Outside the cytoplasm: Biophysical ligand screening for membrane proteins?

September 2010

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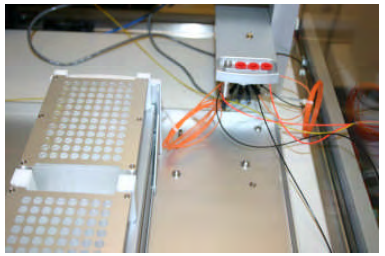

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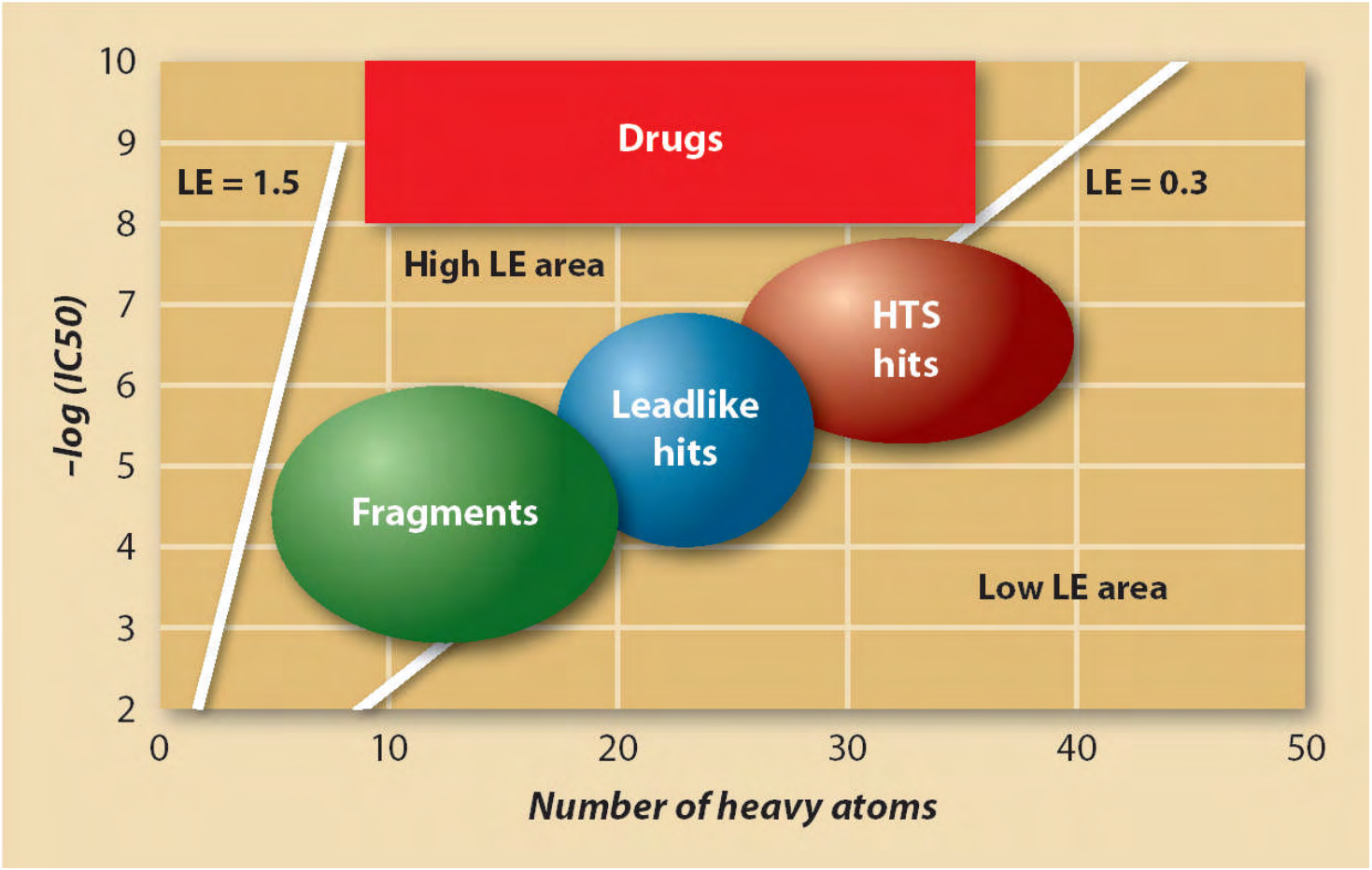
Fragments: Why small is beautiful



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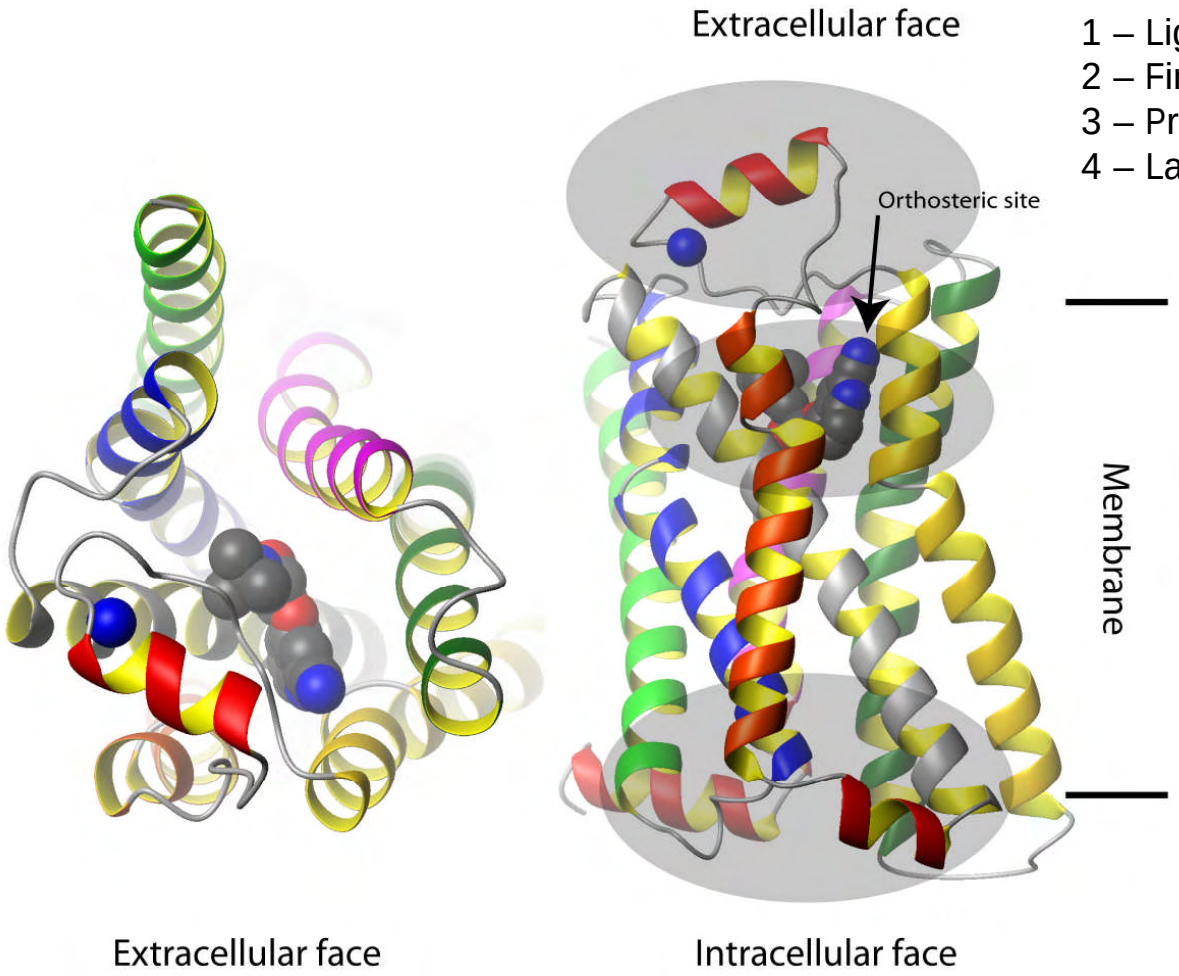
Why biophysical fragment screening for GPCRs?



1 – Ligands for peptide activated GPCRs
2 – Find sites with novel biological function
3 – Provide structural information
4 – Label free

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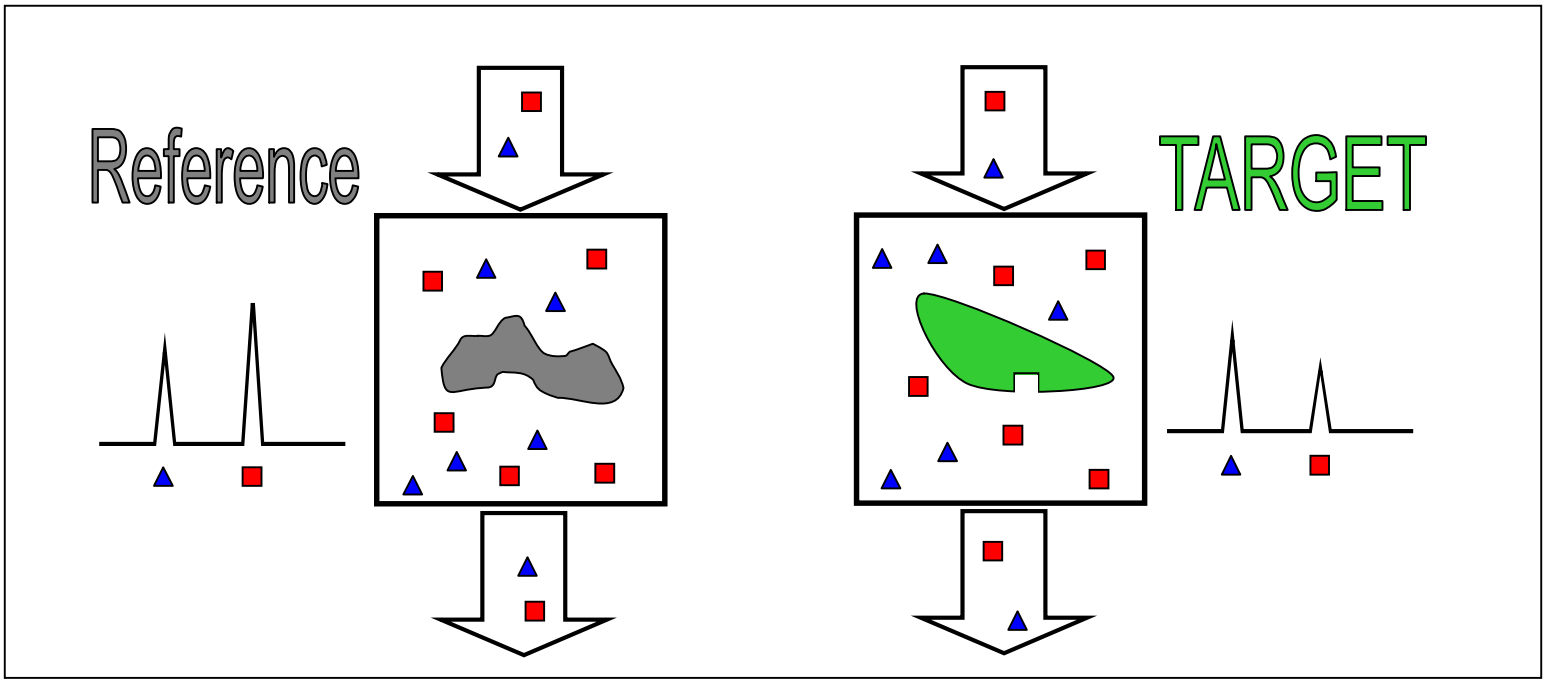
The TINS method for finding hits

