TrkB screening assays for the identification and characterization of small molecule BDNF mimetics and/or modulators

BioFocus
A Galápagos Company

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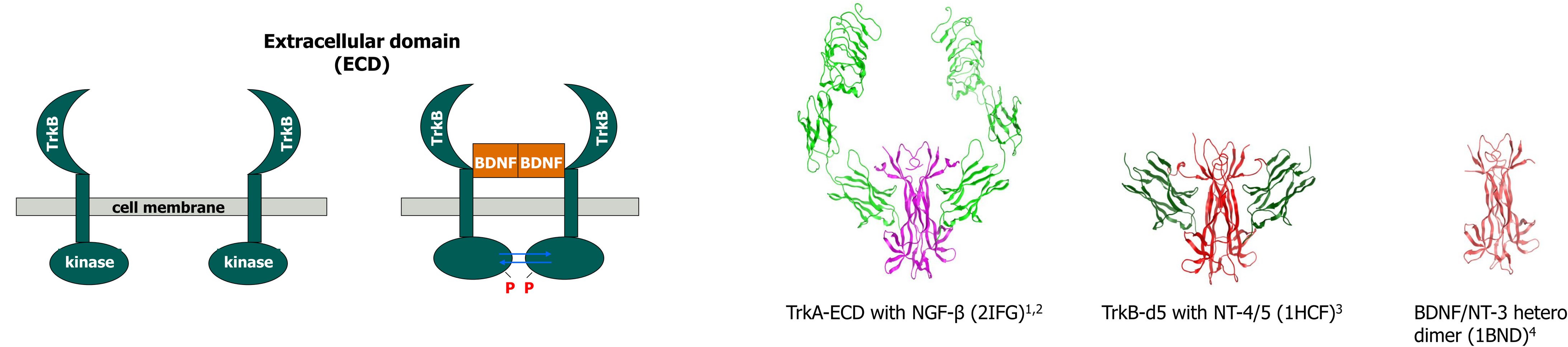
1. Introduction

Brain-derived neurotrophic factor (BDNF) is a secreted protein that promotes neuronal cell survival by activating the TrkB tyrosine receptor kinase. BDNF expression is reduced in Huntington's disease (HD).

A hit identification programe is underway to identify small molecule enhancers of TrkB signalling (*i.e.* either a positive allosteric modulator or a BDNF mimetic (agonist)), and, if successful, establish whether such compounds would show efficacy in a disease-relevant preclinical model.



TrkA-ECD with NGF-β (2IFG)[1,2]

TrkB-d5 with NT-4/5 (1HCF)[3]

BDNF/NT-3 hetero-dimer (1BND)[4]

Schematic of TrkB receptor dimerization on BDNF binding.
Arrows represent trans-phosphorylation of the intracellular tyrosine kinase domains.

