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# Screening of human fructose-2,6-bisphosphatase

— dissection of false positives —

*September 22, 2008*

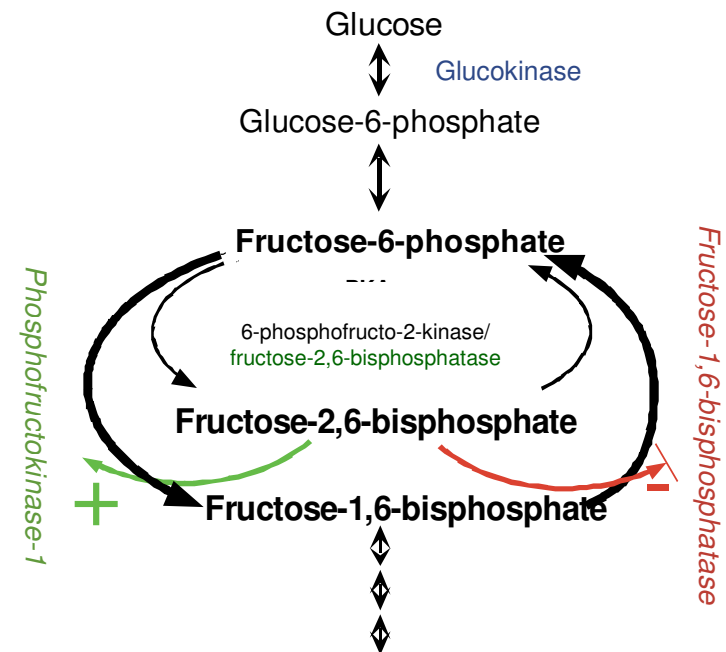


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# Target Rationale

- Altered hepatic glucose utilisation contributes to hyperglycemia in type 2 diabetes
- Modulators of glucose utilisation is broadly pursued as a potential T2D treatment
  - **Fructose-1,6-bisphosphatase** inhibitors reduce hepatic glucose output (HGO)
  - **Glucokinase** activators increase hepatic glucose utilisation (HGU)
  - **Fructose-2,6-bisphosphatase** inhibitors has the potential to positively impact both HGU (↑) and HGO (↓)



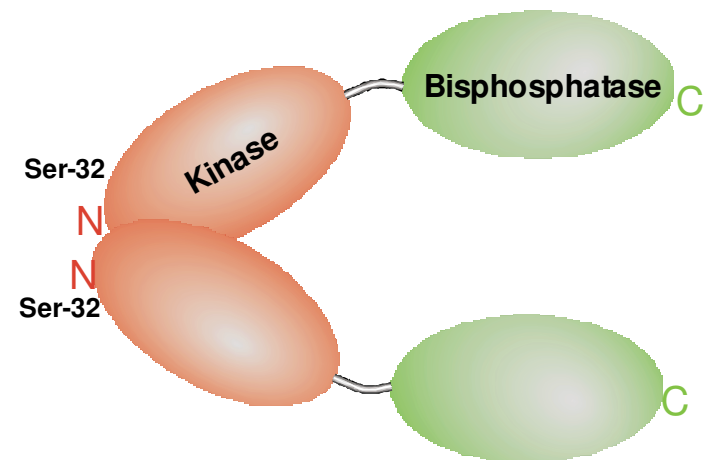
# 6-phosphofructo-2-kinase / fructose-2,6-bisphosphatase

- **Bifunctional enzyme**

- Kinase domain covalently linked to bisphosphatase domain
- Kinase produces F-2,6-P<sub>2</sub> from ATP and F6P
- Bisphosphatase degrades F-2,6-P<sub>2</sub> to F6P and inorganic phosphate
- F-2,6-P<sub>2</sub> levels controlled by kinase / bisphosphatase ratio

