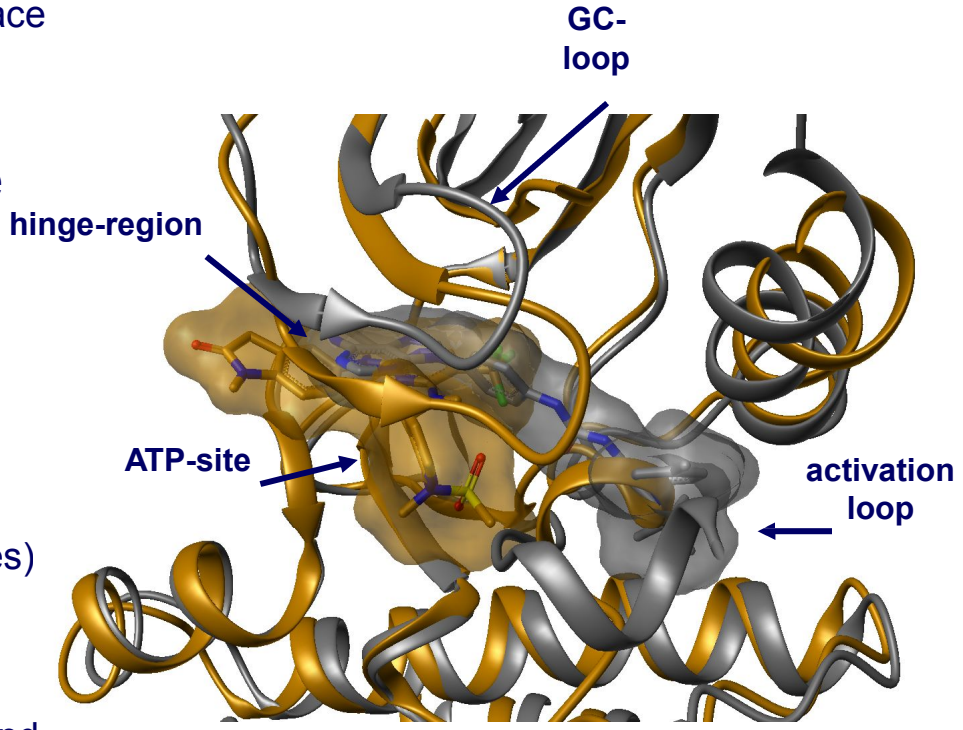
Requirements on Protein Quality

Proteins for Biophysics

- Procedures to identify the suitable constructs
  - Rational design on published precedence or obvious analogies / homologies, Construct library screening
  - Variation of species: elucidation of sequence conservation for surface mutants
  - Limited proteolysis to identify compactly folded domains
- Methods to express the recombinant proteins / isolate native proteins
  - Suitable expression systems / hosts; native sources
  - Timeframe from construct => expression feasibility => upscale
- Quantity
  - Largest consumption in Xray (>5mg) and Calorimetry (>5mg), low for Biacore (<1mg)
  - Ranges from 1mg to 30 mg per production slot (also pooled batches)
  - Several protein variants may be required in parallel
- Quality
  - General protein analytics to confirm identity (e.g. mass, EDMAN) and purity (special: disulfide linkage after refolding)
  - Biochemical behavior with respect to stability (e.g. TSA buffer screen), aggregation/dispersity or app. molecular weight on SEC
  - Xtalisability, Functionality (activity or binding), **PTMs**

Example of PTM relevance

Interaction of inhibitors sensitive to the activation state of a kinase domain



type II inhibitors sensitive to phosphorylation state

Hit Discovery by biophysical methods

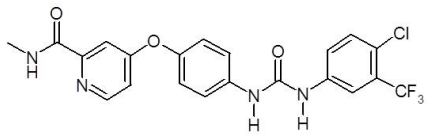
Typical project workflow

1	Assay development phase to establish assay conditions and optimise sample and experiment conditions	Biochemical binding assay, SPR, NMR, HT-MS
2	*Clean screen* to remove not well behaving cpds (e.g. insoluble, slow off, matrix binders, hyper-stoichiometric binders)	SPR, DLS, NMR, ITC
3	Primary screening (*Binding level screen*) Prioritization of hits for profiling, statistical hit identification based on normalized binding response	Biochemical binding assay, SPR, NMR, HT-MS
4	Profiling (*Affinity screen*) Ranking of hits wrt affinity, selectivity based on steady state binding response plots	Biochemical binding assay, Activity Assays, LC-MS, SPR, NMR, MS-Proteomics
5	Analysis of SAR, tractability of hit compounds Hit validation in competition and orthogonal assays Hit expansion	Xray, FMO, DSF, Biochemical binding assay, Activity, SPR, NMR, LC-MS, MS-Proteomics

Residence time in Drug Discovery

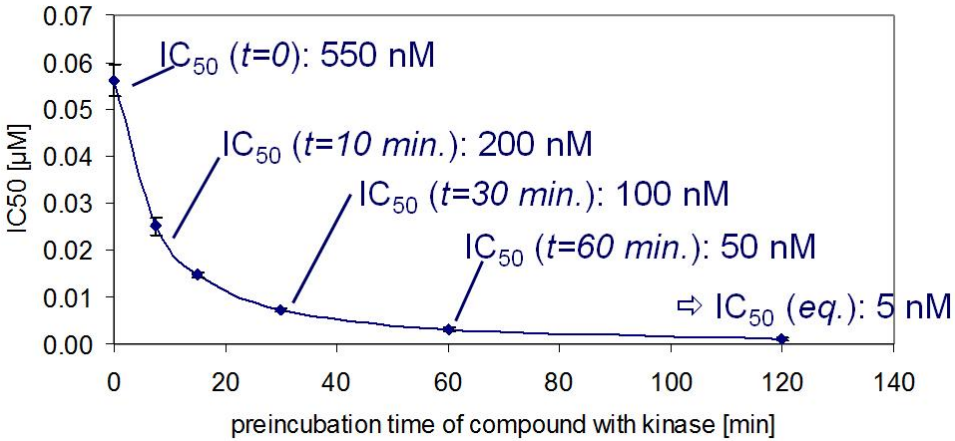
Drive cpd optimization by *in vitro* measurement of K_d and k_{off}

- Critical factor for drug efficacy *in vivo* is not apparent affinity, but rather "**residence time**"
- "Residence time" = binary complex residence time = period for which receptor (R) is occupied by ligand (L)
- ***In vivo:*** compound-target interaction time is dependent and influenced by other determinants rather than compound-target molecular interaction
- **residence time** directly measured by dissociation half-life of compound-target complex ($t_{1/2}=\ln 2/k_{off}$)



Nexavar (Sorafenib, BAY43-9006)