TINS = Target Immobilized NMR Screening



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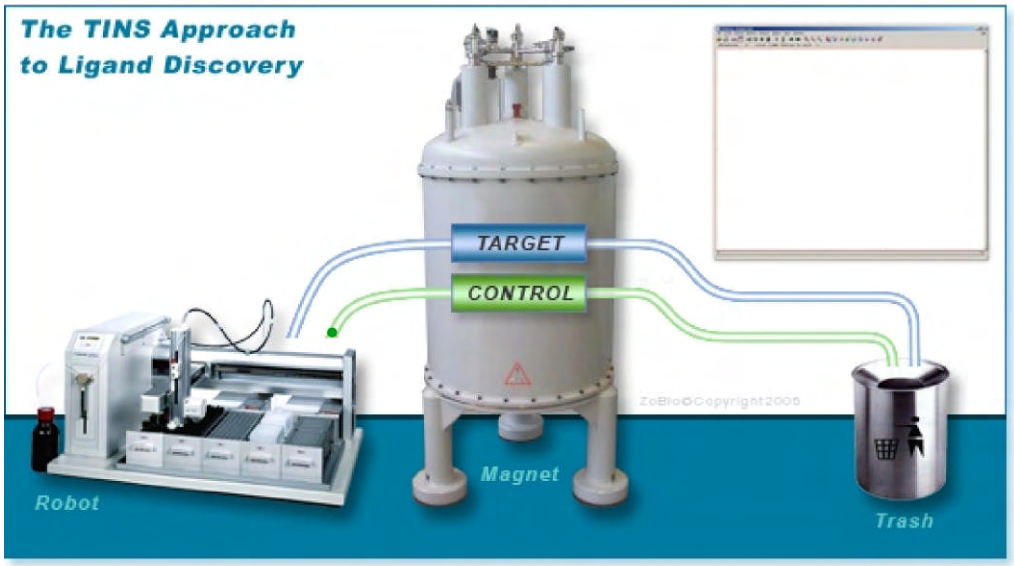
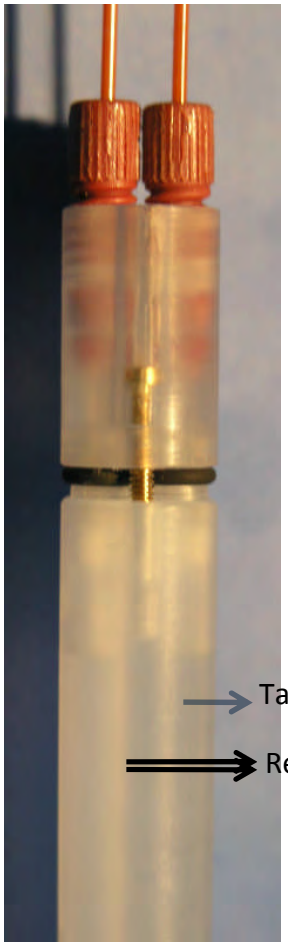
The TINS Screening Station



Dual cell
Sample holder



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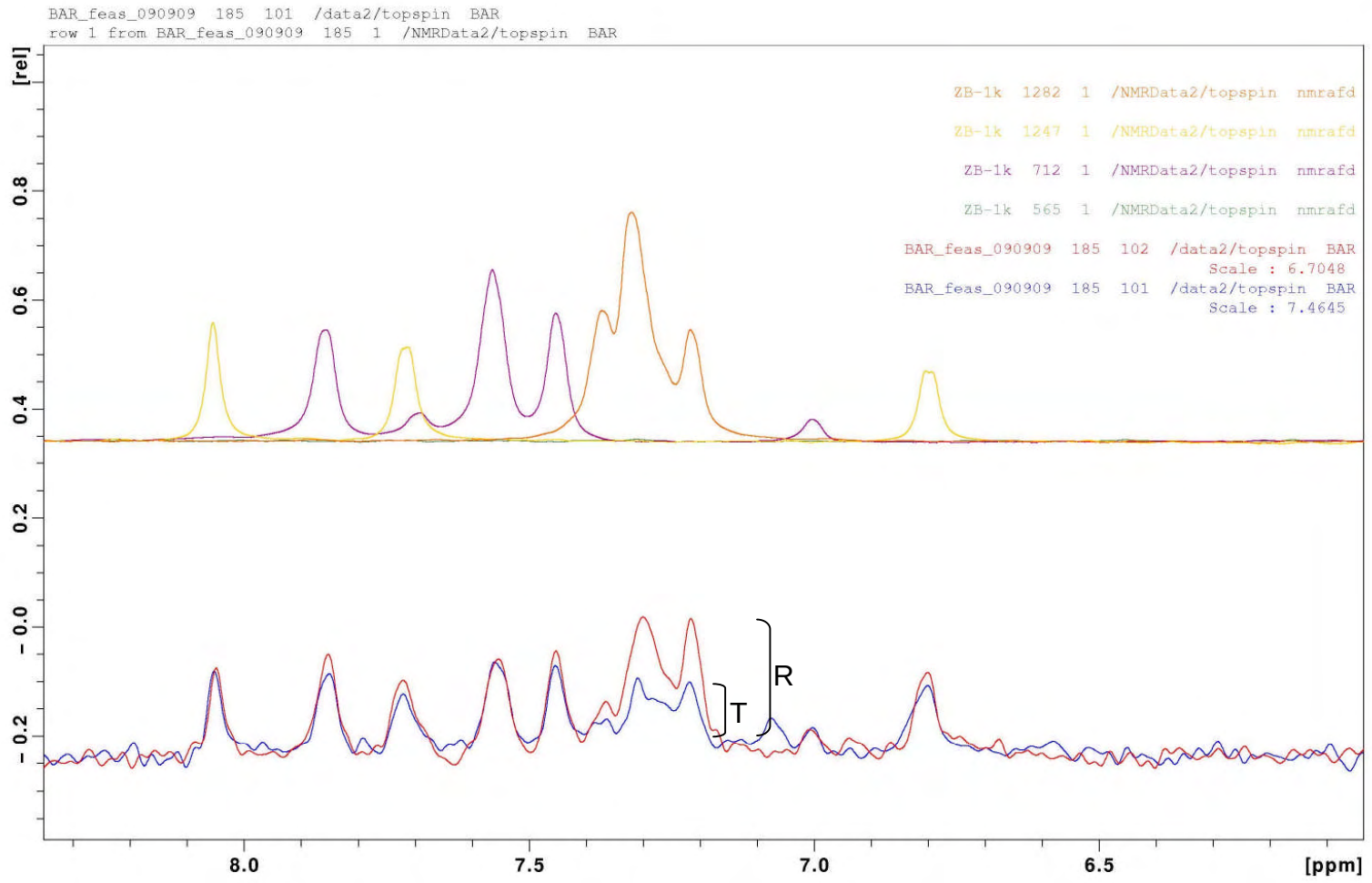
An example of raw data



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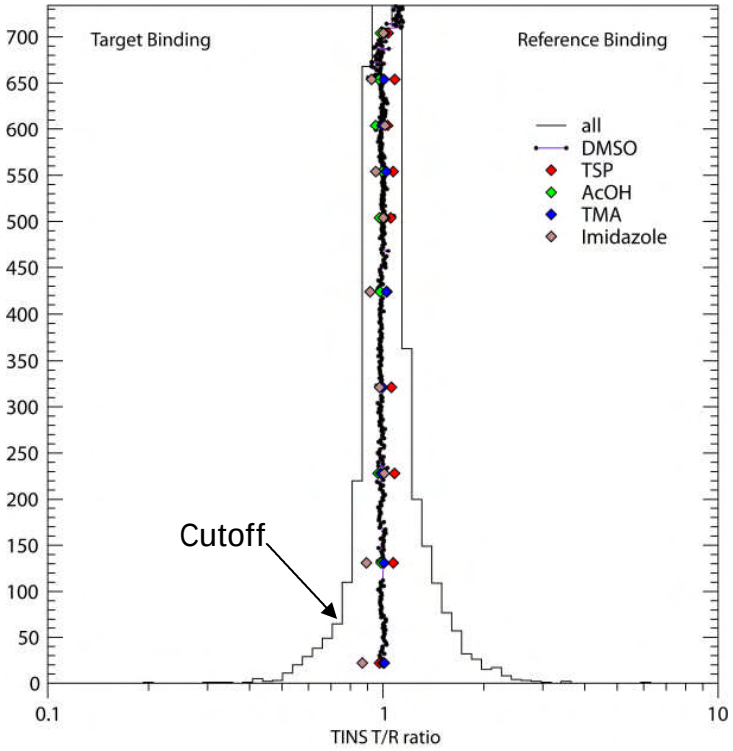
Summary of Selected Immobilized Targets

