Analysis of specific targeting of kinetoplastid ERK8 and GSK3β by kinase inhibitors

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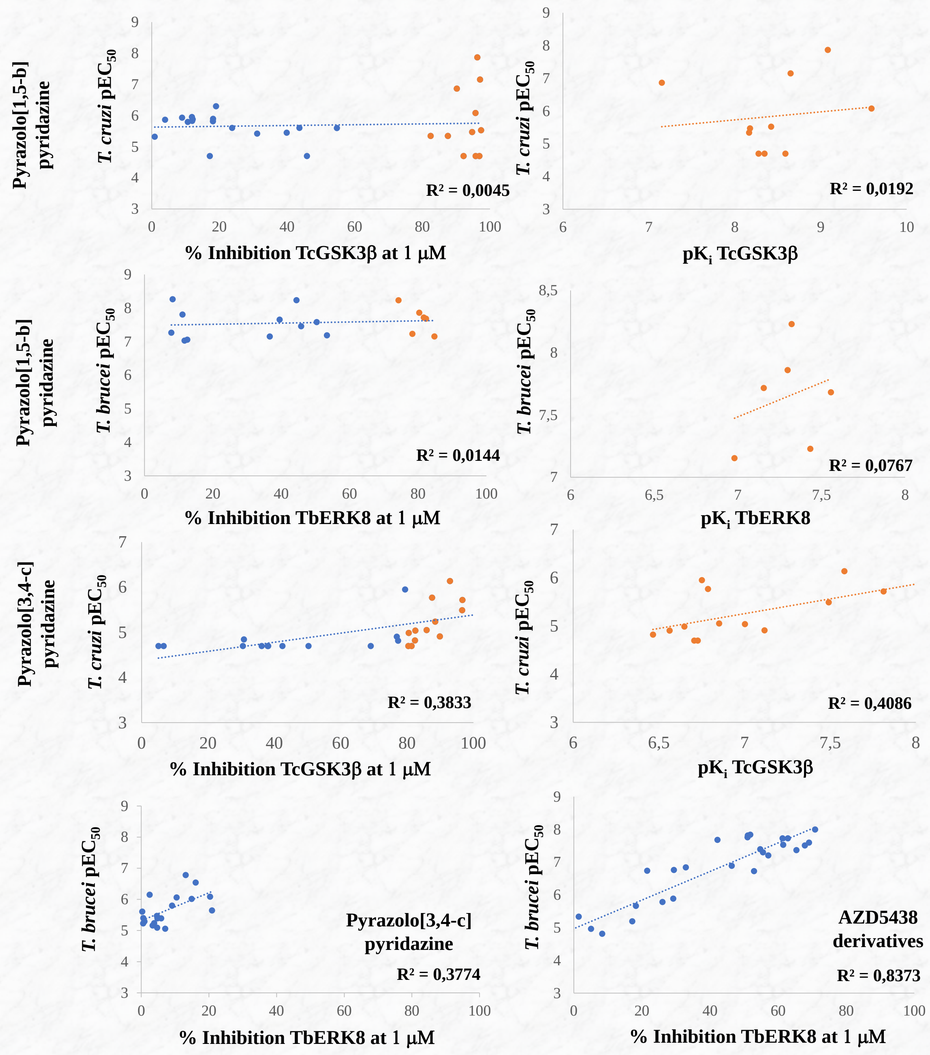
Kinase inhibitors have been described as a tool for rapid compound progression in the discovery of new treatments against parasitic diseases. We present data aimed at target identification for a series of compound classes with anti-kinetoplastid activity and potential kinase inhibitory activity. ERK8 and GSK3β, belonging to the CMGC superfamily of kinases, are known validated targets for therapeutic purposes in kinetoplastids. Existing data point towards these two kinases as potential targets of the selected compound clusters. Recombinant purified TbERK8 and TcGSK3β were obtained and the *in vitro* inhibition profiles of the two enzymes were established. *In vitro* assays were also performed with HsGSK3β for assessment of enzyme selectivity. Moreover, we generated a *T. brucei* line over-expressing TbERK8 and exposed it to a selected set of inhibitors. Kinase over-expression causes a severe growth defect in the parasite upon induction. This toxicity is reverted in a dose dependent manner by certain TbERK8 inhibitors, suggesting that the over-expressed kinase is the main target for this subset of compounds.