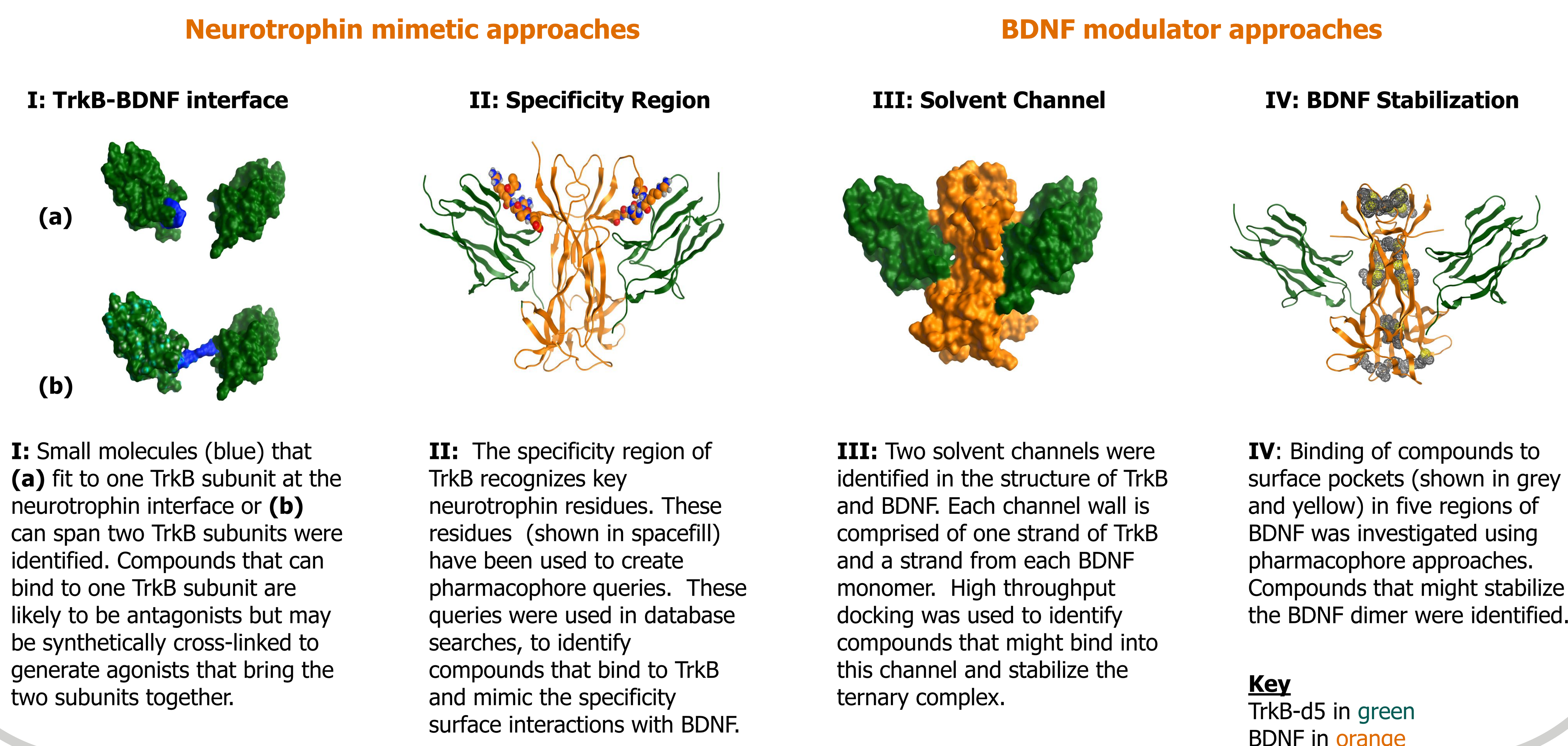
Key crystal structures.
TrkB interactions with neurotrophins mediated by d5 subunit.

Project strategy:

- **Modelling the TrkB interaction with BDNF for virtual compound selections**
- **Development of binding and functional assays for Trk receptors**
- **Testing of compounds selected by virtual screen**

2. TrkB compound selection using a structure-based *in silico* approach

- **TrkB-d5 models generated with BDNF**
- **Models used for virtual screen of compound libraries**
- **Compounds were selected to test the following hypotheses**



I: Small molecules (blue) that **(a)** fit to one TrkB subunit at the neurotrophin interface or **(b)** can span two TrkB subunits were identified. Compounds that can bind to one TrkB subunit are likely to be antagonists but may be synthetically cross-linked to generate agonists that bring the two subunits together.

II: The specificity region of TrkB recognizes key neurotrophin residues. These residues (shown in spacefill) have been used to create pharmacophore queries. These queries were used in database searches, to identify compounds that bind to TrkB and mimic the specificity surface interactions with BDNF.

III: Two solvent channels were identified in the structure of TrkB and BDNF. Each channel wall is comprised of one strand of TrkB and a strand from each BDNF monomer. High throughput docking was used to identify compounds that might bind into this channel and stabilize the ternary complex.

