The acetogenin-based bis-tetrahydropyran 1,4-triazole, JM425 potentially targets the trypanosomatid mitochondrial complex V

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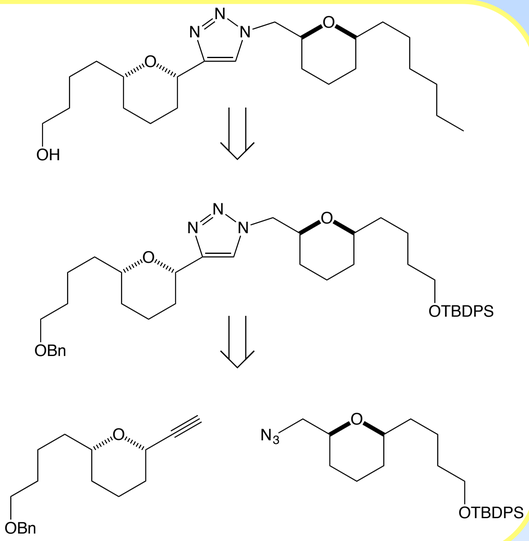
Introduction

i. Human African Trypanosomiasis (HAT) – African Sleeping Sickness.

- Caused by infection with the protozoan parasite *Trypanosoma brucei gambiense* or *T. b. rhodesiense*.
  - Early stage infection (haemolymphatic stage):
    - fever and malaise as parasites invade subcutaneous tissues.
  - Late stage infection (encephalitic stage):
    - brain injury as parasites cross blood-brain barrier.
  - Fatal within 3 months (*T. b. rhodesiense*) or 3 years (*T. b. gambiense*) if untreated.
- Endemic in sub-Saharan Africa where it is transmitted by the bites of infected tsetse flies.
  - Estimated 20,000 people infected.
  - 65 million people at risk.
- Current therapies are inadequate:
  - Most are ineffective for late-stage infection.
  - Drug toxicity (e.g. melarsoprol) kills 5 % of those treated.
  - New treatments are urgently required.

ii. Retrosynthesis of JM compounds

- We recently reported [1] the synthesis and trypanocidal activity of bis-tetrahydropyran 1,4-triazoles (B-THP-T compounds, scheme opposite).
- B-THP-T compounds are active against bloodstream form and procyclic form (PF) *T. brucei*.



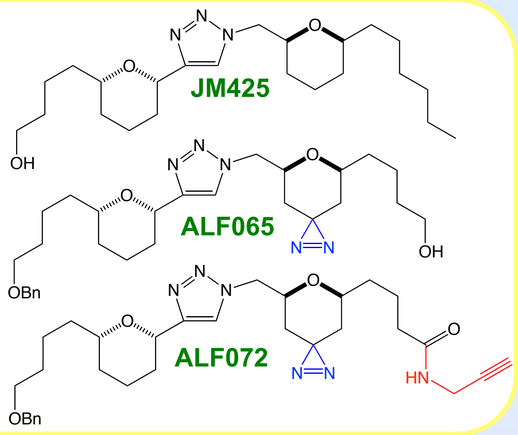
iii. Study aim

- **To identify the target of these inhibitors to allow us to improve inhibitor potency and selectivity.**

[1] Florence *et al* (2014) *Chem. Med. Chem. 9; 2548-2556*

Compounds used

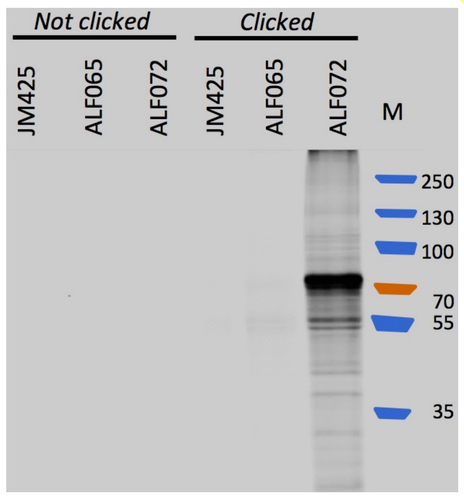
- We generated a compound (ALF072) with additional tags to identify the target of our inhibitors:
  - A diazirine forms a reactive carbene under UV and covalently attaches the inhibitor to its target.
  - An alkyne handle allows subsequent addition of reporter tag (Cy5.5 or biotin) via Cu^{2+}-catalysed click reaction.
- A cross-linkable, non-clickable inhibitor (ALF065) was generated as negative control.