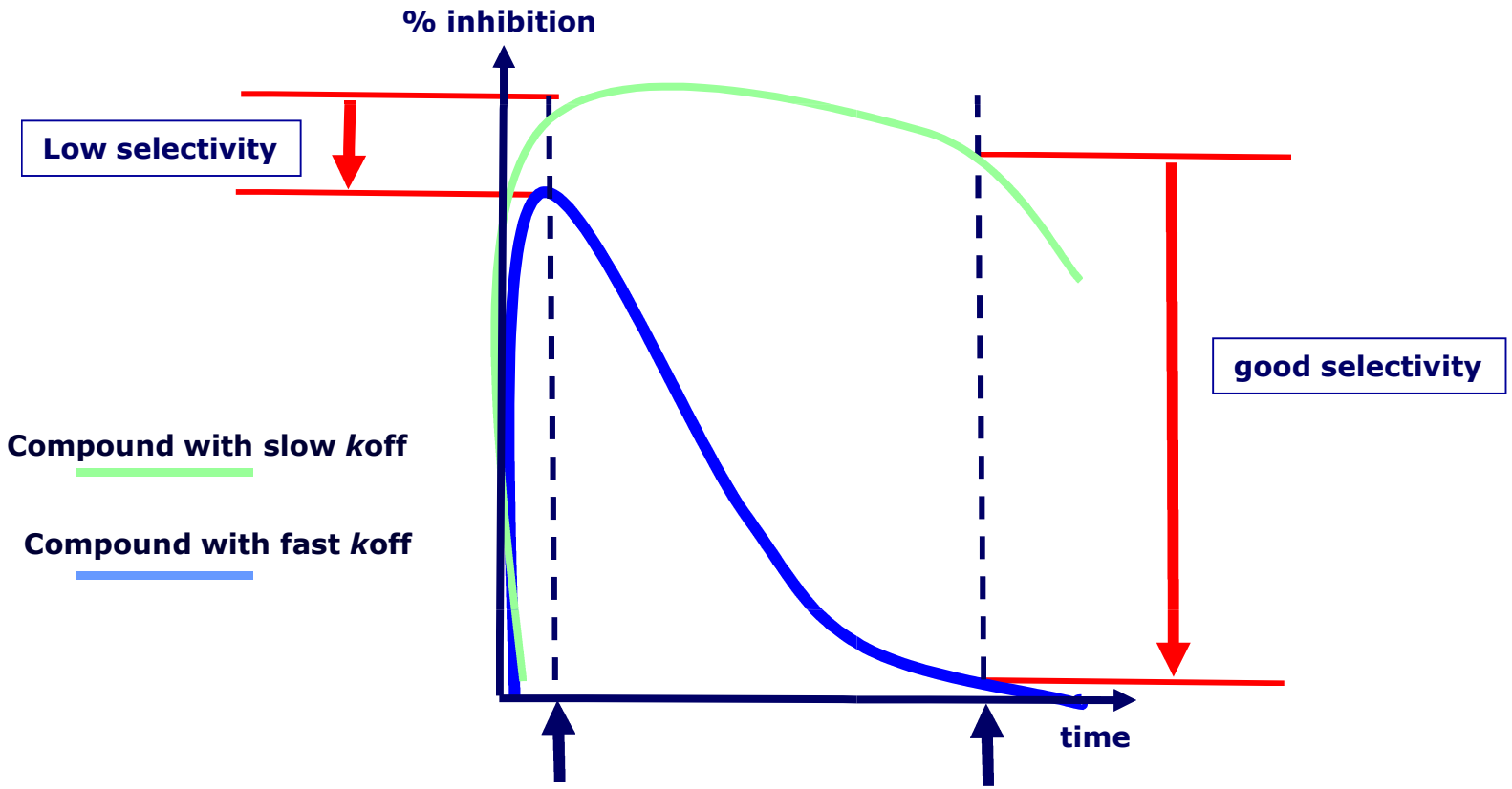
- IC50 – a multiparameter value



Copeland et al. (2006) Nat. Rev. Drug Discov. 5, 730

Residence time determines selectivity

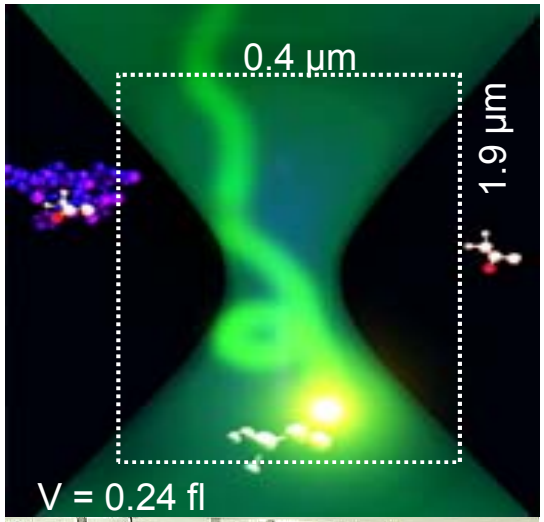
Demand for kinetic selectivity panels



In vivo Selectivity can be gained through residence time and is best predicted through kinetic selectivity panels

FCS+plus provides multiple read-out options

The target selects the optimal read-out for binding and activity assays



Portfolio of read-out options for FBDD

- Translational diffusion / FCS
- Rotational diffusion / FP
- Fluorescence lifetime / FL
- Fluorescence intensity / FI
- Molecular particle brightness / FIDA

Fluorescence-intensity distribution analysis and its application in biomolecular detection technology

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*EVOTEC BioSystems AG, Schnackenburgallee 114, D-22525 Hamburg, Germany; and †Institute of Experimental Biology, Instituudi tee 11, EE3051 Harku, Estonia

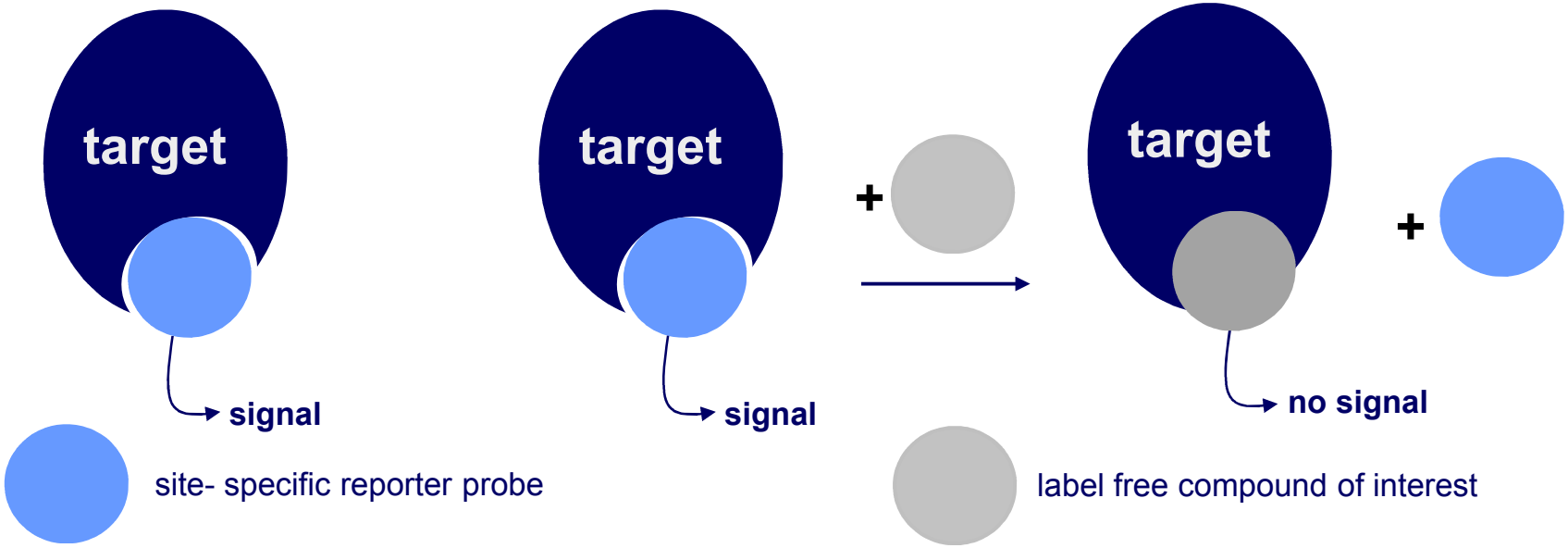
Communicated by Manfred Eigen, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, October 4, 1999 (received for review February 16, 1999)

A methodology, fluorescence-intensity distribution analysis, has been developed for confocal microscopy studies in which the fluorescence intensity of a sample with a heterogeneous bright-

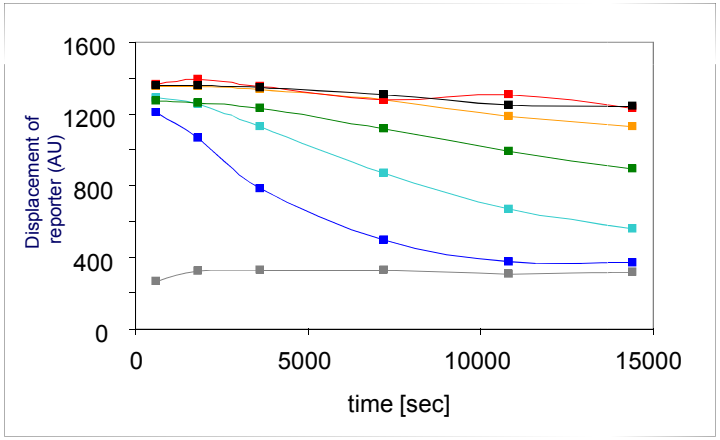
photon count number distributions. An appropriate theory and realization of this method of analysis is introduced in this paper and has been designated fluorescence-intensity distribution

Site-specific biochemical reporter displacement

Determines k_{obs}, k_{on}, k_{off}, K_d and residence time $t_{1/2}$



Multiple readouts possible incl. FCS+plus



Full reporter binding

increasing concentrations of label free compound

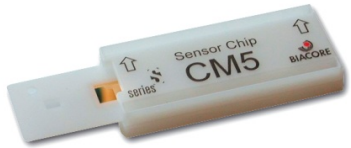
Full reporter displacement

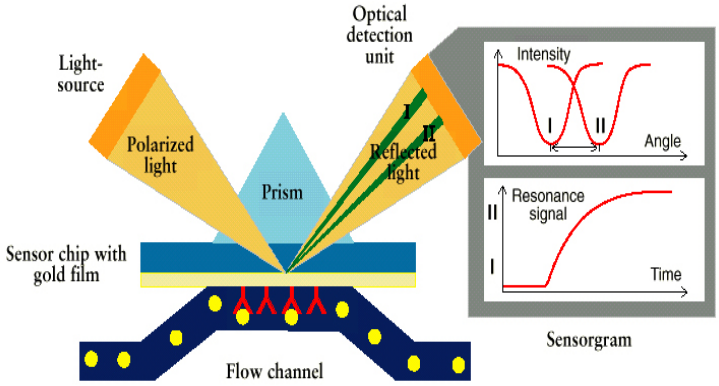
Neumann L.; von König K. & Ullmann D. (2011) HTS Reporter Displace ment Assay for Fragment Screening and Fragment Evolution Towards Leads with Optimized Binding Kinetics, Kinetic Selectivity, and Thermodynamic Signature, In: Methods in Enzymology, Vol. 493, 299-320.