- **Four isoforms encoded by separate genes PFKFB1 through to PFKFB4**

- PFKFB1 encodes liver isoform
- K / B ratio altered by phosphorylation of Ser32 in response to glucagon
- Glucagon lowering of F-2,6-P<sub>2</sub> levels results in reduced glycolysis and increased gluconeogenesis



# Literature Conditions

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- Crystal structure determined
  - Active BPase domain expressed in *E. coli*
  - Active site in defined binding pocket
- Enzymatic mechanism of action well characterised
  - Commonly studied using  $^{32}\text{P}$  based assay at 37°C
- Lack of HTS compatible assay
  - $^{32}\text{P}$  assay based on ion exchange chromatography
  - No small molecule inhibitors available
  - Both products are endogenous inhibitors with known potency

# HTS Assay Desirables

- Predictive - reflects the desired biological activity
    - Appropriate response to known inhibitors
  - Robust and reproducible
  - Stable solutions of all components - ideally for 24h
    - DMSO tolerance after mixing solutions
  - Simple and homogeneous - automation feasible
  - Minimal number of "false negative" and "false positive" hits
  - Finances, sensitivity, and many more...
- High biological interest: Compromise?

# Protein Production

- Yields of soluble protein depended on several factors
  - *E. coli* strain
  - Chaperone co-expression
  - Low temperature at induction phase

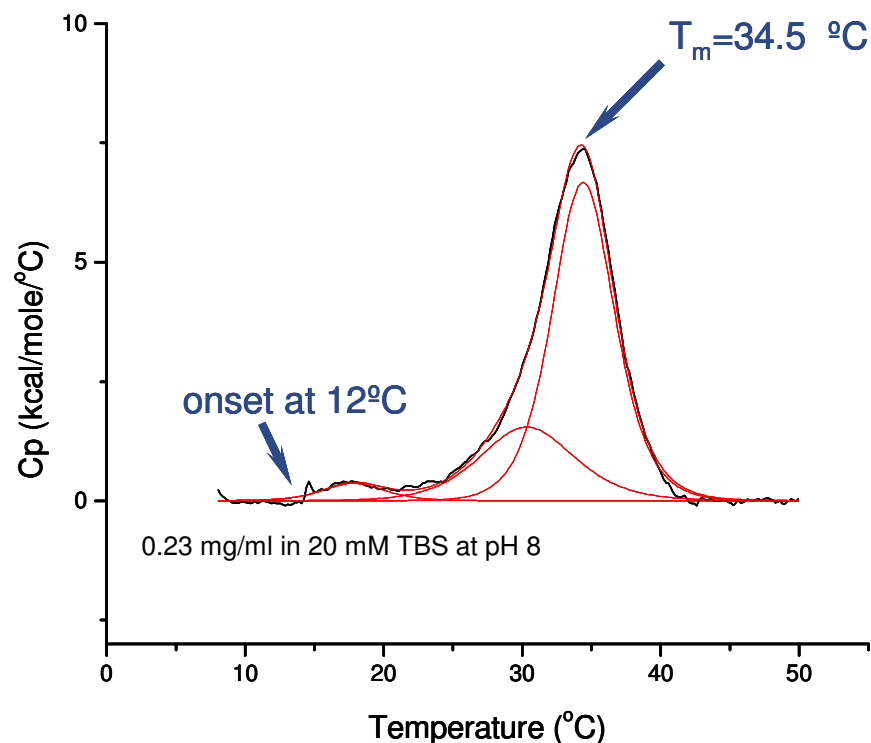


- Other findings were not easily predicted from literature
  - Enzyme activity deteriorated at room temperature
  - Significant non-precipitating aggregation at room temperature over time

# Protein Characterisation

- Extensive characterisation commenced, *e.g.*:

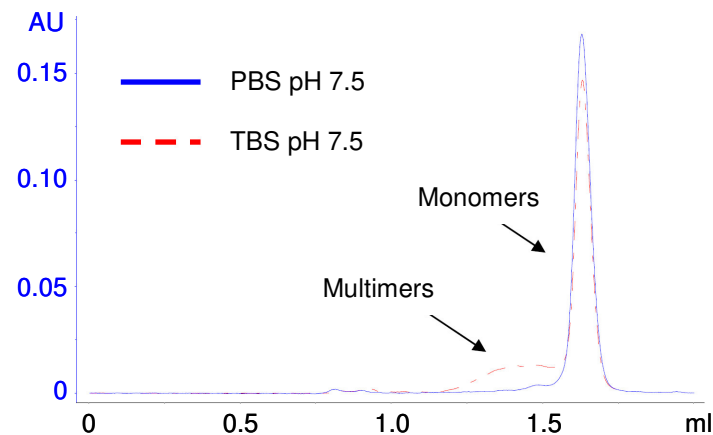
- Analytical gel filtration
- Activity assay
- ESI-MS
- Differential Scanning Calorimetry (DSC)



- Confirming poor thermal stability and DMSO tolerability
- Improving material and/or buffer conditions was key to enable screening

# Buffer Screening

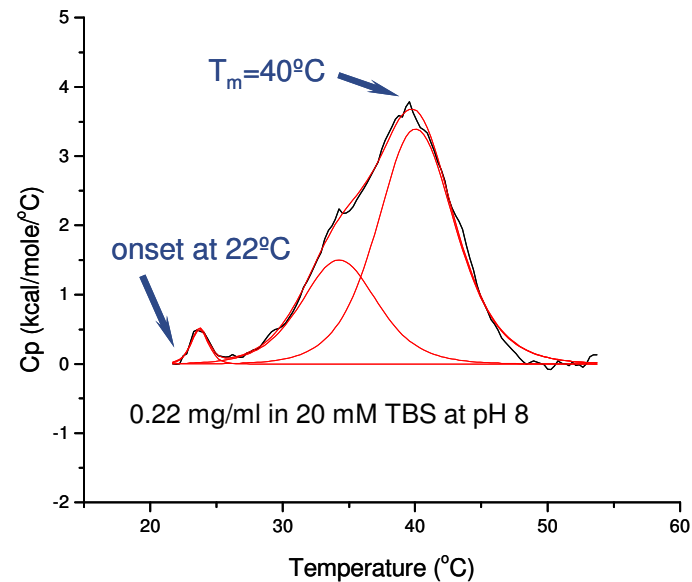
- Identification of appropriate buffer for storage and assay purposes
  - Design-of-experiments approach recommended for rational buffer selection
  - Identifies key buffer components for activity and stability (during assay and storage)
  - Disregards non-essential components
  - Inorganic phosphate found to stabilise BPase – but cannot be used due to its inhibitory properties



# Protein Modification

- Removal of the his-tag was key to improve stability
  - Still not a simple two-state transition, but:
  - Increased  $T_m$  and onset of melting in the absence of inorganic phosphate
  - Significantly improved DMSO tolerability
  - Reduced aggregation propensity

See Tjernberg *et al.* 2006 J. Biomol. Screen.



# Hit Confirmation

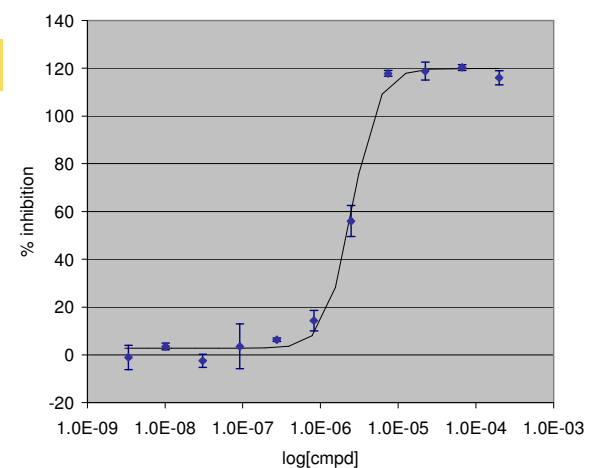
## I. Dose-response characterisation using screening assay