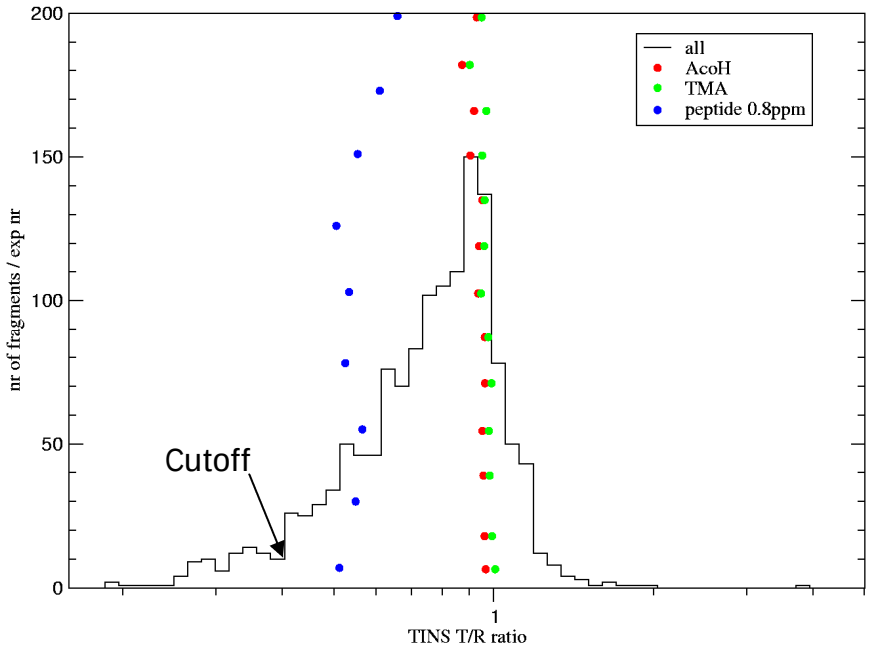
Protein	Size	Functional	TINS hits
Protease	44 kDa	LB	5.9%
HSP90	24 kDa	LB	6.5%
Small GTPase	20 kDa	LB/BA	9% apo form 3% NDP form
Viral enzyme	67 kDa	LB/BA	9.5%
DsbB (Bacterial mem. Prot.)	14 kDa	BA	7.3%
Various kinases (pY,pS/T)	30-35 kDa	LB/BA	3.8-5.1%
KcsA (Ion channel)	57 kDa	LB	Feasibility only (95 cmpds, 7%)
Metalloproteins	105 kDa homotrimer	LB/BA	5-8.5%
Prot-Prot Interaction (5)	14-100 kDa	LB	3-6%

LB ligand binding
BA biochemical assay

Fragment screening with TINS



Undruggable Target
11 hits



Druggable Target
89 hits

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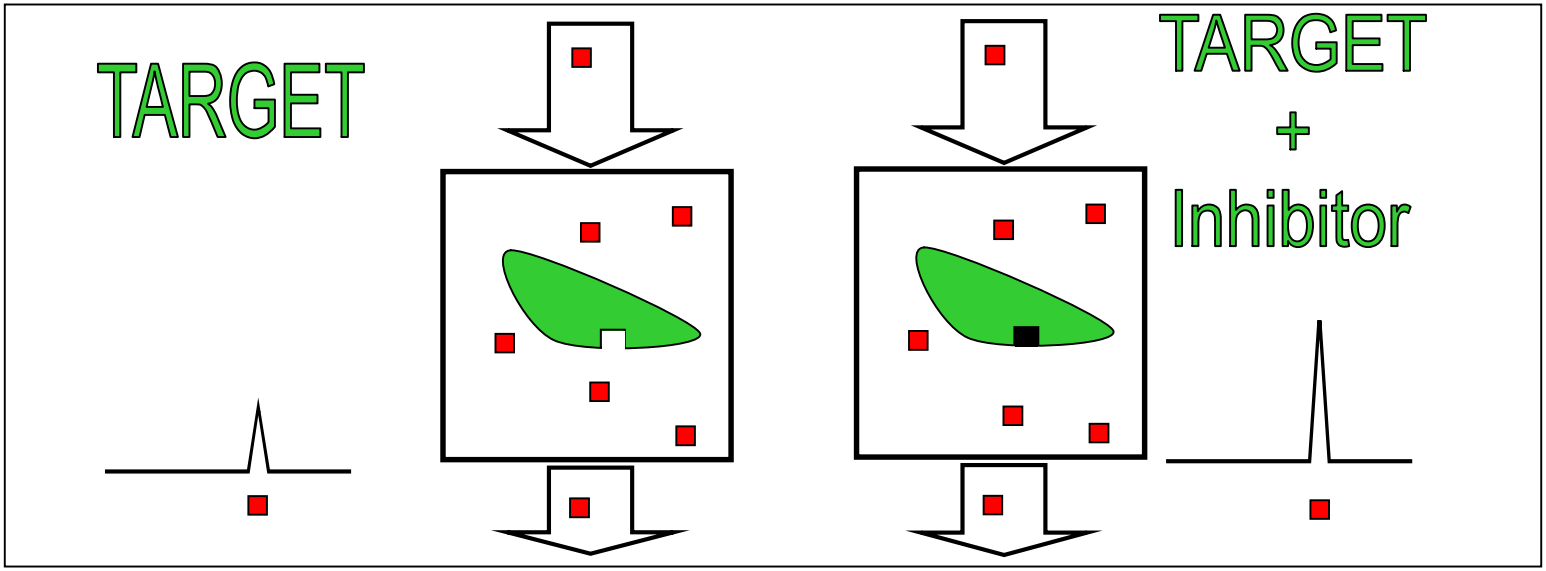
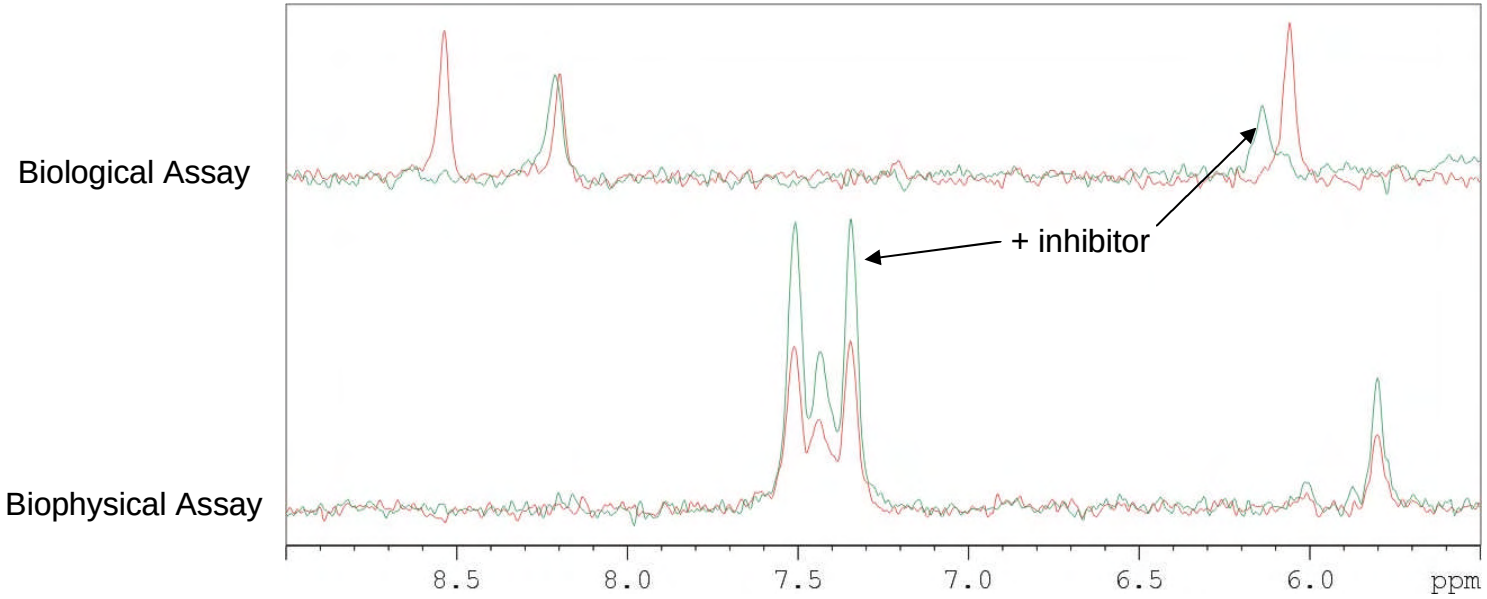
Methods for Hit Validation