Surface Plasmon Resonance (SPR)

Label free screening and profiling

- Powerful tool for studying biomolecular interactions in a sensitive and label-free detection format
  - Secondary screening of compound libraries and hit conformation
  - Primary screening of larger fragment libraries for hit identification
- Principle
  - Measurement of the refractive index near the senor surface
  - Immobilization of a ‘ligand’ (i.e. enzyme, receptor) on the sensor surface
  - Monitoring of binding kinetics of analytes in solution passing the surface under continuous flow

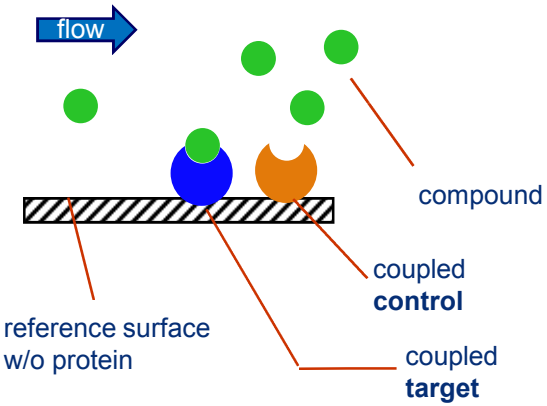


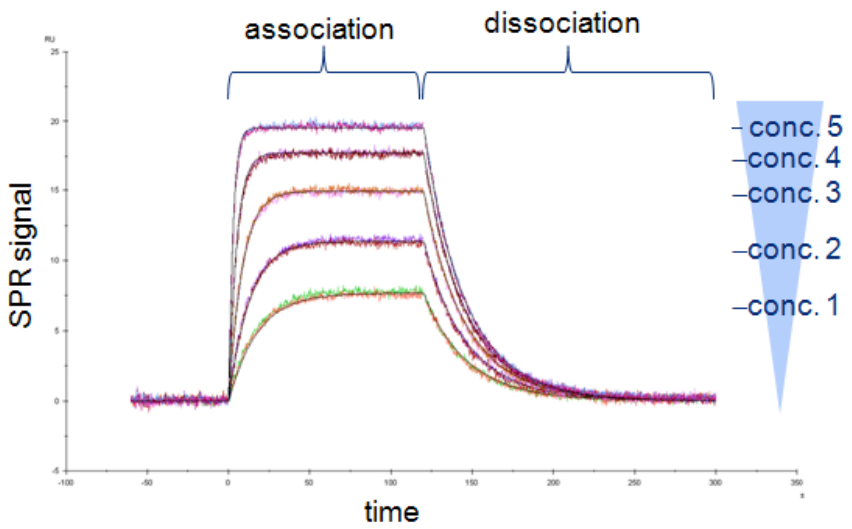


Surface Plasmon Resonance (SPR)

Direct Binding Assay

- Monitoring biomolecular interactions in real-time
  - Target protein and unrelated control protein is immobilized to sensor chip
  - Analytes (compounds/fragments) are flown over enzyme surface
    - Binding of compound to enzyme results in increase of SPR signal (association phase)
    - Wash out of bound compound with buffer result in decrease of SPR signal (dissociation phase)
- Fitting of sensorgrams recorded at different compound concentrations to an appropriate interaction model
  - Extraction of kinetic parameters (k_a, k_d → K_D)
- Requirements/limitations
  - k_a / k_d values in reasonable ranges
  - Protein size (< 100 kDa)
- Excellent method to screen fragments and profile/ validate hits identified in HTS campaigns
  - BC4000 used for primary screening (up to 4,000 data pts/week)
  - Biacore T100 suitable for hit profiling and validation



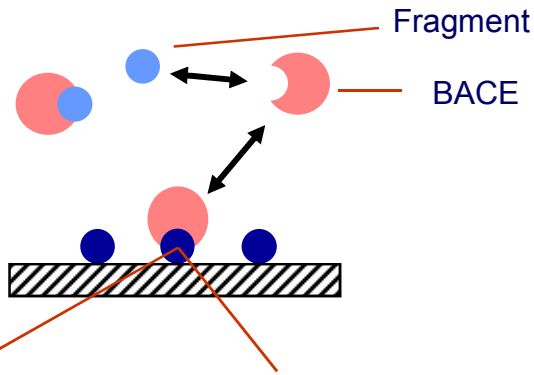


Surface Plasmon Resonance (SPR)

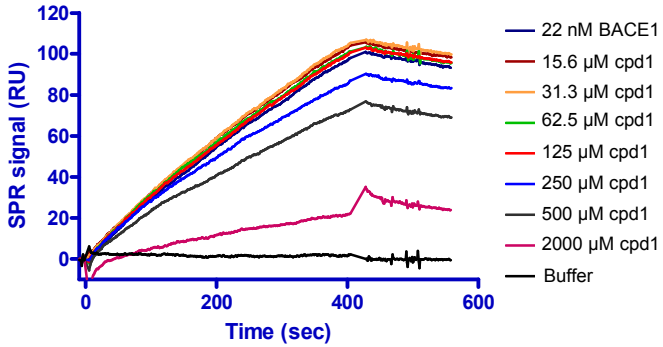
In-solution Competition Assay (ISA)

- Validation of fragment inhibitors of target enzyme by label-free SPR-based in-solution competition assay (ISA) – case study BACE

Solution competition. Competitor fragments (light blue) bind to BACE (red) obstructing binding to the high affinity peptide ligand (blue). Binding of free analyte (BACE) is detected.



Sensorgrams of hit fragment profiling



Calculated IC_{50} of ~ 0.9 ± 0.1 mM is spot on with value determined in biochemical assay (0.87 ± 0.26 mM)

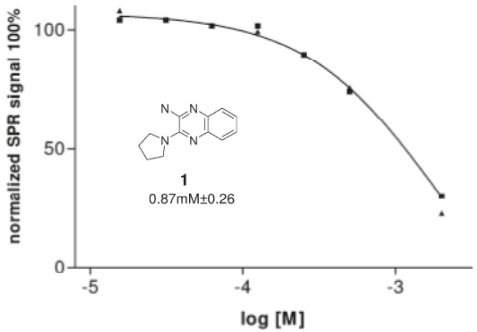


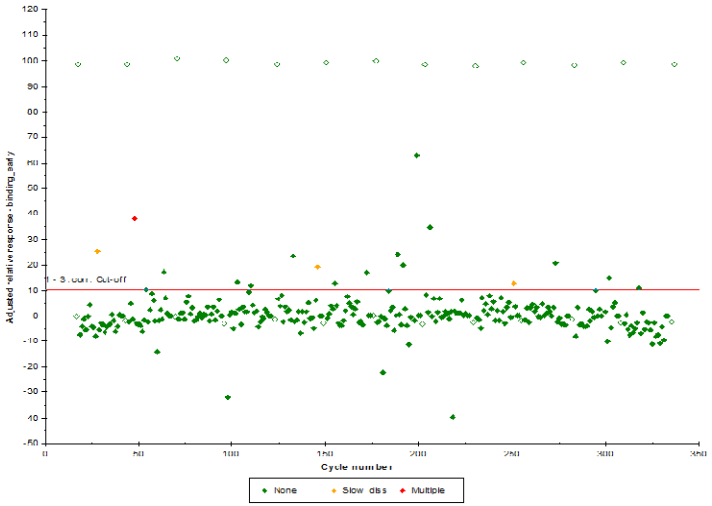
FIGURE 4: Inhibition in solution of interaction of BACE1/KEEISEVN-statin-VAEF with Compound 1 on a Biacore device. Data were fitted by nonlinear regression, and IC_{50} is estimated to be 0.9 ± 0.1 mM (n = 2). The maximum applied compound concentration is 2 mM; therefore, only a partial sigmoidal concentration response curve was recorded.

Godemann et al. (2009) Biochemistry 48, 10743-51

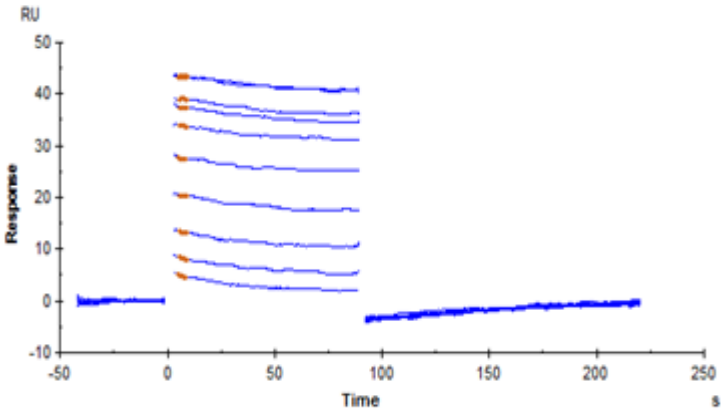
Surface Plasmon Resonance (SPR)

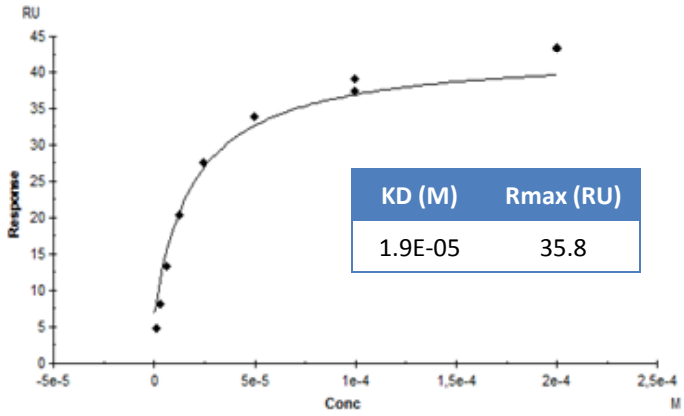
Case study fragment screen cytokine target