Results

1. Pull-down of cross-linked protein target.

- Compounds were UV-cross-linked to their target(s) in live cells and proteins were extracted by MeOH/$CHCl_3$ precipitation.
- For visualisation by SDS-PAGE, Cy5.5 azide was clicked onto the alkyne handle, proteins separated by SDS-PAGE and labelled proteins detected at 700 nm with Licor Odyssey.
- Several proteins successfully fluoresced following crosslinking with ALF072 (containing both diazirine and alkyne) and clicking with Cy5.5 azide.
- No protein fluoresced when the alkyne was missing (JM425 and ALF072) or the diazirine was missing (JM425), **indicating we had added reporter specifically to the ALF072-cross-linked proteins**.



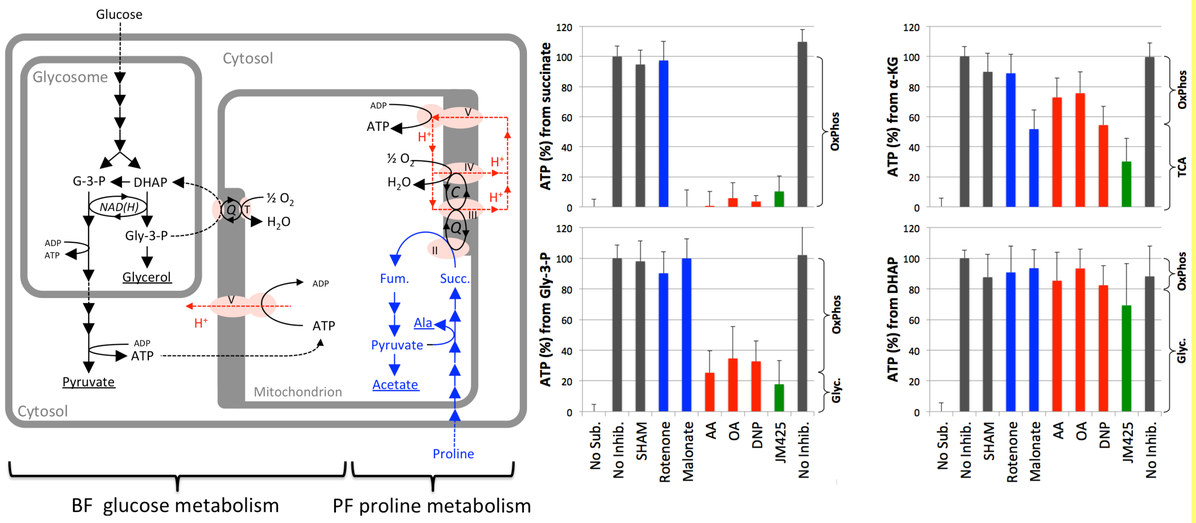
Protein	Mass	Nucleotide as cofactor?	No pulldown detected		Total peptides	Total score
			-ve control	ALF072		
Heat shock protein 70-4	71391		0	3	28	1266
Enolase	46563		0	3	13	824
Heat shock protein 70	71430		0	3	20	677
Pyruvate phosphate dikinase	101256	✔	0	3	13	386
Tryparedoxin	15881		0	3	11	295
Heat shock protein 83	80712		0	2	18	640
Fructose-bisphosphate aldolase	41130		0	2	10	458
S-adenosylhomocysteine hydrolase	48416	✔	0	2	11	391
ATPase β subunit	**55552**	✔	**0**	**2**	**4**	**239**
δ-1-pyrroline-5-carboxylate dehydrogenase	61997	✔	0	2	9	230
Arginine kinase	44670	✔	0	2	9	227
Putative immunogenic protein	15101		0	2	4	194
Glycosomal malate dehydrogenase	33917	✔	0	2	6	191
Triosephosphate isomerase	26818		0	2	6	181
Paraflagellar rod protein	68640		0	2	6	177
ATPase α subunit	**63589**	✔	**0**	**2**	**4**	**171**
Ribosomal protein S14	15503		0	2	3	160
Trypanothione reductase	53250	✔	0	2	4	151
Threonine 3-dehydrogenase	36918	✔	0	2	4	150
Mitochondrial phosphate transporter	34273		0	2	3	132
Phosphofructokinase	53484	✔	0	2	5	121