- Affinity ranking

- Competition Binding
- SPR
- HSQC Binding Site Determination

Hit Validation: Competition Binding in TINS





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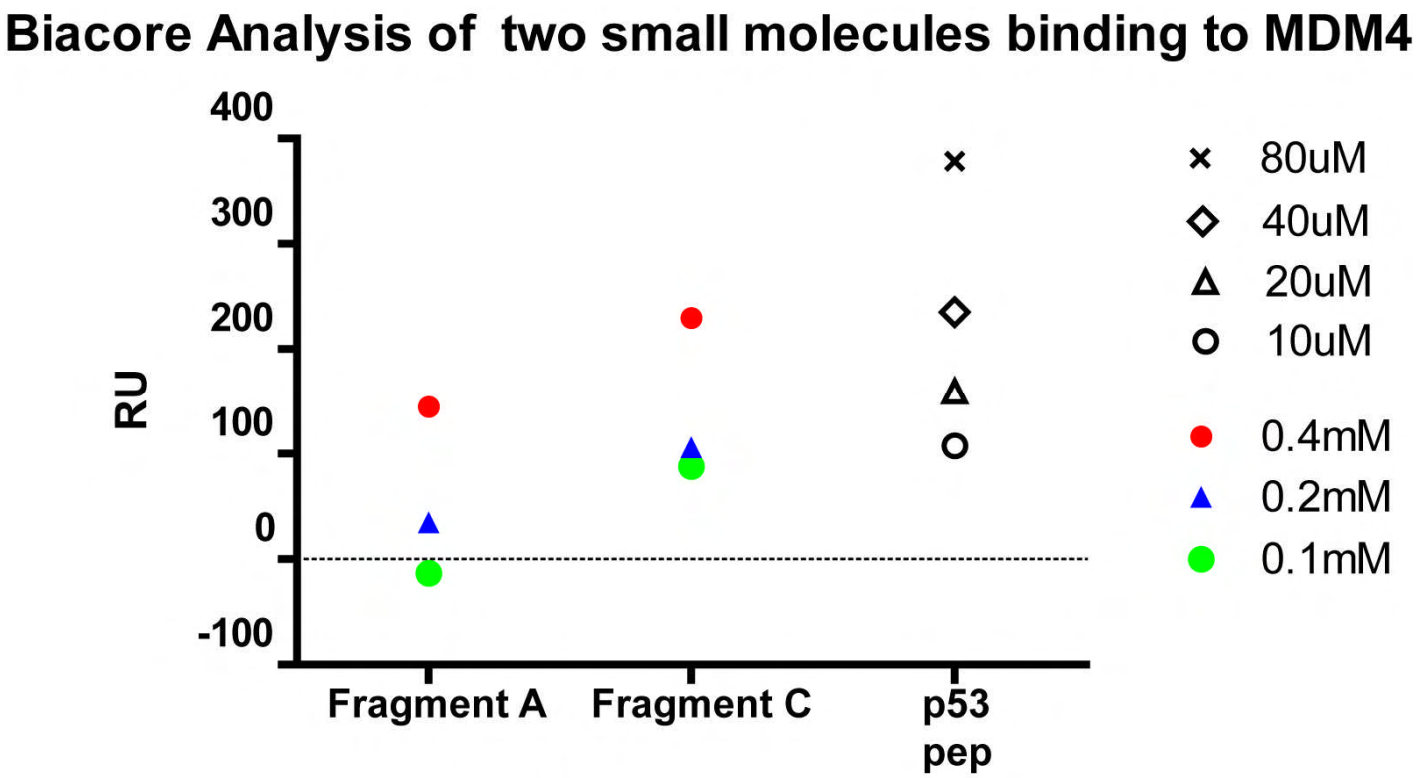
Hit Validation: SPR (Biacore T200)



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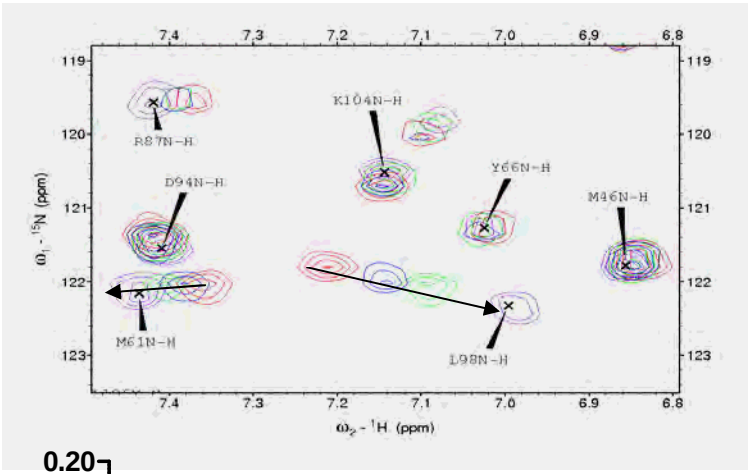
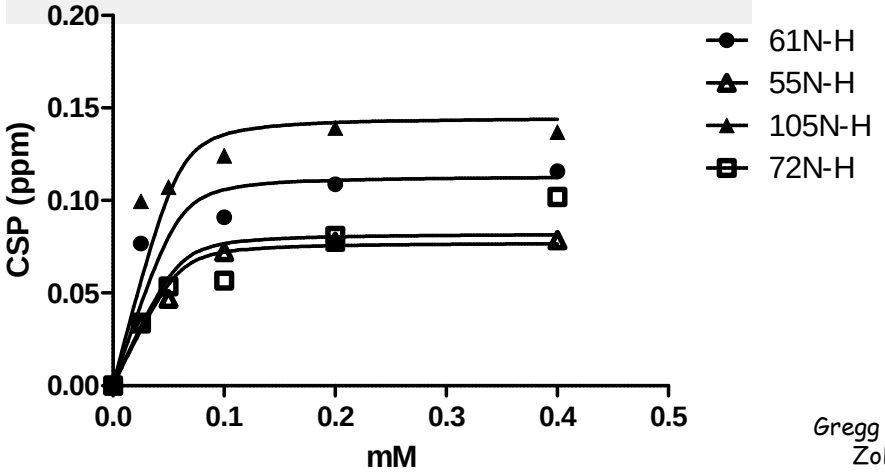
Hit Validation: K_D and Binding Site Characterization by HSQC

- Chemical shift perturbation for each HN
  – magnitude of resonance shift due to compound binding

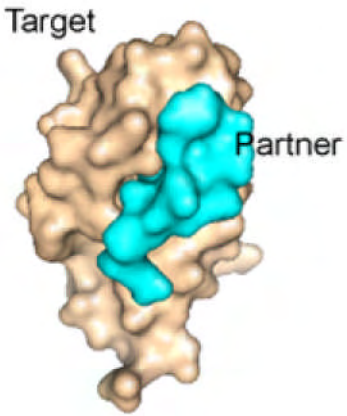
- CSP = sqrt [$\Delta H_{ppm}^2 + (\Delta N_{ppm}/6.5)^2$]

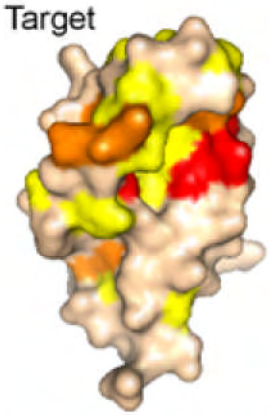




CSP observation on 80 TINS Hits

Low resolution binding site detemination





CSP: ■ > ■ > ■

Hits bind in the vicinity of PPI site

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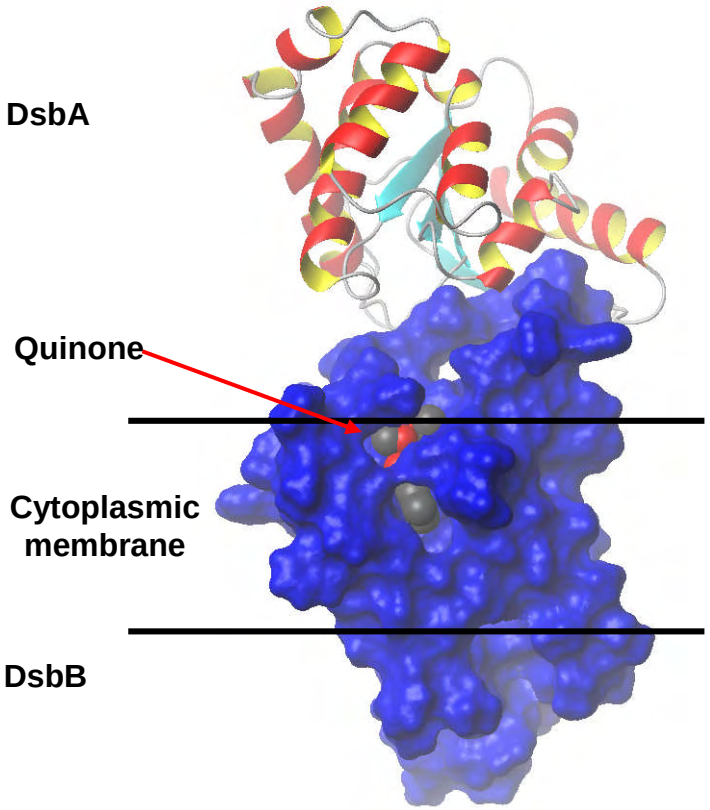
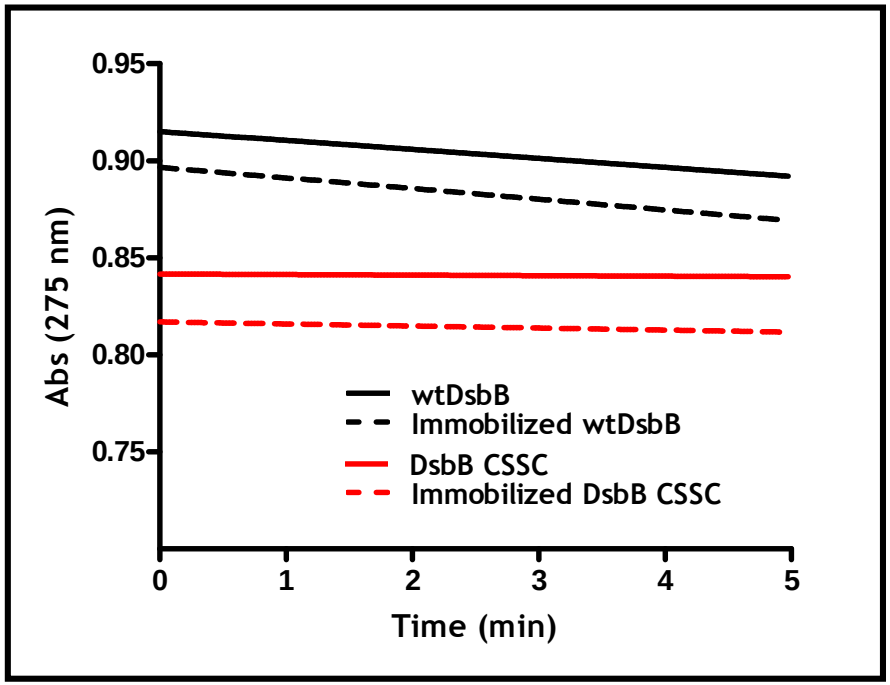
The Challenges of Fragment Discovery on MPs

- Protein production/solubilization/stability
- Non-specific binding
- Slow kinetics
- Fragment size

FBDD on Membrane Proteins: DsbB





Immobilized DsbB has 90% the activity of the soluble protein.