- 10k of the Evotec fragment library screened at 100 µM fragment conc. using BC4000
  - Interactions with an unrelated control protein monitored in parallel
  - Replicate injections of control inhibitors throughout the run to ensure surface performance: Z‘: 0.6 - 0.9
  - Hit identification using 3σ method; hit threshold calculated for each plate and each protein surface separately
  - Prioritization of fragments based on binding level, sensorgram shape and selectivity (using curve markers)
- Profiling of prioritized fragment hits returned specific, well behaved binders
  - 8-pt CRCs, incl. 2 conc. in duplicates, 2-fold dilution



high affinity fragment hit

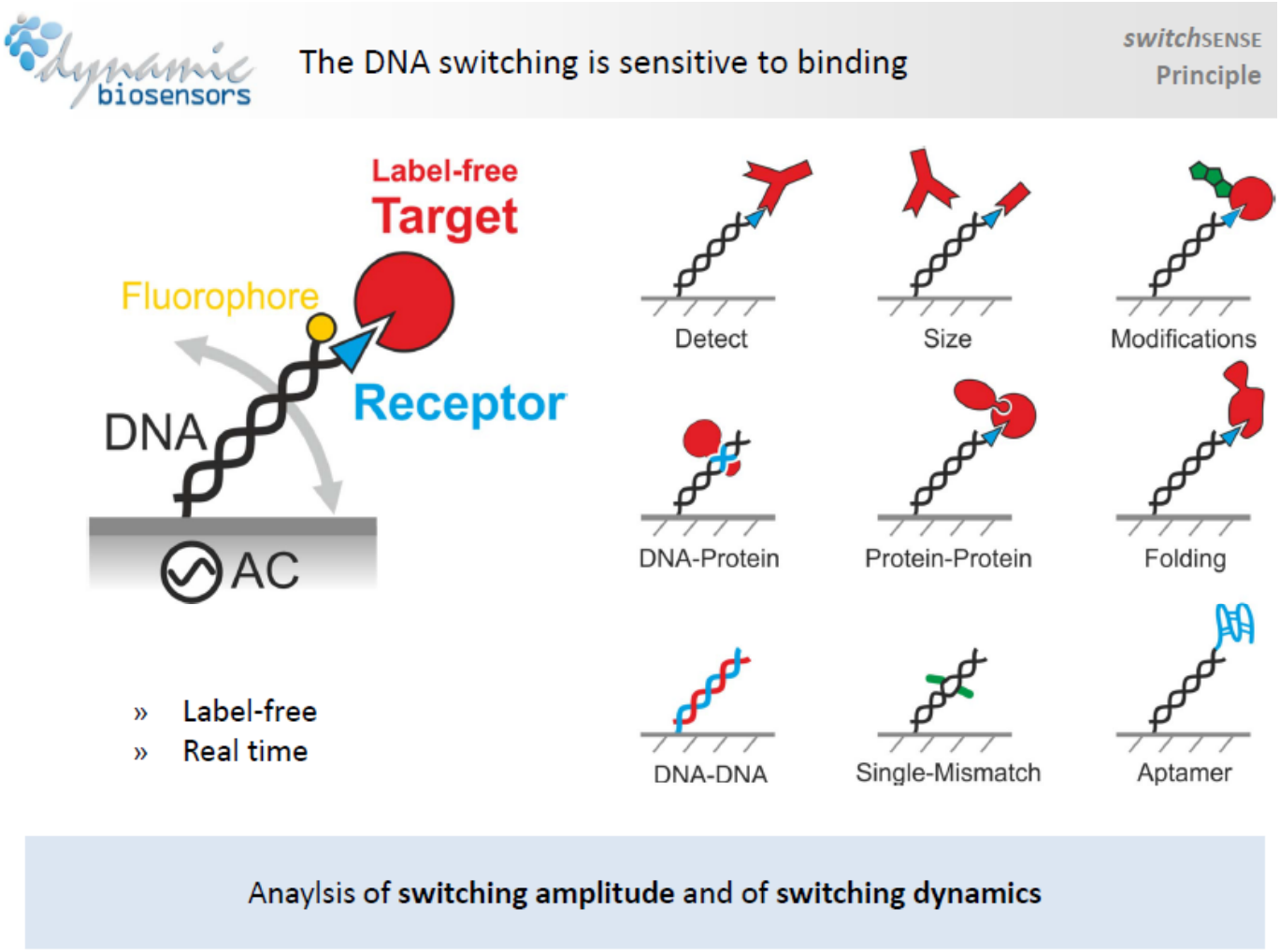




KD (M)	Rmax (RU)
1.9E-05	35.8

switchSENSE Technology

Novel multiparamter “SPR-like” approach



- Principle is based on electrically switchable DNA interfaces
- New additonal parameters: Molecular Size and shape
- Located in Munich, Germany

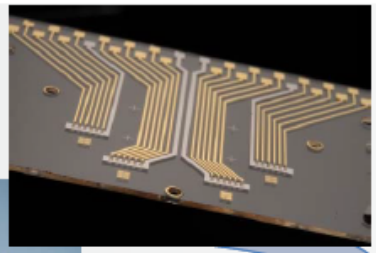
www.dynamic-biosensors.com/

switchSENSE Technology

Technology Potential

*switch*SENSE is the only label-free technology allowing the measurement of molecular dynamics

*switch*SENSE Principle



switchCHIP
24 x parallel
4 flow channels, V=1µL
re-usable

***switch*SENSE analyzer**
label-free, real-time,
variable temp.,
molecular dynamics

k_{on}, k_{off}
K_D
$T_{denature}$
size & shape

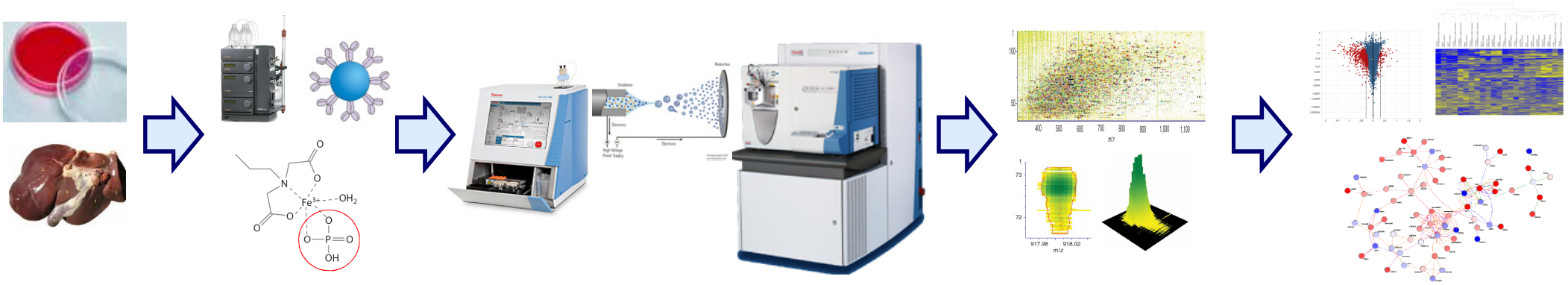
Measurement of **molecular dynamics** allows **unrivaled sensitivity** and **determination of size and shape** (e.g. conformational changes, aggregation)

- Sensitivity ca. 1000x higher than SPR
- Perfect method for high affinity interactions (pM-fM, Biologics)

*switch*SENSE Analyzer

Mass Spectrometry based Proteomics

Binding Dynamics within Cells and Proteome



Sample | **Proteins or Peptides** | **Separation** | **Ionization** | **Mass Spectrometer** | **Data Analysis** | **Data Interpretation**

Isotopic Labeling
(SILAC, DiMe, mTRAQ, iTRAQ, TMT)

Pre-fractionation / Enrichment
(Affinity beads, SCX-IMAC/TiO_2, anti-bodies, SAX, high pH RP)

Liquid Chromatography (LC)
(EASY nLC1000, 20-50 cm, 75µm ID, 1.9 µm bedas)

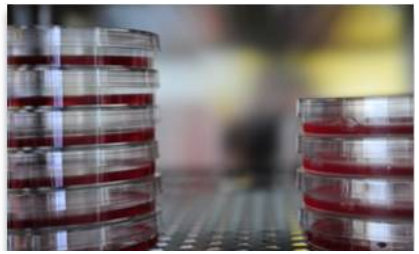
Mass Spectrometry (MS)
(LTQ-Orbitrap Velos, Q-Exactive, TSQ Vantage)

Identification/Quantification
(MaxQuant)

Bionformatics/Statistiks
(Spotfire, in-house programs, e.g. SubExtractor)