- For pull-down of the target, biotin azide was clicked on, and the tagged proteins enriched with streptavidin-agarose.
- Proteins were digested with trypsin and peptides analysed by LC-MS-MS. Peptides were identified by a Mascot search of the *T. brucei* genome.
- ALF065 or JM425 were used as negative controls in three independent pull-down pairs (ALF072 Vs negative control).
- Hit criteria:
  - Not detected in negative pull-down.
  - Score >50 in 2+ ALF072 pull-downs

- **A large number of hits use ATP or NADH as a substrate/cofactor, suggesting our B-THP-Ts may mimic nucleotides (e.g. ATP/ADP).**
- We were particularly interested in the α- and β-subunits of the F1 ATPase which form the ATP-binding regulatory and catalytic subunits of the FoF1 ATP synthase (mitochondrial complex V, highlighted above).

2. B-THP-Ts inhibit oxidative phosphorylation in isolated PF mitochondria.

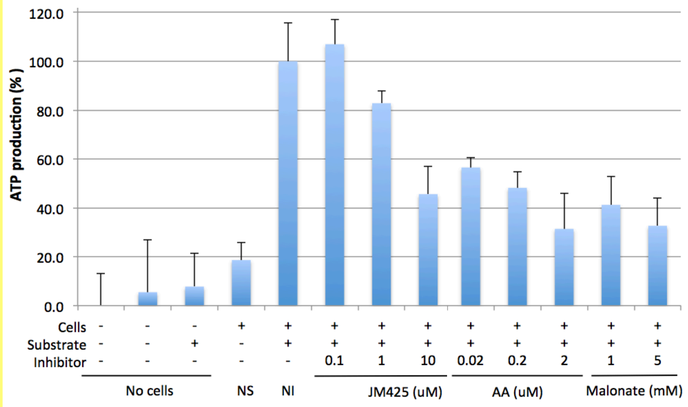
- We set out to determine if B-THP-Ts inhibited ATP production using digitonin-permeabilised PF cells. In this assay the mitochondrion and glycosome remain intact and can be probed independently using specific substrates: succinate (Ox-Phos); α-ketoglutarate (TCA); glycerol-3-phosphate (Ox-Phos and glycolysis); dihydroxyacetone phosphate (glycolysis).



- **Our compounds abolished oxidative phosphorylation (Ox-Phos) from succinate and Gly-3-P**:
  - Residual ATP production from Gly-3-P was glycosomally-produced as a result of its conversion to DHAP, and this was not significantly inhibited by our compounds or oxidative phosphorylation inhibitors.
- Our compounds inhibit ATP production from α-KG:
  - α-KG is converted to succinate and our B-THP-T compounds abolish the Ox-Phos ATP production.
  - They inhibit ATP production in addition to oxidative phosphorylation, and we note that α-ketoglutarate dehydrogenase was pulled down specifically in all ALF072 pull-downs, but with low score, perhaps suggesting weak interaction with this protein..
- **These data indicate that our B-THP-Ts target a specific component of oxidative phosphorylation.**

Results (continued)

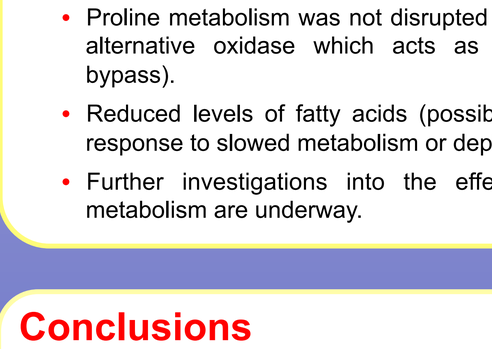
3. JM425 reduces [ATP] in live cells.