Inaba et al., Cell, 2006, **127**, p.789

Früh et al, Chem. Biol., 2010, v. 17, p. 881

Target: E. coli DsbB
Ref: E.coli OmpA
Both proteins DPC solubilized
Cmpds Screened: 1,270
Protein used: 2 mg
Hits: 93

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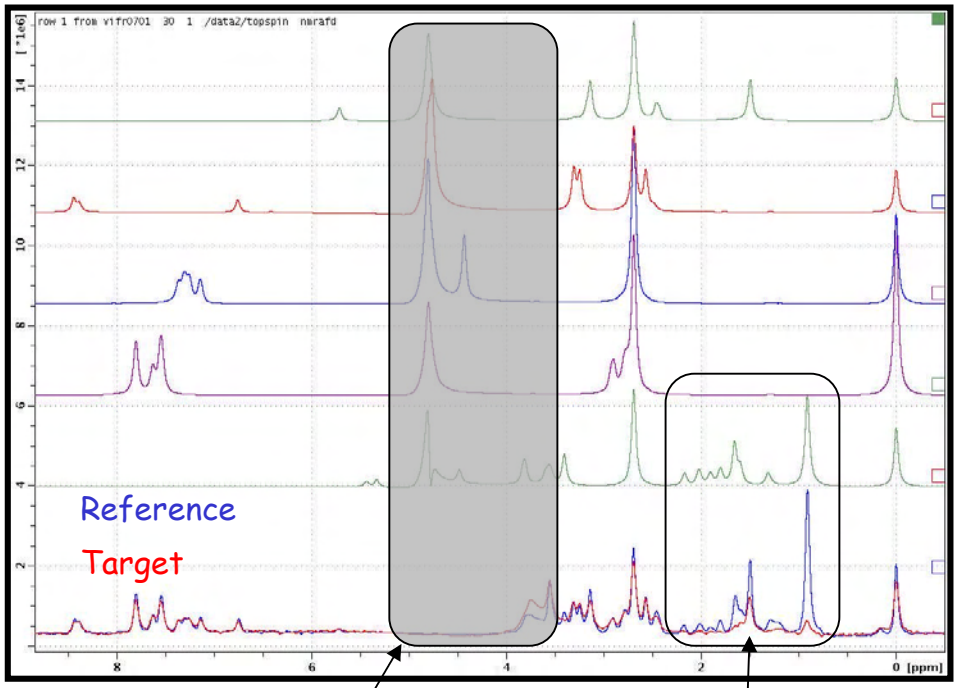
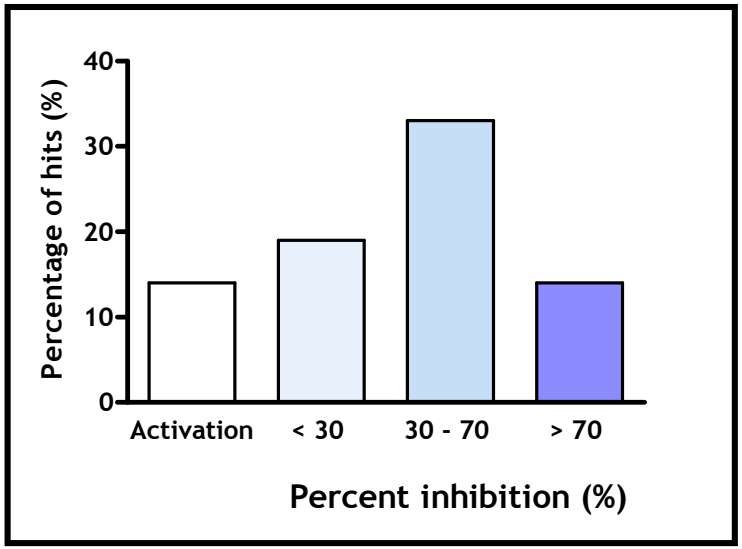
TINS Ligand Screening on Membrane Proteins





1,270 fragments tested → 7.3 % hit rate ➔ Validation/characterization

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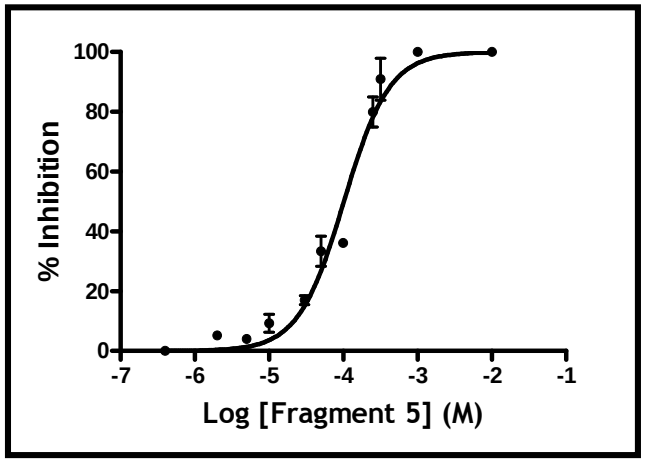
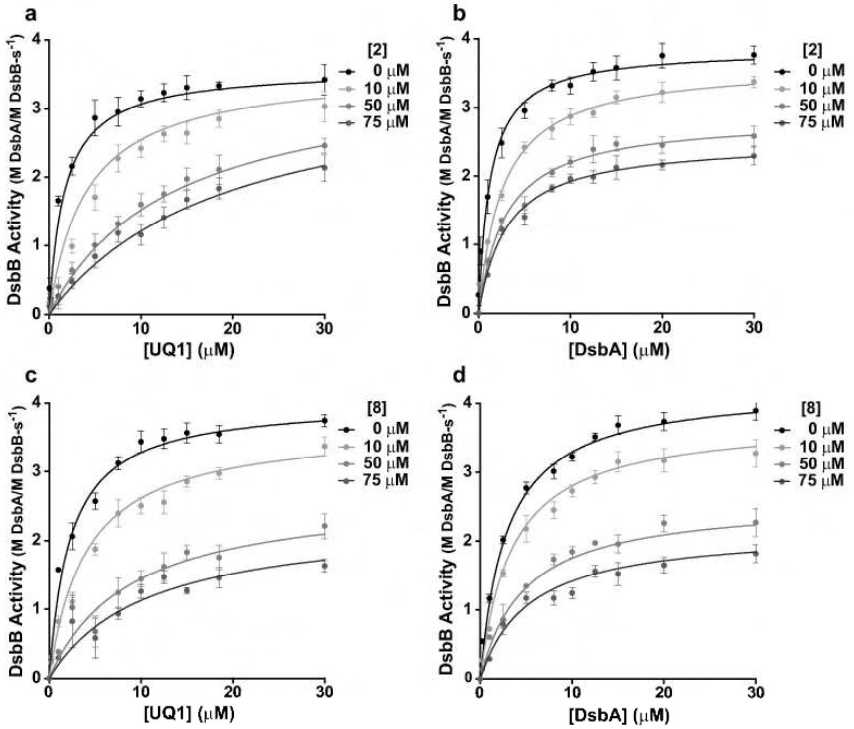
Membrane Protein Inhibitors: Mode of Action



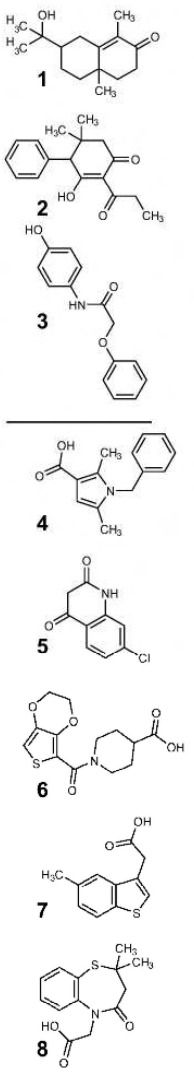
Frag	IC50 (uM)	Hill Slope
1	7 ± 1	0.80 ± 0.10
2	10 ± 1	0.80 ± 0.10
3	50 ± 10	0.80 ± 0.10
4	70 ± 10	1.00 ± 0.10
5	100 ± 10	1.40 ± 0.10
6	115 ± 15	1.15 ± 0.05
7	170 ± 10	1.40 ± 0.10
8	190 ± 10	1.20 ± 0.10



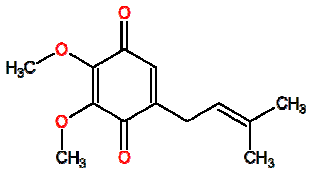
e

Fragment Number	Fragment [Conc]	Substrate	K_{cat} (M DsbA/ M DsbB-s^{-1})	K_m (μM)
2	0 μM	UQ1	3.7 ± 0.1	1.6 ± 0.1
	75 μM		3.1 ± 0.2	13.2 ± 1.7
	0 μM	DsbA	4.3 ± 0.1	2.4 ± 0.2
	75 μM		2.5 ± 0.1	2.8 ± 0.1
8	0 μM	UQ1	4.0 ± 0.1	2.2 ± 0.0
	75 μM		2.3 ± 0.1	9.7 ± 1.5
	0 μM	DsbA	4.3 ± 0.1	1.9 ± 0.1
	75 μM		2.4 ± 0.2	4.1 ± 0.5