Cellular Target Profiling®

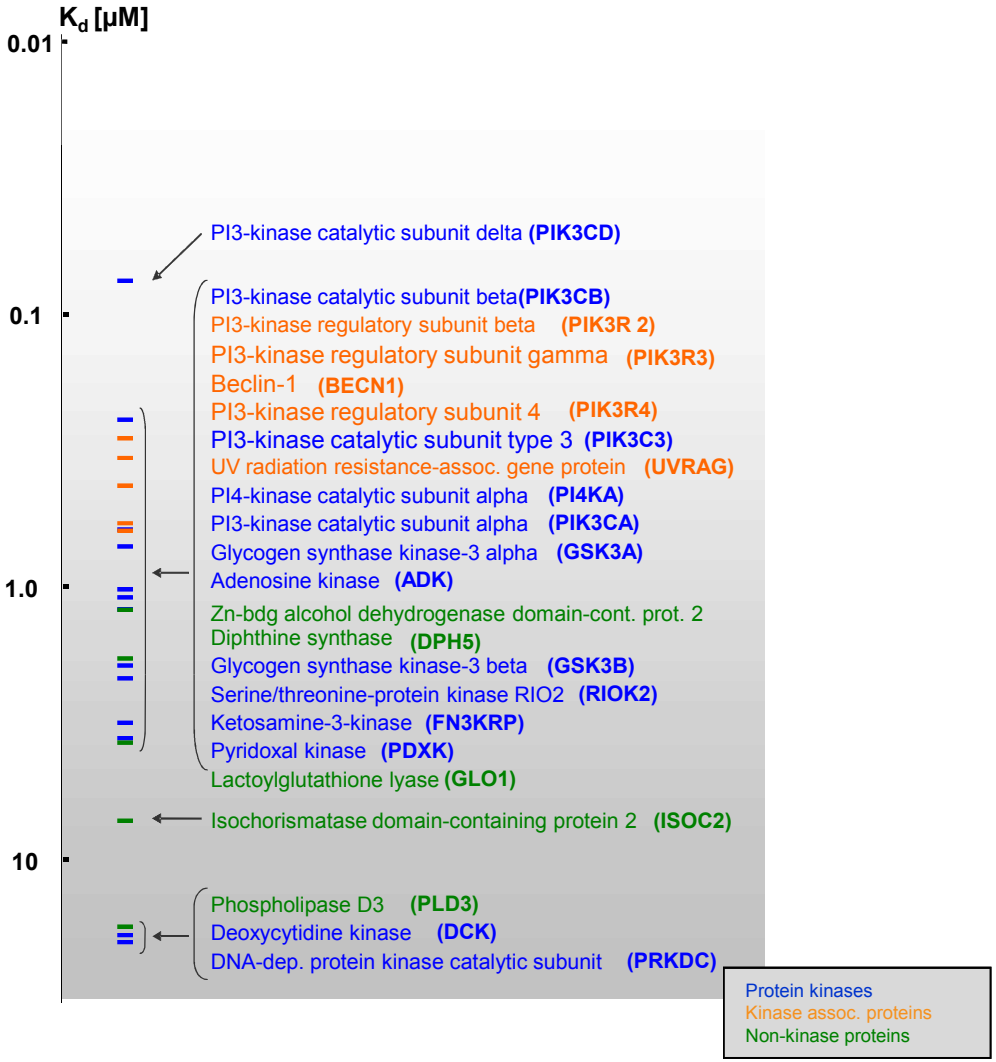
Revelation of a compound's cellular target spectrum



- Determines a compound‘s proteome-wide binding affinities in **any cell line or tissue of choice**
- State-of-the-art chemical proteomics facilitates **unbiased native profiling** against endogenously expressed, full length proteins in the presence of cellular co-factors and complex partners
- Enables **target deconvolution** of hit compounds from phenotypic screens and thus broadens the available chemical space for drug discovery
- Provides cellular selectivity data and identfies off-target liabilities to inform decisions in lead optimization and clinical candidate selection
- Extensive, non-target class restricted track record in **target deconvolution** and **profiling** of various small molecule compounds (e.g. kinase inhibitors, antibiotics, epigenetic drugs, HDM2 inhibitors & small molecules targeting metabolic enzymes, ligases, reductases, transferases, heat shock proteins)

Cellular Target Profiling®

Profiling of a kinase inhibitor



PIK3CD ⟷ $K_{d,free}$ = 87 nM

PIK3CB ⟷ $K_{d,free}$ = 300 nM

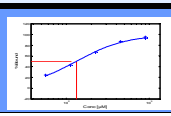
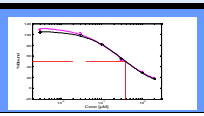
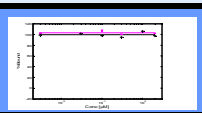
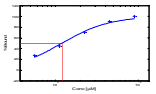
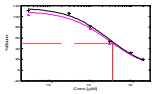
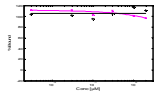
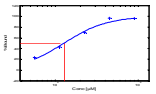
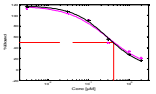
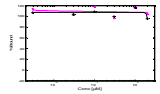
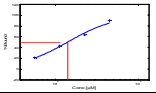
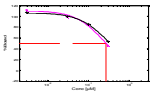
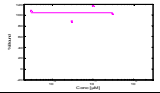
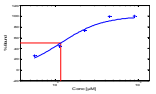
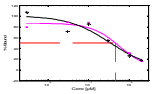
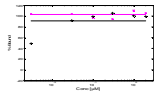
PIK3C3 ⟷ $K_{d,free}$ = 800 nM

PIK3CA ⟷ $K_{d,free}$ = 1366 nM

UCB's cpd discriminates PI3K isoforms within a cancer cell line

Cellular Target Profiling®

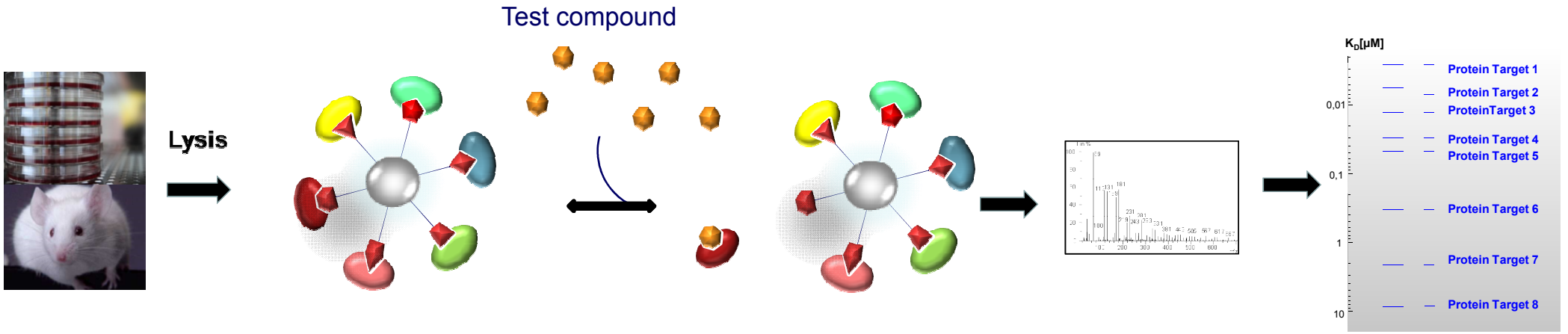
Target deconvolution study with Johnson&Johnson

Protein Name	Sequence Coverage [%]	Binding Curve (Linker Compound)	$K_D$immo [µM]	Competition Active Compound	Competition Inactive Compound	$K_D$free [nM]
Protein X	**80.7**		**132.8**			**49.7**
Protein A	**1.9**		**120.7**			**44.7**
Protein B	**3.5**		**127.9**			**51.0**
Protein C	**2**		**141.1**			**40.8**
Protein D	**0.1**		**116.9**			**47.8**

- Compound was identified from a phenotypic screens with differential effect on cancer cell lines
- Cellular Target Profiling® identified protein X as main cellular target
- Gene expression profiling and target validation by Johnson&Johnson confirmed key role of protein X as critical target in sensitive cancer cells

Chemical proteomics for sub-proteome analysis

KinAffinity® & Epigenetics Target Profiling®



Cell or tissue sample

Enrichment of selected subproteomes by suitable small molecules

Competition of target proteins by test compound

LC-MS/MS analysis

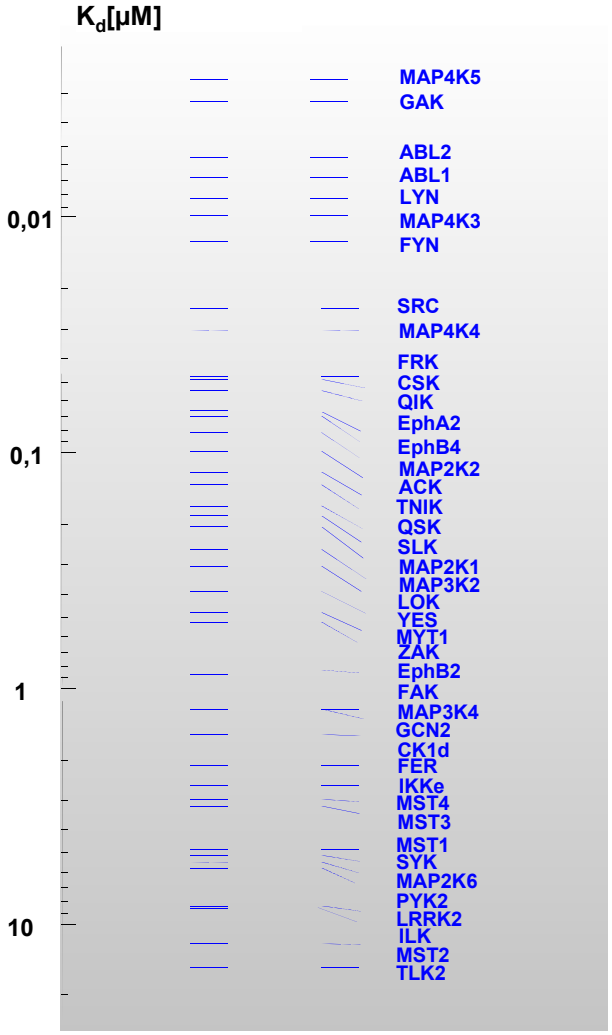
Subproteomic K_d protein target list

Mixture of beads of broadly selective kinase or HDAC inhibitors

- Rapid native selectivity assessment against endogenous kinases HDACs without need for linker compound synthesis
- Correlation of *in vivo* data with cellular target information to support the selection of drug candidates