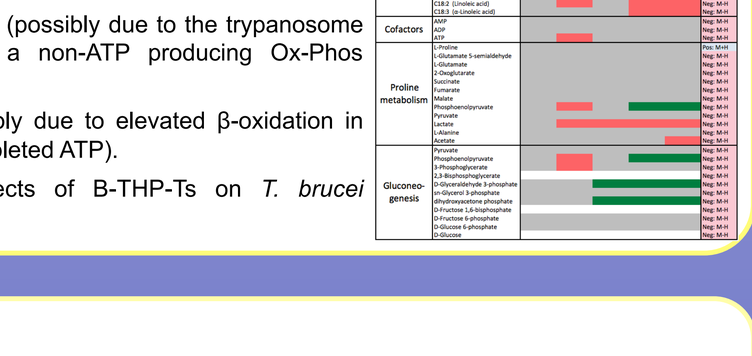
- We determined whether B-THP-T inhibition of Ox-Phos was relevant in live PF cells.
- PF *T. brucei* were cultured for 1 h in buffered PBS with proline +/- inhibitors.
- The ATP content of cells was determined using a bioluminescence assay.
- **JM425 decreased the levels of ATP in cells and was comparable with inhibitors of oxidative phosphorylation, confirming that our inhibitors target ATP production *in vivo*.**

4. B-THP-T compounds elevate mitochondrial membrane potential.

- To determine the part of oxidative phosphorylation inhibited by our compounds we measured the mitochondrial membrane potential (MMP) in the presence of various inhibitors with MitoTracker Red (MTred), which accumulates in active mitochondria (the greater the accumulation the greater the activity and MMP).
- PF *T. brucei* were cultured in glucose-free SDM-79 +/- inhibitors for 1 h.
- MTred was added to 100 nm, unincorporated MTred was washed away, and fixed cells were analysed by microscopy (DeltaVision) while fluorescence was quantified with a plate reader (normalised to Mitotracker green).



Mitotracker red staining is specific to the mitochondrion

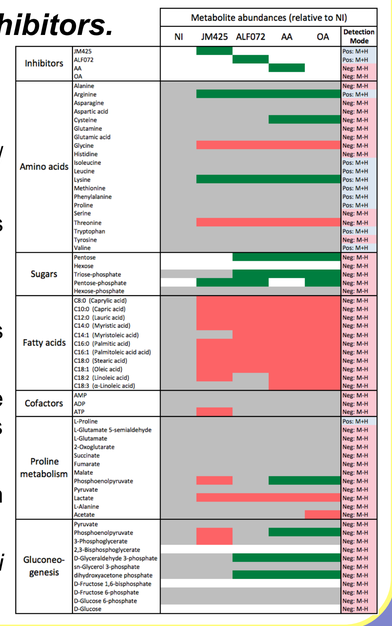


- ETC inhibitors (DNP and AA) decrease the MMP, while complex V inhibitors (OA) elevate the MMP.
- Our compounds elevate the MMP, indicating that they target mitochondrial complex V.
- **This supports our pull-down of the ATPase α- and β-subunits of FoF1-ATPase mitochondrial complex V.**

5. B-THP-Ts have similar metabolic effects as Ox-Phos inhibitors.

- We determined the effects of inhibitors on PF metabolism using MS.
- PF *T. brucei* were incubated with inhibitors at EC10 concentrations for 24 h.
- Cells were washed thoroughly in PBS and metabolites extracted in dH_2O / MeOH / $CHCl_3$ (ratio 1/3/1).
- Metabolites were analysed by orbitrap MS and identified to 3 d.p. (mass +/- 0.0005 Da) and abundances relative to no inhibitor control (NI) calculated:

  < 50 % (red); 50-200 % (grey); >200 % (green); not detected (white).
- The general trend was that B-THP-Ts had the same effect on metabolism as Ox-Phos inhibitors AA and OA:
  - Proline metabolism was not disrupted (possibly due to the trypanosome alternative oxidase which acts as a non-ATP producing Ox-Phos bypass).
  - Reduced levels of fatty acids (possibly due to elevated β-oxidation in response to slowed metabolism or depleted ATP).
  - Further investigations into the effects of B-THP-Ts on *T. brucei* metabolism are underway.



Conclusions

- Diazirine- and alkyne-tagged ALF072 was used to identify the target of B-THP-T compounds in PF *T. brucei* by crosslinking it to its target with subsequent biotin pull-down.
- Among the pull-down hits were the F1 ATPase α- and β-subunits, which among other functions, form part of the mitochondrial complex V FoF1-ATP synthase.
- We showed that complex V is inhibited by B-THP-Ts: they reduce cellular ATP levels; inhibit oxidative phosphorylation; and elevate the mitochondrial membrane potential before cell death.
- Furthermore, complex V is essential in the bloodstream form and JM425 is thus a suitable lead compound for development of a new drug to combat African Sleeping Sickness.

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