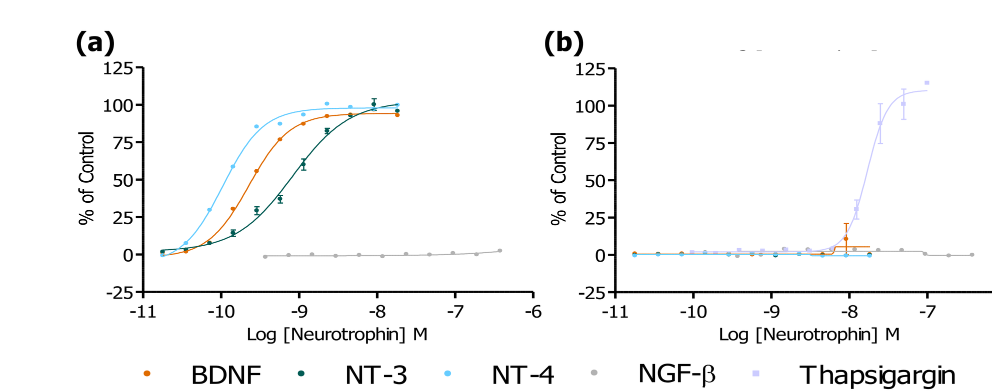
IV: Binding of compounds to surface pockets (shown in grey and yellow) in five regions of BDNF was investigated using pharmacophore approaches. Compounds that might stabilize the BDNF dimer were identified.

Key
TrkB-d5 in green
BDNF in orange

3. Primary screening assays – hit identification

Compounds will be tested in parallel using a cell-based functional reporter gene assay (Invitrogen™ CellSensor®) and a biophysical binding assay (Biacore™).

The CellSensor TrkB assay was validated using neurotrophins and agonist, antagonist and positive allosteric modulator modes were configured. Selectivity (TrkA, C) and specificity (NFAT reporter without Trk receptor) formats were also validated. Neurotrophin pharmacology and assay performance (Z′ >0.6, S:B >5) correlated well with published data.[5]

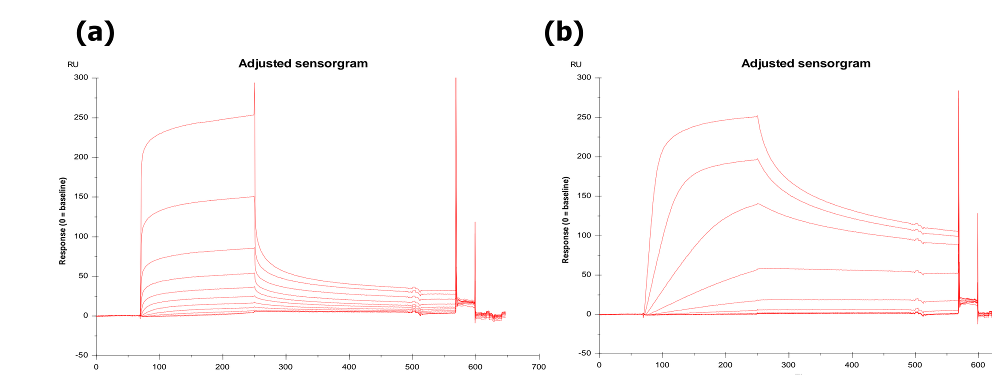


CellSensor reporter gene pharmacology.
(a) TrkB CellSensor assay and **(b)** NFAT CellSensor specificity assay. The NFAT beta-lactamase reporter gene responds to activation of the Trk>PLCγ>PKC/Ca^{2+} pathway. Thapsigargin is used to activate the specificity cell line by elevating intracellular Ca^{2+}.