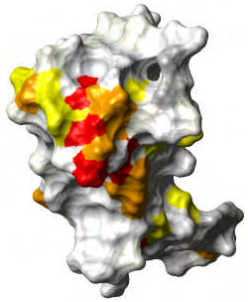
f

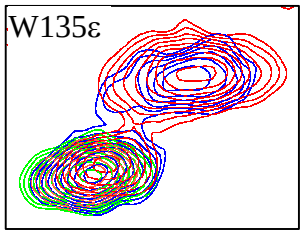


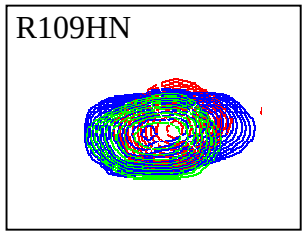
Structural Model of Fragment Binding

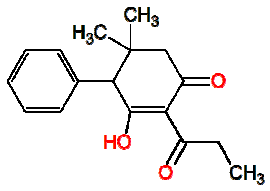


Competitive

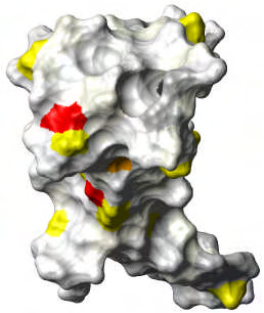


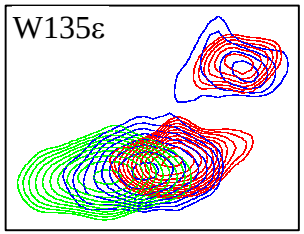


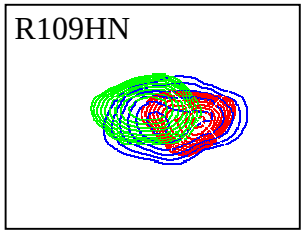


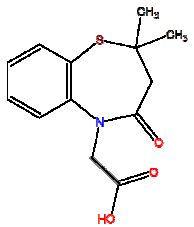


Competitive

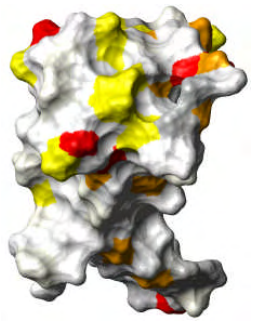


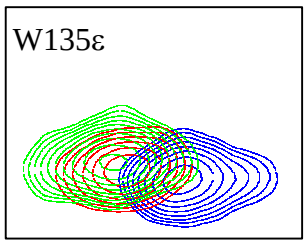


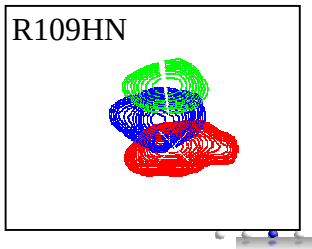




Mixed Model







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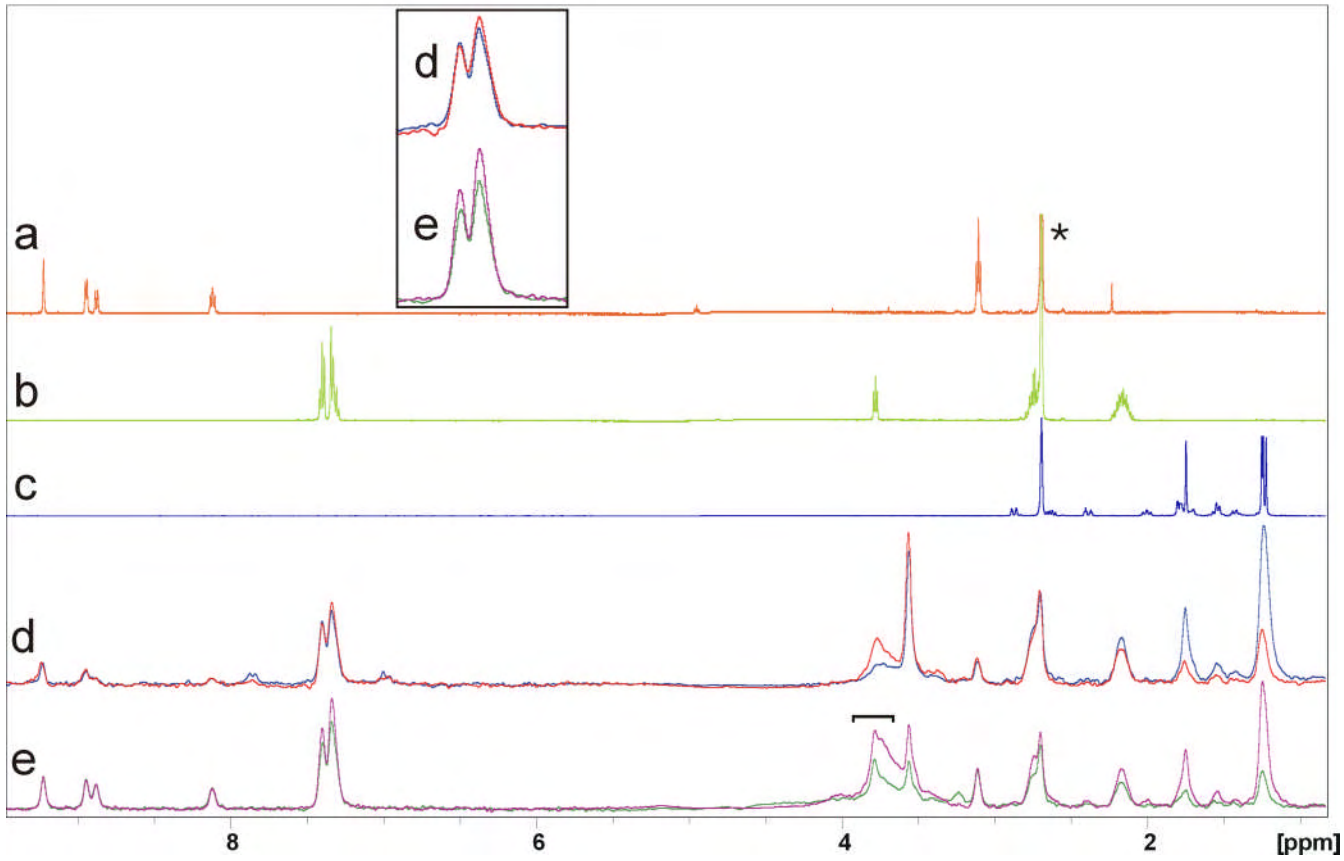
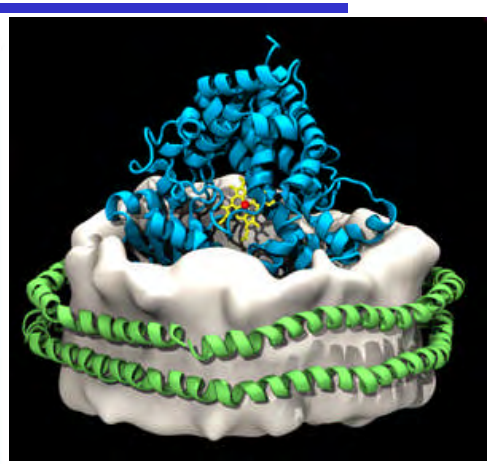
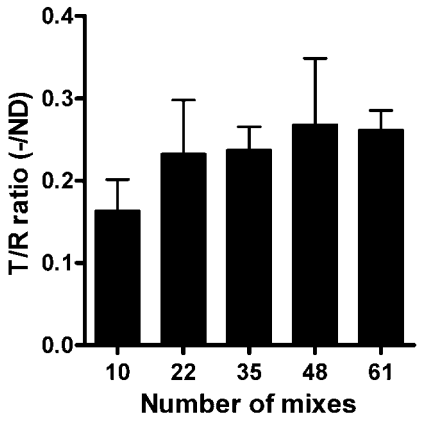
Effects of the micelle on screening







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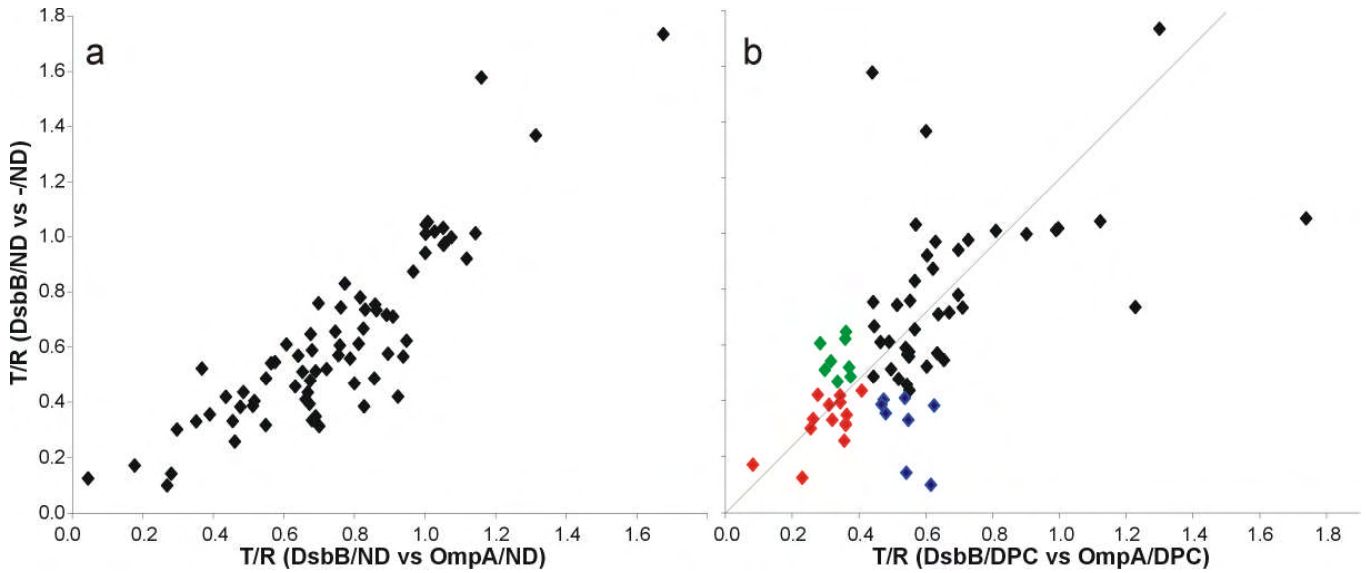

	Obs/Unobs (cLogP)	Hits	cLogP	BioAssay in ND	BioAssay in DPC
Micelle	127/56 (0.9/1.8)	8	1.34	-	+
NanoDisc	164/19 (1.1/1.6)	8	2.21	++	++
Both	-	14	2.13	++	++