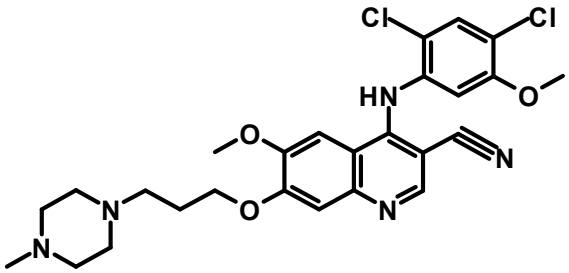
KinAffinity®

Native kinome profiling



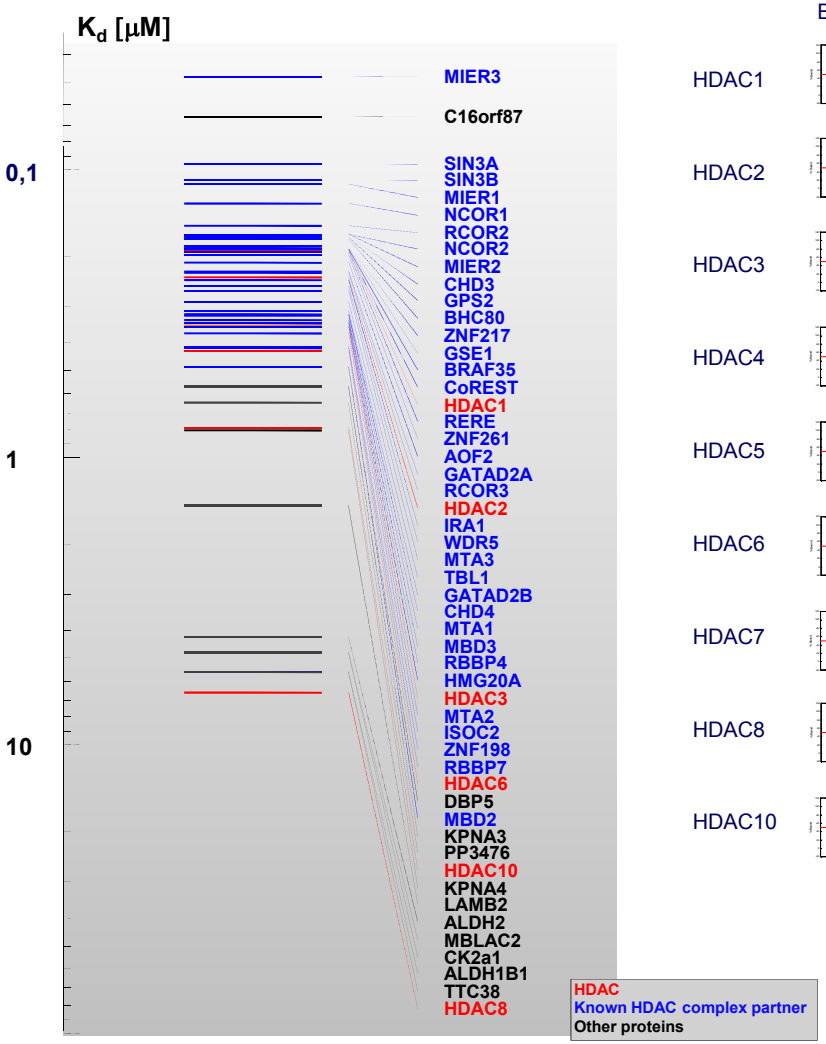
Target profile of bosutinib in PC3 cells

- **Bosutinib** (SKI606, Wyeth) is currently tested in breast cancer and CML
- KinAffinity® enriched nearly 200 endogenously expressed kinases from human prostate cancer (PC3) cells
- 45 of these kinases were identified as molecular targets of bosutinib



Epigenetics Target Profiling®

Profiling of SAHA (Vorinostat) using a generic matrix



	Binding	Competition	K_d [µM]
HDAC1			0,19
HDAC2			0,24
HDAC3			0,35
HDAC4			-
HDAC5			-
HDAC6			0,43
HDAC7			-
HDAC8			> 6,5
HDAC10			0,79

Target profile in human ovarian cancer (A2780) cells

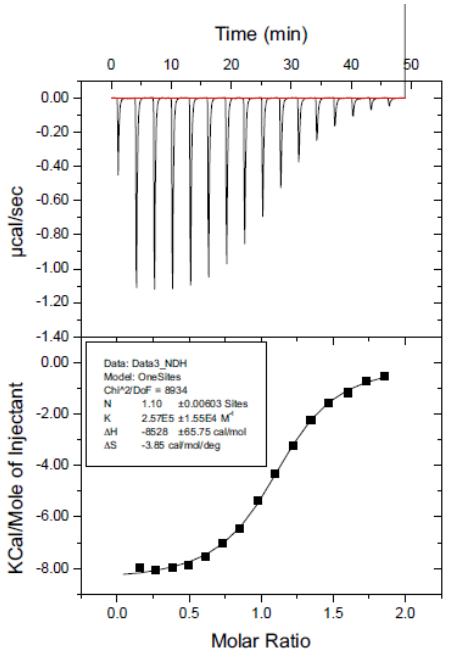
Epigenetics Target Profiling® allows for maximum HDAC coverage and constitutes a native assay under conditions that preserve the integrity of HDAC complexes

Isothermal Titration Calorimetry (ITC)

Gold standard in binding thermodynamics

- ITC simultaneously determines all binding parameters (N, Kd, ΔH and ΔS) in a single experiment
- High protein consumption, low throughput (MicroCal iTC 200 as a sensible compromise)

XX0088XXXX



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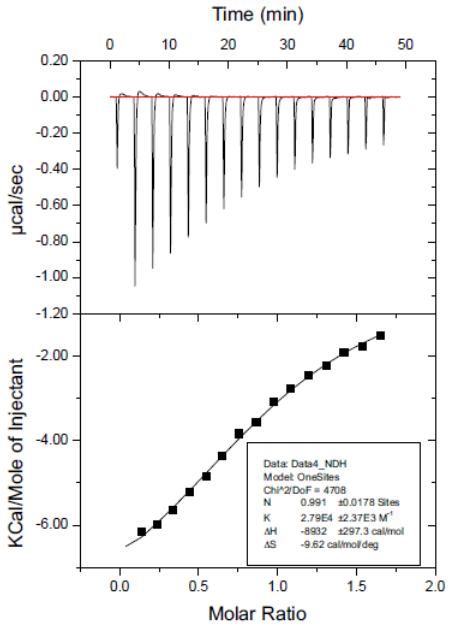

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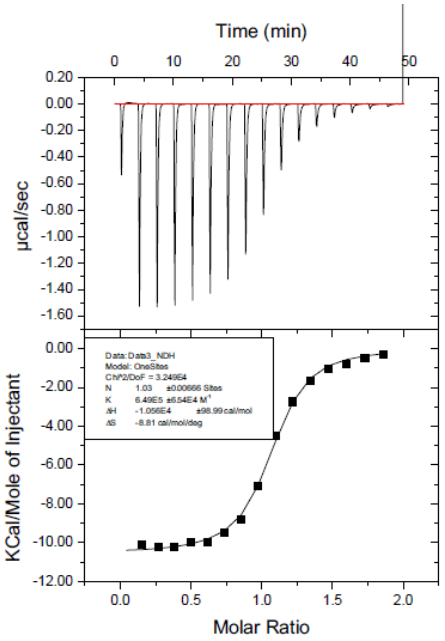
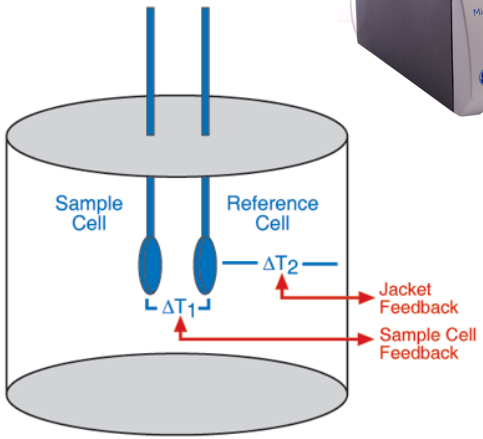


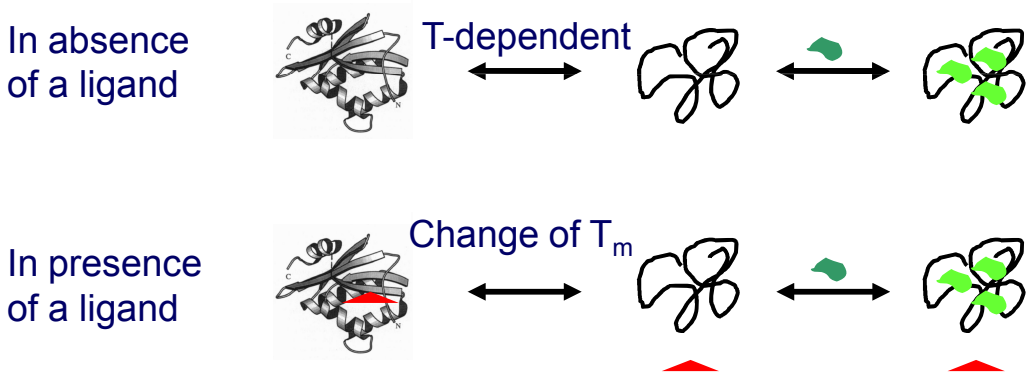


- Power required to ΔT_1 maintained during titration of ligand into protein solution equal to Heat Change of Binding ΔH
- Slope defines $1/K_d$
- Molar ration defines Number of binding sites