- 69 cmpds yielded reproducible dose-response curves with  $IC_{50}$  values
- Several hits gave high Hill slopes
- Cmpd A used here as illustrative example

Potential killer 1

## II. Generally applied filters

- Hit statistics, identity, purity, stability, solubility etc.
- Structural clustering of compounds - SAR?
- Medicinal chemistry experience

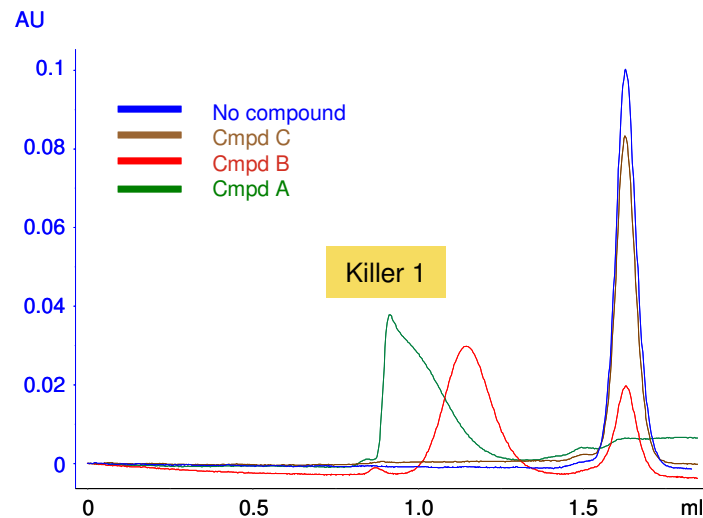


## III. Potential filters for removal of false positives

- Orthogonal assay to remove compounds that affect assay signal
  - Option for enzymatic assays to apply compounds after enzymatic incubation
- The “detergent test” for removal of promiscuous inhibitors
  - Option to prolong compound incubation prior to reaction to observe  $IC_{50}$  shifts
- Biophysical studies to confirm binding and mechanism-of-action

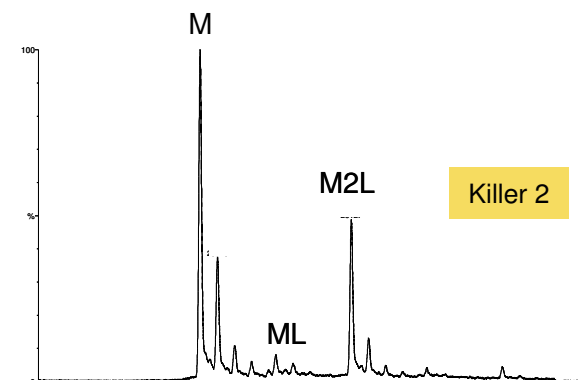
# Promiscuity test and protein aggregation studies

- Cmpds confirmed dose-dependent activity both in orthogonal assay and in the primary assay applied in the presence of 5.9  $\mu\text{M}$  Tween-20
- Analytical gelfiltration
  - Cmpd A promotes aggregation of BPase
  - Cmpd C does not