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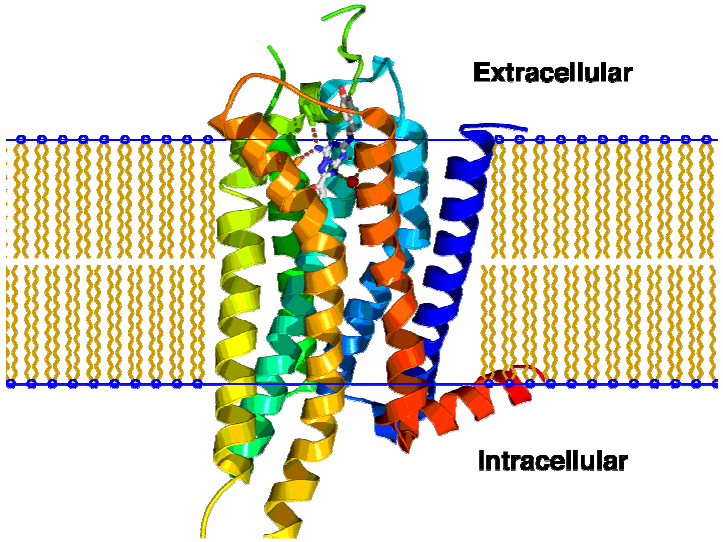
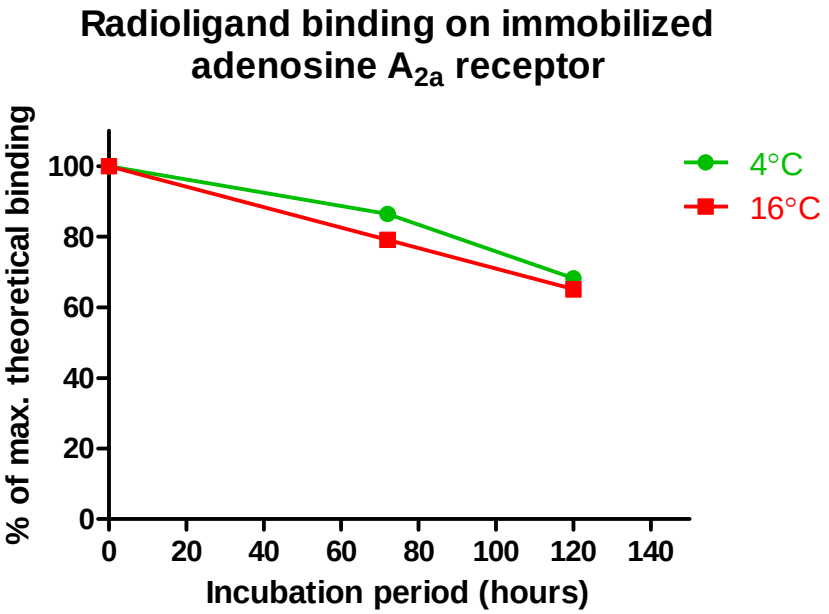
Fragment Screening of GPCRs: Adenosine 2a & β1 Adrenergic receptors





$A_{2a}R$ agonists – anti-inflammatory therapeutic potential

$A_{2a}R$ antagonists – used to treat Parkinson's disease as $A_{2a}R$ dimerises with dopamine D_2 receptor

Immobilized hA2aR maintained 60% the activity in five days.

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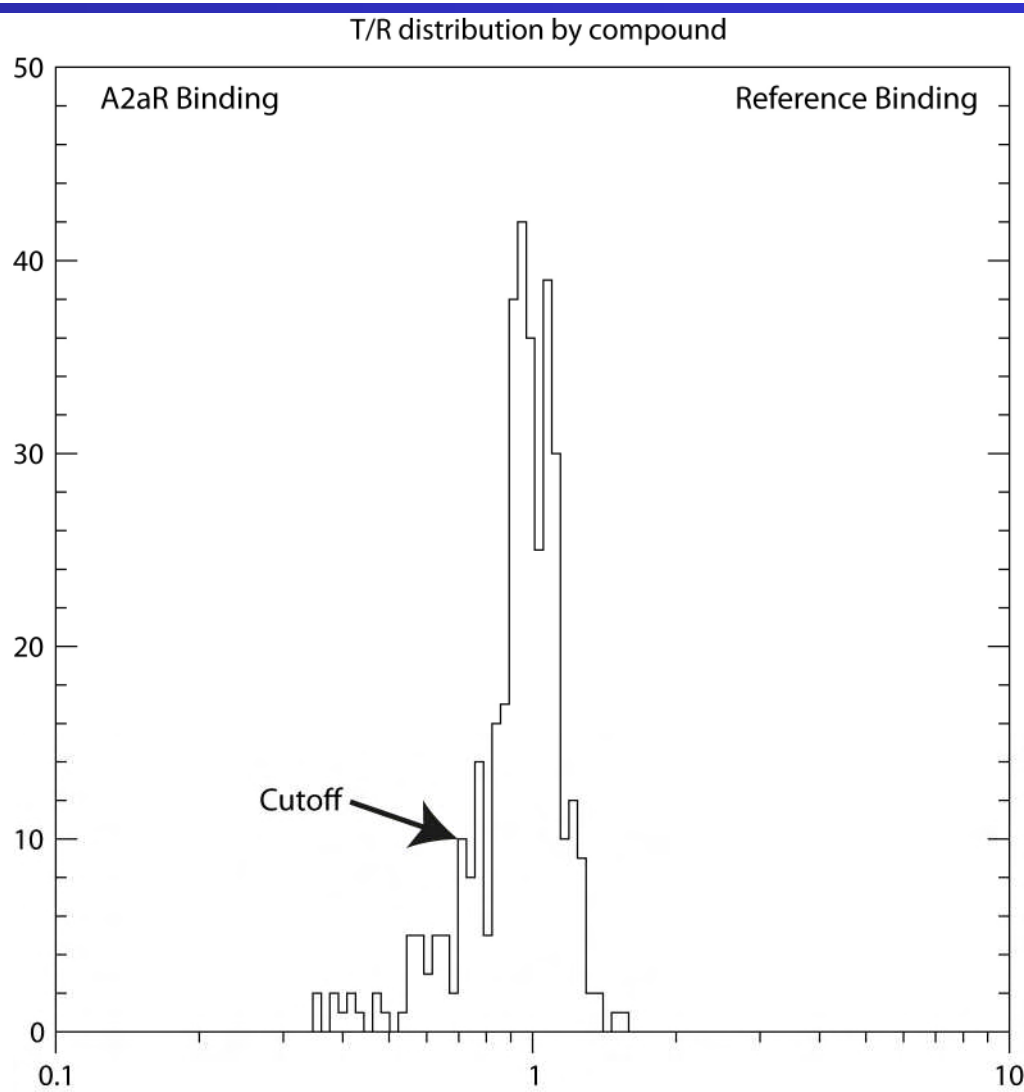
Profile of Ligand Binding in the Screen

531 fragments assayed against A2aR
94 hits



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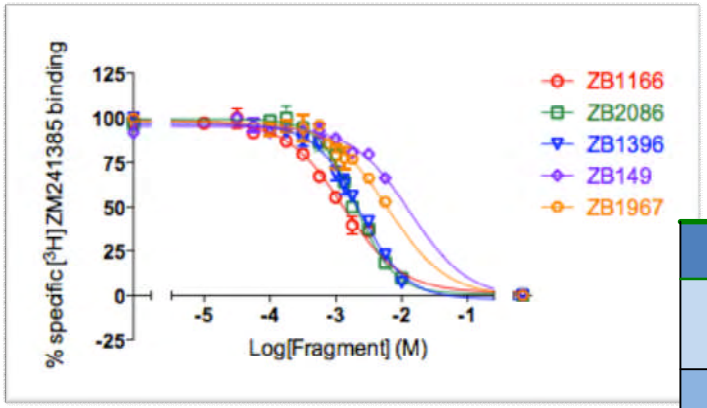
Hit validation by equilibrium radioligand displacement



Each hit assayed at 500 μM for ^{3}H- ZM241385 displacement using wild type A2aR.

ID	K_i (M)	EC_{50} (M)	Hill Slope	T/R ratio
ZB643	$3.0 \pm 0.1 \times 10^{-6}$	$7.0 \pm 0.3 \times 10^{-6}$	-0.5 ± 0.1	0.65
ZB418	$3.0 \pm 0.1 \times 10^{-5}$	$5.5 \pm 0.1 \times 10^{-4}$	-2.0 ± 0.5	0.44
ZB1703	$4.1 \pm 0.1 \times 10^{-5}$	$6.4 \pm 0.1 \times 10^{-4}$	-1.1 ± 0.4	0.60
ZB1166	$1.2 \pm 0.1 \times 10^{-4}$	$1.5 \pm 0.1 \times 10^{-3}$	-0.9 ± 0.1	0.61
ZB2086	$1.2 \pm 0.2 \times 10^{-4}$	$2.4 \pm 0.3 \times 10^{-3}$	-0.9 ± 0.3	0.74
ZB114	$8.2 \pm 0.3 \times 10^{-5}$	$1.2 \pm 0.1 \times 10^{-3}$	-2.2 ± 5.4	0.59
ZB1605	$8.7 \pm 0.3 \times 10^{-5}$	$1.4 \pm 0.3 \times 10^{-3}$	-1.1 ± 0.5	0.37
ZB1967	$3.2 \pm 0.2 \times 10^{-4}$	$6.1 \pm 0.1 \times 10^{-3}$	-0.8 ± 1.3	0.61