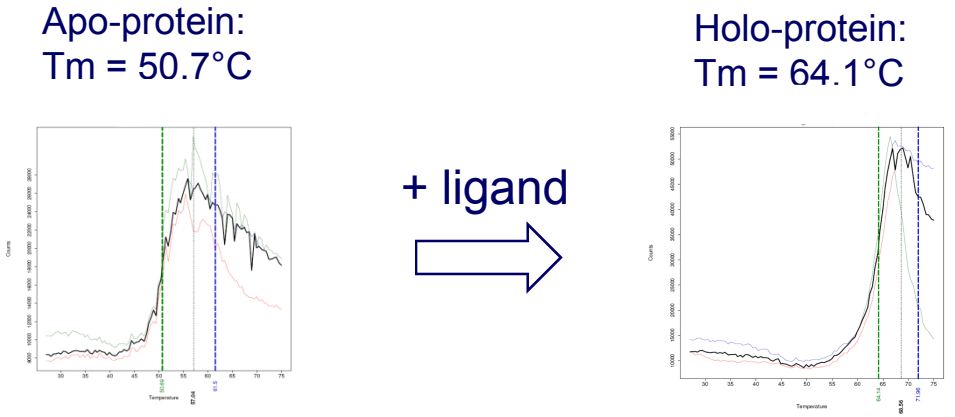
Thermal Shift Analysis

Protein folding and thermodynamic equilibrium analysis

- Thermal shift analysis
  - Based on energetic coupling between ligand binding and protein unfolding
  - Detects thermal unfolding of a protein (melting temperature T_m)
  - Assumes that a ligand changes the thermal stability of a protein (+/- ΔT_m)
  - Environment-sensitive dyes selectively interact with the unfolded state



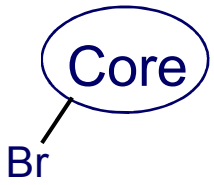
- Evotec‘s capacity:
  - FluoDia T70, Photon technology international
  - 384-well format
  - 1 plate/day
  - Detection range: 25 – 75°C
  - Routinely applied in hit validation phase and as early filter for X-ray crystallography

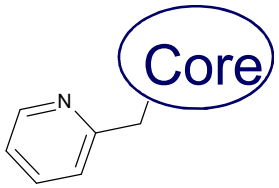


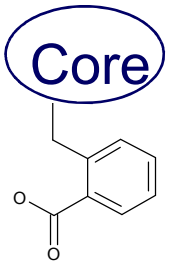
Example: Hit to Lead Project Support

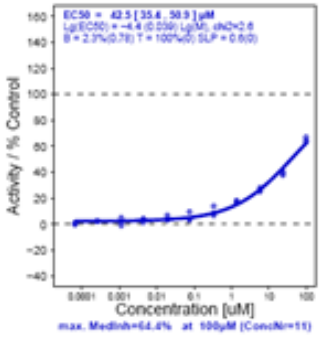
Thermal shift drived compound series prioritization

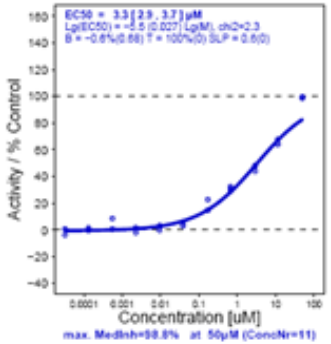
- Increase of thermal stabilization and potency within a compound series

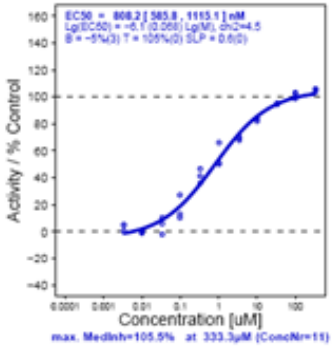










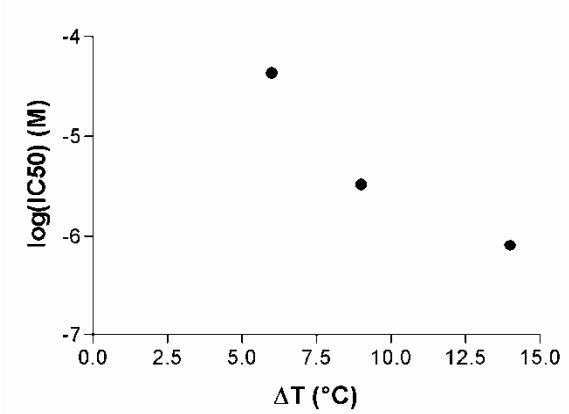


IC_{50}: 43 µM
T_m: ΔT = 6°C

IC50: 3.3 µM
Tm: ΔT = 9°C

IC50: 0.81 µM
Tm: ΔT = 13.4°C

Correlation between TS and potency



Increase of potency and thermal shift

Example: Multi-equilibrium target inhibitors

Interpretation of thermal-shift data

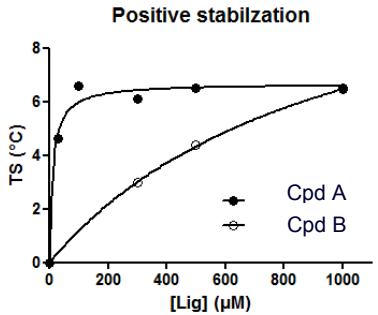
- Observations: compounds may stabilize or destabilize target (Prot)
- Proposed mechanism:

Stabilization

$Prot_{native} + Lig \overset{K_D}{\rightleftharpoons} Prot_{native}Lig$

T_M ~50°C | T_M >52°C

$Prot_{unfold} + Lig$ $Prot_{unfold}Lig$

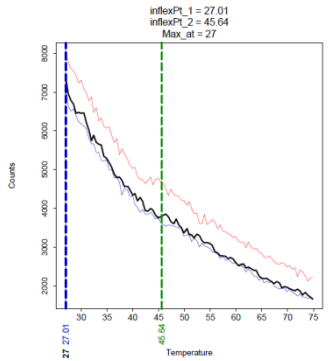


Destabilization by unfolding

$Prot_{native} + Lig$ $Prot_{native}Lig$

T_M ~50°C | T_M <30°C

$Prot_{unfold} + Lig \overset{K_D}{\rightleftharpoons} Prot_{unfold}Lig$



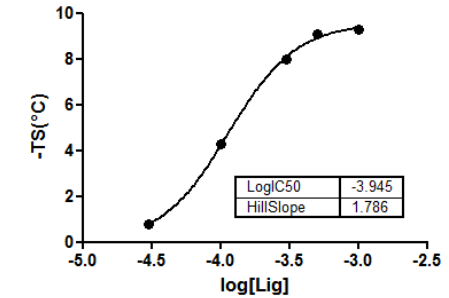
Destabilization by forming monomers

$Prot_{dimer} + 2xLig$ $Prot_{native}Lig$

$2xProt_{monomer} + 2xLig \overset{K_D}{\rightleftharpoons} 2xProt_{monomer}Lig$

T_M <48°C

$2xProt_{unfold}Lig$



Application of FMO Calculations

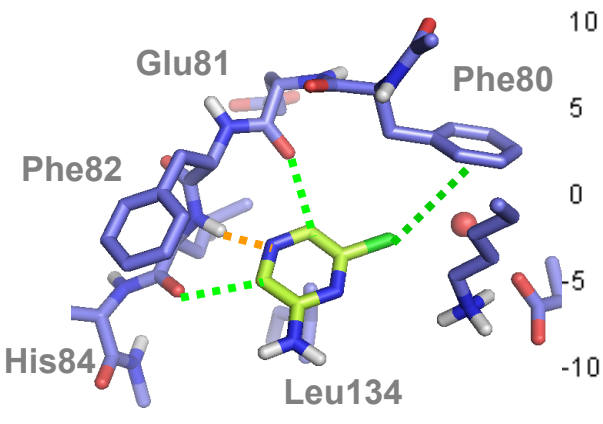
Example of a fragment in complex with CDK2

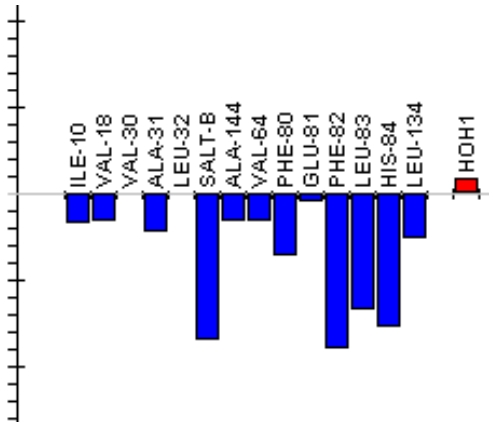
- Fragment Molecular Orbital (FMO) QM calculations can be used to assess the interaction enthalpy between a small molecule and each amino acid residue in the binding site of the protein
  - Analysis of Paired Interacting Orbitals (PIO) and by Pair Interaction Energy Decomposition Analysis (PIEDA) can give valuable insight into what are the key interactions
  - FMO results may not correlate directly with activity data as solvation and entropy effects are not considered