# Biophysical characterisation

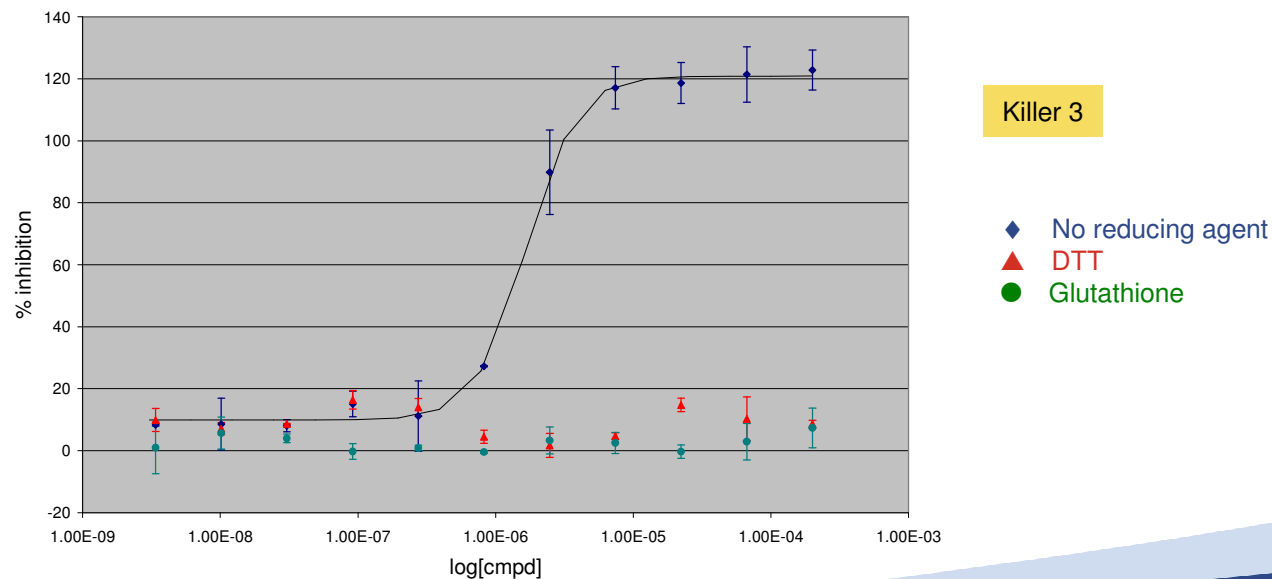
- Binding confirmed using several techniques – but the use of different conditions resulted in revealing results
  - Non-denaturing ESI-MS demonstrated 2:1 binding for Cmpd C
  - Binding was not reproduced in NMR binding studies in the presence of reducing agent
  - Cmpd C was reduced to a 2 Da larger molecule in the presence of reducing agents
  - Cmpd dimer formation suspected



- Different technologies have different benefits as well as constraints:
  - Sensitivity impacts ligand/protein concentrations
  - Sample volumes impact material consumption
  - Buffer tolerability impacts buffer composition
  - Ability to detect stoichiometry and protein/ligand integrity
  - Hence they are applied at different concentrations of protein, ligand and buffer compositions making them highly complementary

# Compound Properties

- Additional cmpds gave conflicting results in various buffers
  - Explained by the presence/absence of reducing agents for some series
  - However, inconsistent results were obtained also for cmpds that passed glutathione oxidation assays
  - Low concentrations of an oxidised form was responsible for the activity – disappeared upon addition of reducing agents
- Cmpds were rerun in the presence of glutathione or DTT to confirm loss of activity



# Conclusions

- Prevent progression of false positives by cautious exercise of appropriate filters:
  - Essential to save costs downstream – while allowing serendipity
  - Orthogonal assay or kinetic studies remove “assay signal” artifacts
  - Medicinal chemistry experience, hit statistics, and assay modifications help to remove promiscuous inhibitors
  - Preclinical characterisation eliminates unstable and reactive cmpds
  - A combination of biophysical technologies is required to cover all cmpds (ligand-related constraints)
- Down-stream cell-assays can and are used as a substitute
  - But need to account for serum binding, permeability and endogenous substrate concentrations
  - Some cmpds impact generic pathways and thus various functional assay readouts
  - Thus confirmation does not always guarantee appropriate mechanism-of-action
- Consider using a “dirty plate” to test assay for cmpd robustness
  - A reasonable hit rate in pre-screening activities does not guarantee sound hits
  - Use collection of colored, fluorescent, reactive, precipitating, promiscuous etc inhibitors

# Acknowledgements

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