Cell line	hBDNF EC_{50} (nM)		hNT-3 EC_{50} (nM)		hNT-4 EC_{50} (nM)		hNGF-β EC_{50} (nM)	
	BioFocus data	Invitrogen data	BioFocus data	Invitrogen data	BioFocus data	Invitrogen data	BioFocus data	Invitrogen data
TrkA	>18	>50	5*	16*	10*	>50	4.1±3.1	0.04**
TrkB	0.5±0.3	0.39	1.3±0.5	2.5	0.1±0.02	3.5	>400	>50
TrkC	>18	28*	0.4±0.2	0.09	>18	>50	>400	>50
NFAT	>18	>50	>18	>50	>18	>50	>400	>50

CellSensor neurotrophin potency summary.
Comparison of BioFocus potency data with that described by Invitrogen.[5] * Values may not be accurate as the curves did not reach saturation. ** Invitrogen used mNGF 2.5s.

Biacore assay configured using TrkB-d5 and TrkB-ECD. Neurotrophin affinities were comparable to literature precedence and the CellSensor data.[5,6] BDNF and specificity surfaces are in development.



Biacore sensorgram profiles.
Concentration-dependent binding of **(a)** NGF-β and **(b)** NT-4 to TrkB-d5.

Neurotrophin	BioFocus K_D (nM)		Naylor K_D (nM)
	TrkB-d5	TrkB-ECD	TrkB-d5
BDNF	1.0	1.0	0.8
NT-3	5.6	5.5	18.0
NT-4	0.7	2.4	0.3
NGF-β	264.0	97.9	nd

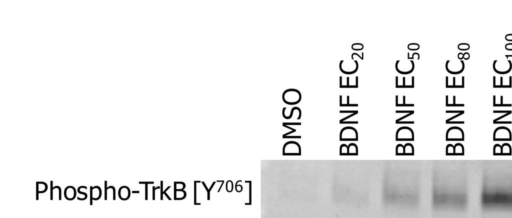
Biacore TrkB affinity analysis.
Comparison of BioFocus affinity data with that described by Naylor.[6]

4. Secondary screening assays to further validate hit compounds

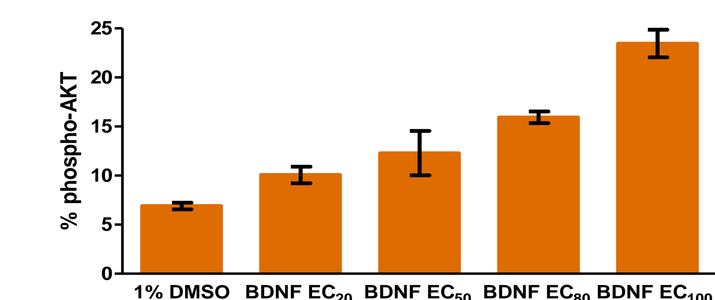
Secondary screens will utilize retinoic acid differentiated SH-SY5Y cells,[7] a neuronal-like cell line, to characterize endogenous TrkB receptor mediated signalling by hit compounds.

Compounds will be tested in the following assays:

- **Phospho-TrkB western blot – direct proximal readout**
- **MesoScale Discovery phospho/total-ERK1/2 & -AKT – quantitative multiplex readout for TrkB downstream kinases**



Western blot showing BDNF induced phosphorylation of TrkB in SH-SY5Y cells.
Anti-phospho-TrkB (Santa cruz, #sc-135645) applied.



MSD showing BDNF induced phosphorylation of AKT in SH-SY5Y cells.

5. Conclusions

- **Modelling used to identify screening deck of ~7,000 compounds**
- **Primary cell-based assay validated**
- **Biacore assay development on-going**
- **Secondary screening assay cascade selected**

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References

1. Berman *et al*, (2000), *Nucleic Acids Research*, 28, 235-242; 2. Wehrman *et al*, (2007), *Neuron*, 53, 25-38; 3. Banfield *et al*, (2001), *Structure*, 9, 1191-1199; 4 Robinson *et al*, (1995), *Biochemistry*, 34, 4139-4146; 5. Wang *et al*, (2008), Current Chemical Genomics, 1, 1-7; 6. Naylor *et al*, (2002), *Biochem.Biophys.Res.Comm*, 291, 501-507; 7. Kaplan *et al*, (1993), *Neuron*, 11, 321-331