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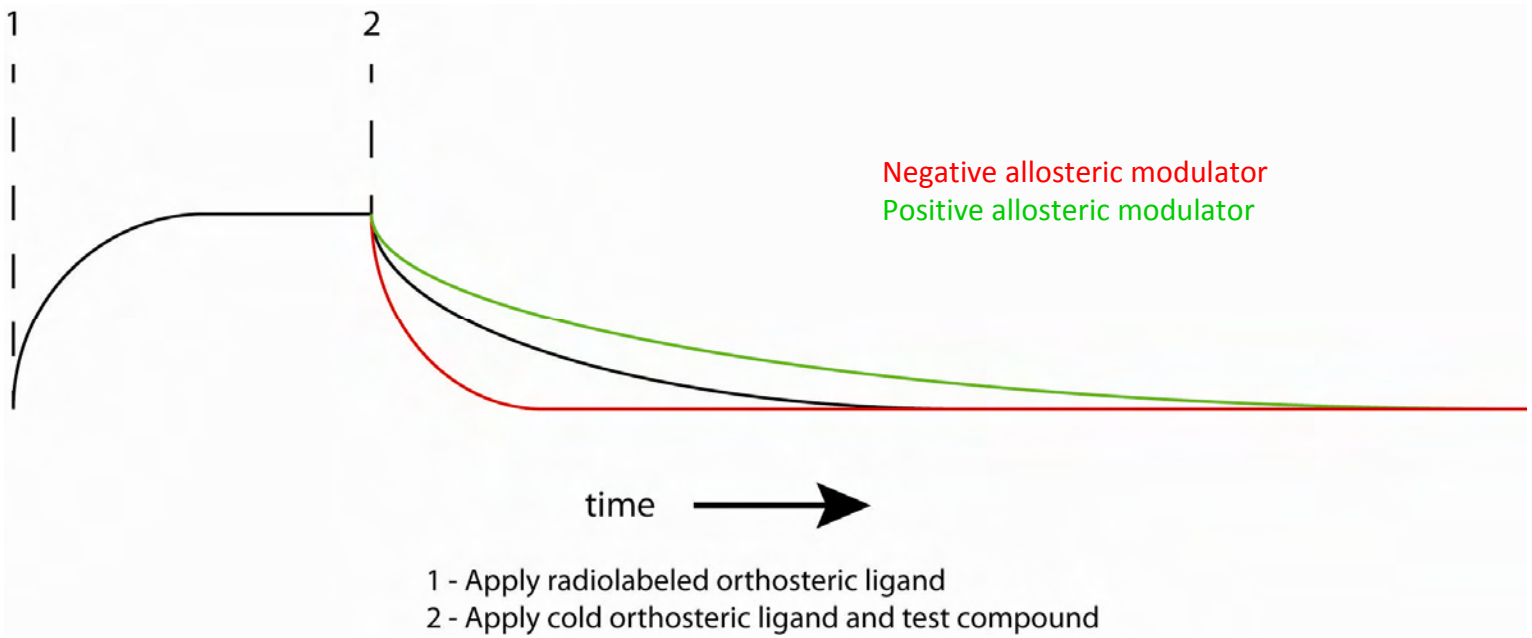
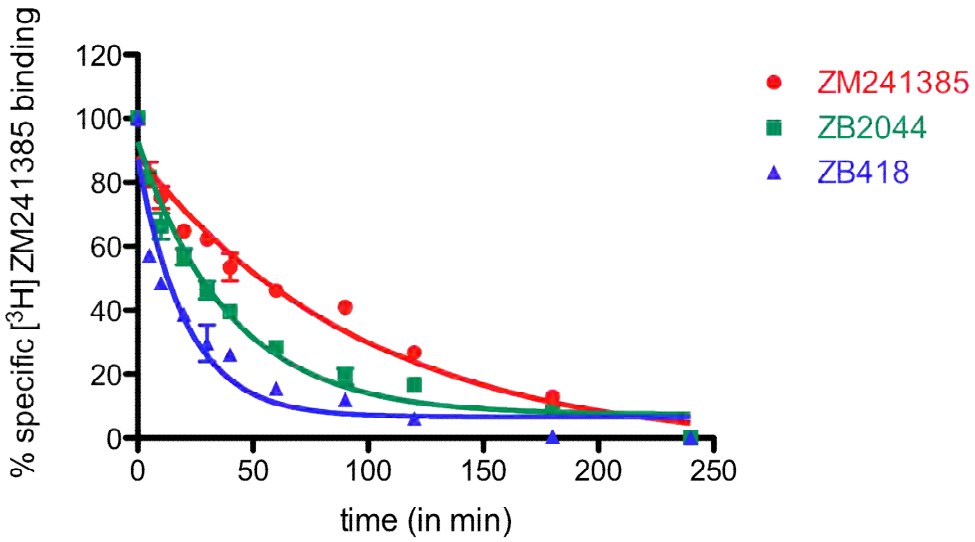
Mode of action: Orthosteric vs Allosteric modulators



Screen all hits at t = 50% ZM bound.

TINS hits as negative allosteric modulators (NAMs) of A2aR



	ZM241385	ZM241385 + 2.5mM ZB2044	ZM241385 + 2.5mM ZB418
k_{off} of ZM241385 (min^{-1})	0.010 ± 0.003	0.025 ± 0.004	0.048 ± 0.011
Half Life (min)	69	27	15

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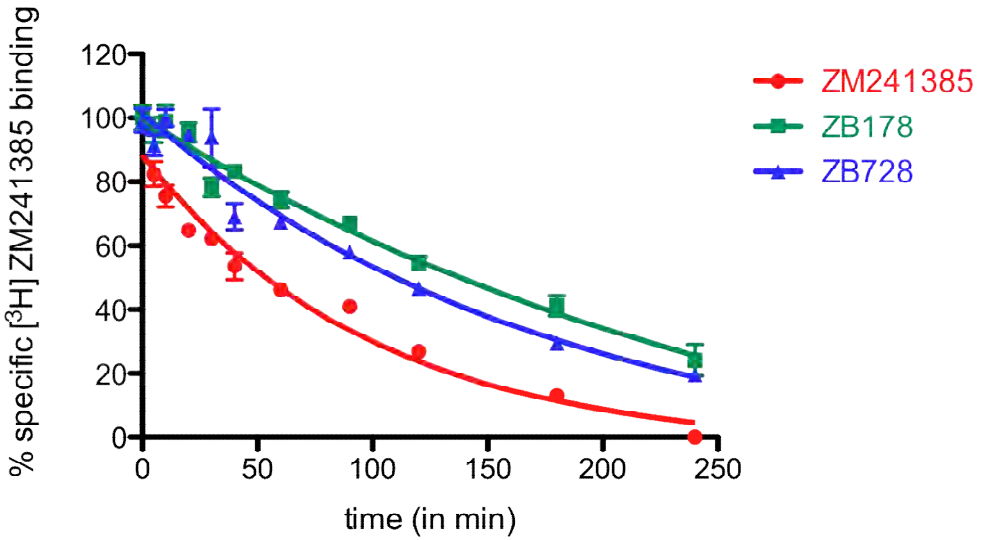
TINS hits as positive allosteric modulators (PAMs) of A2aR



	ZM241385	ZM241385 + 2.5mM ZB178	ZM241385 + 2.5mM ZB728
k_{off} of ZM241385 (min^{-1})	0.0100 ± 0.0026	0.0034 ± 0.0016	0.0057 ± 0.0023
Half Life (min)	69	205	123

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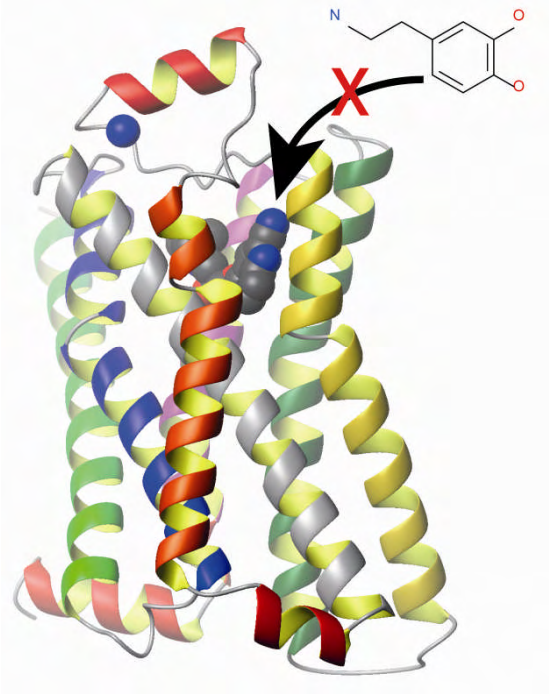
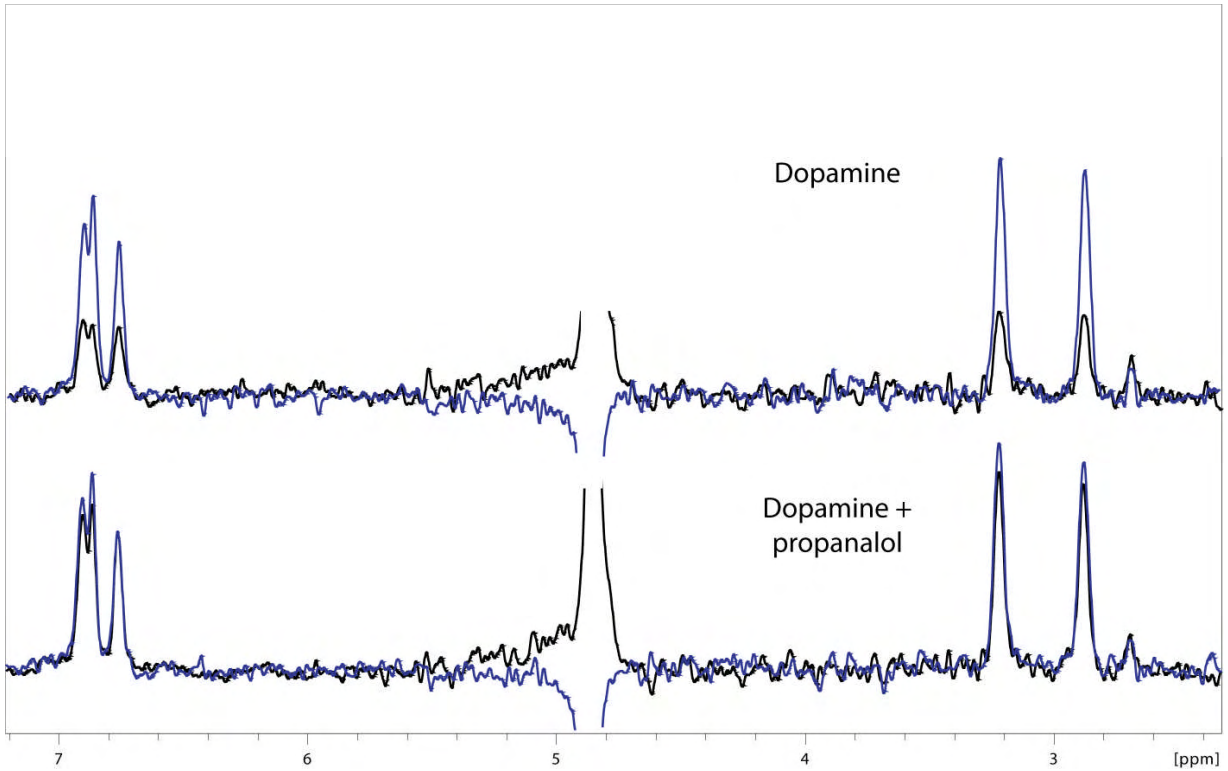
Structural information from TINS?



β1AR



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Summary

- True fragment screening (e.g. biophysical) can be successfully applied to GPCRs.
- Novel orthosteric ligands can be found using FBDD.
- Novel allosteric modulators of GPCR function can be found using FBDD.

Applications

- Primary screen for novel matter
- Scaffold hopping
- Allosteric modulators

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β1AR/A2aR

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