PDB: 1WCC

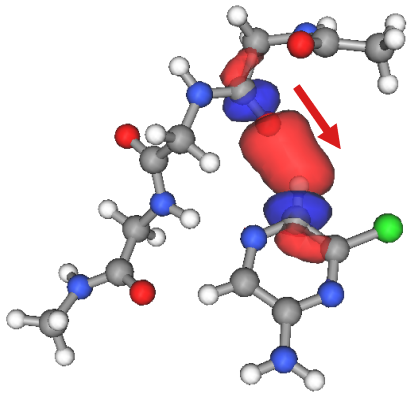
IC_{50} = 350 μM

-48.40 kcal/mol

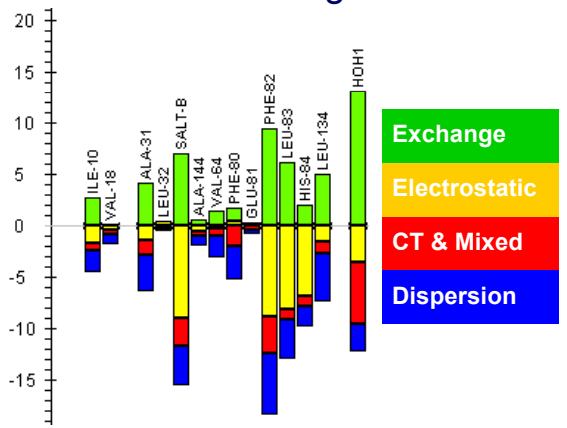




PIO analysis



PIEDA diagram

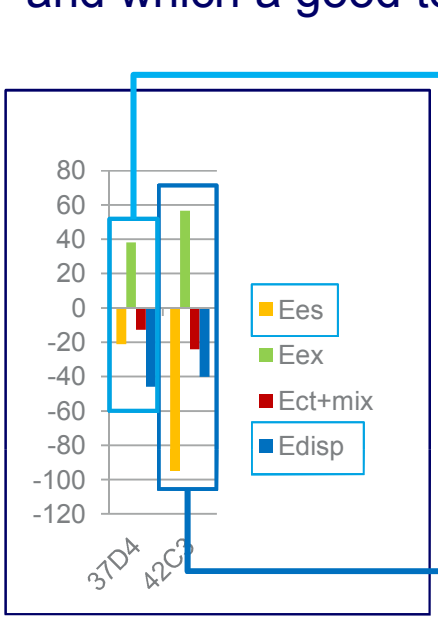


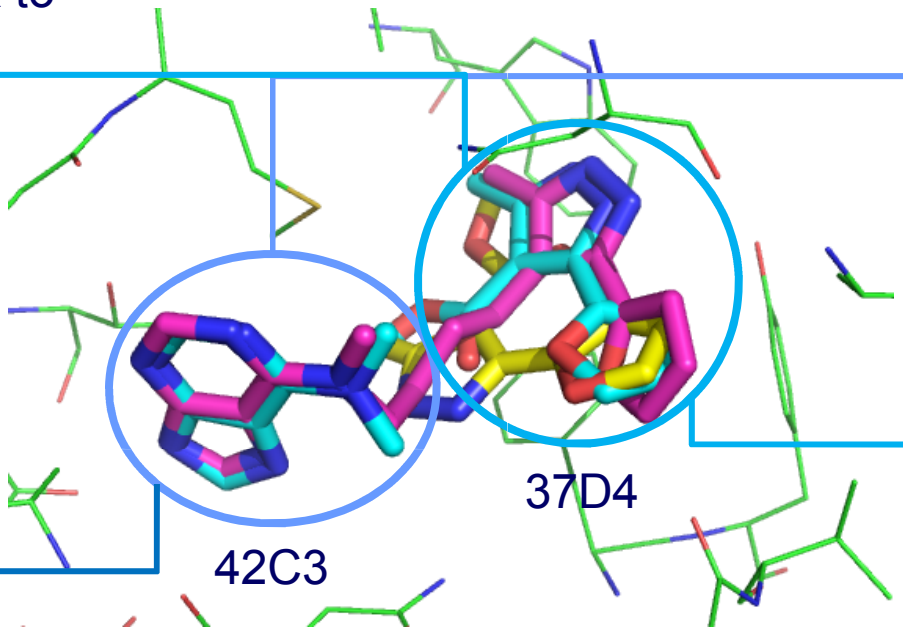
Mazanetz M.P. *et al., J. Cheminform.*, 2011, **3**:2.

Use of FMO analysis to select fragments

Which to expand on, which to link to? Hsp90 example

- FMO can be used to select/prioritize fragments for expansion or linking
- Ratio of electrostatic and dispersive interactions predicts which fragments are good to expand on, and which a good to link to





- Good elec/disp. ratio
- Good for expansion – low RMSD

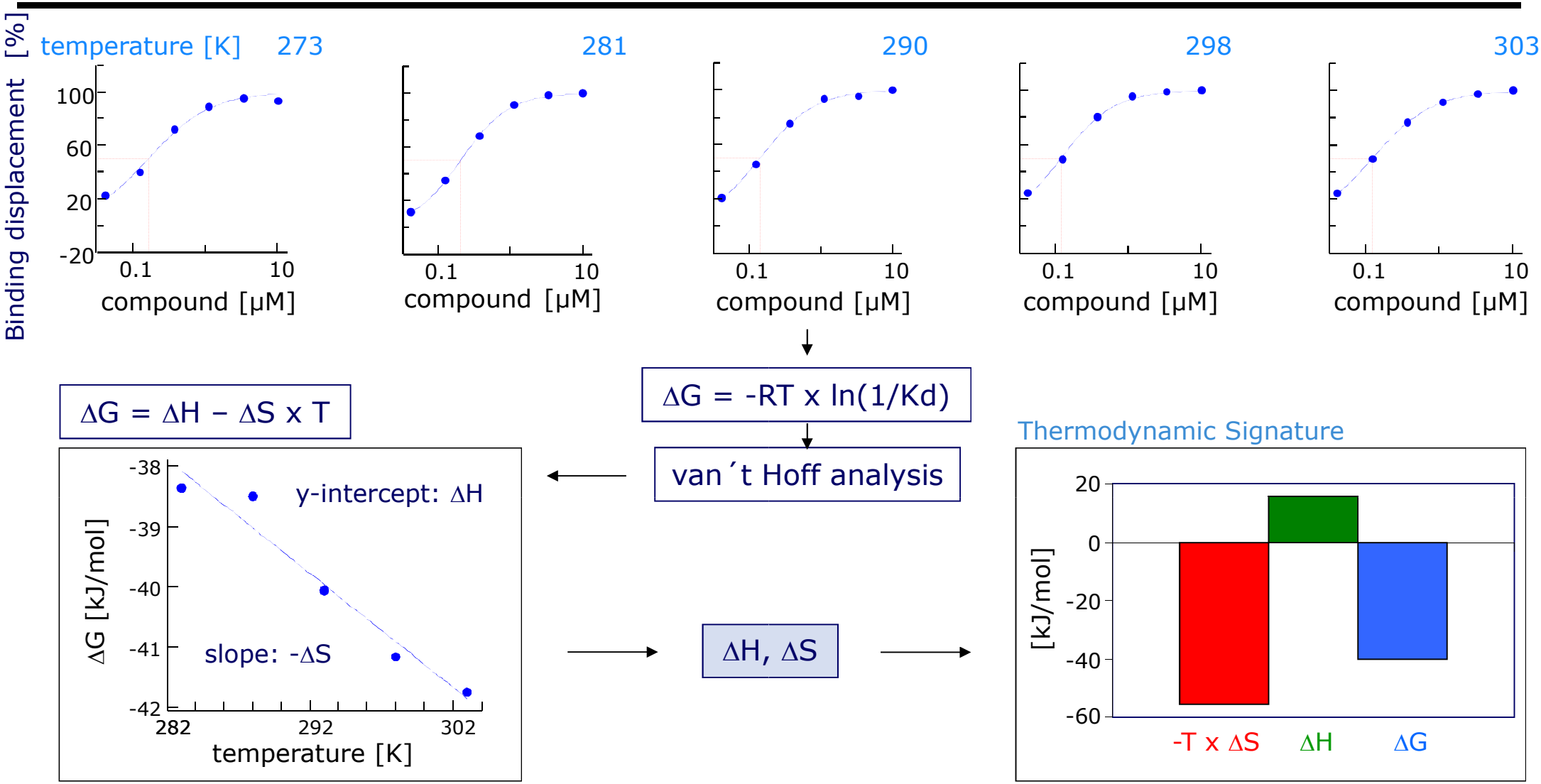
- Relatively highly dispersive
- Not good for expansion
- Good for linking to from other good ratio fragment

- Maintaining the electrostatic/dispersive balance in medicinal chemistry is important for maintaining potency (too high elec – high desolvation penalty)

Ichihara O. *et al., Molecular Informatics*. 2011, **30**, 298-306.

Thermodynamic signatures - STR

Impacting MedChem optimization



Neumann L.; von König K. & Ullmann D. (2011) HTS Reporter Displace ment Assay for Fragment Screening and Fragment Evolution Towards Leads with Optimized Binding Kinetics, Kinetic Selectivity, and Thermodynamic Signature, In: Methods in Enzymology, Vol. 493, 299-320.

Summary and conclusions

1. There is tremendous and emerging panel of biophysical methods and technologies available impacting more and more on drug discovery
2. Kinetics and thermodynamics are appreciated parameters by an increasing number of medicinal chemists in hit and lead optimization
3. Many technologies show high-throughput capabilities e.g. enable the introduction of biophysical methods earlier, some have even primary screening capabilites
4. Kinetics and thermodynamics are of increasing relevance to more complex models and networks (cellular, proteome)
5. There is no single technology – a thorough understanding of biophysics will drive the selection of the right method

Acknowledgments

Key Contributors to Evotec's Biophysics technology platform

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Stefan Mueller
Christoph Schaab
Andreas Tebbe

Protein Production

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Richard Hitchman
Simone Mueller
Christoph Scheich

X-Ray Crystallography

Ole Anderson
John Barker
Myron Smith

Computational Chemistry

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Richard Law
Michael Mazanetz
Inaki Morao

Medicinal Chemistry

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Stephen East
David Hallett
Craig Johnstone
Clive McCarthy
Mark Whittaker

DYNAMIC BIOSENSORS

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proteros

Lars Neumann

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‘RESEARCH NEVER STOPS’

Building innovative drug discovery alliances

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