In vitro inhibition of kinases by compound clusters



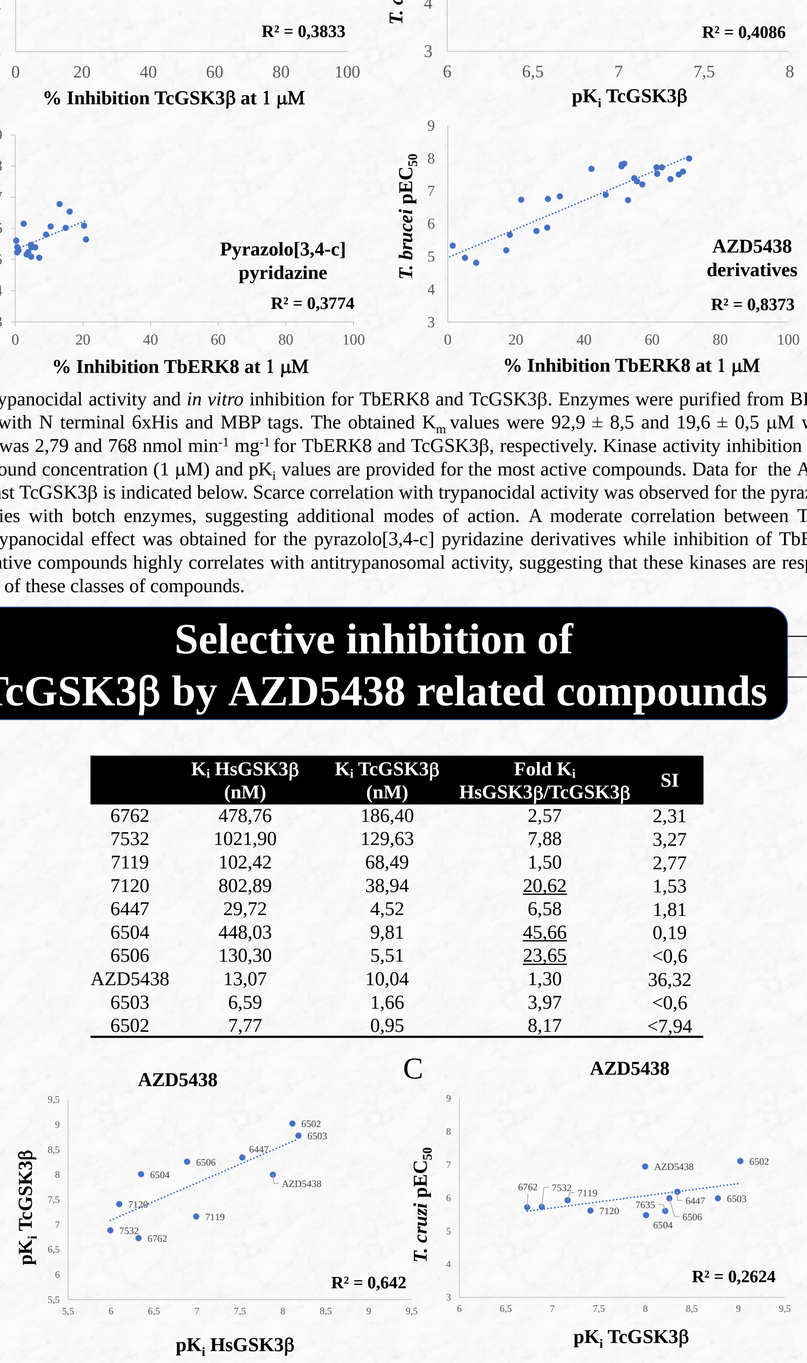
Correlation of trypanocidal activity and *in vitro* inhibition for TbERK8 and TcGSK3β. Enzymes were purified from BL21 DE3 *E. coli* bacteria with N terminal 6xHis and MBP tags. The obtained K_m values were 92,9 ± 8,5 and 19,6 ± 0,5 μM while the specific activity was 2,79 and 768 nmol min^{-1} mg^{-1} for TbERK8 and TcGSK3β, respectively. Kinase activity inhibition is shown at a single compound concentration (1 μM) and pK_i values are provided for the most active compounds. Data for the AZD5438 derivatives against TcGSK3β is indicated below. Scarce correlation with trypanocidal activity was observed for the pyrazolo[1,5-b]pyridazine series with botch enzymes, suggesting additional modes of action. A moderate correlation between TcGSK3β inhibition and trypanocidal effect was obtained for the pyrazolo[3,4-c] pyridazine derivatives while inhibition of TbERK8 by AZD5438 derivative compounds highly correlates with antitrypanosomal activity, suggesting that these kinases are respectively the major targets of these classes of compounds.

Selective inhibition of TcGSK3β by AZD5438 related compounds

A

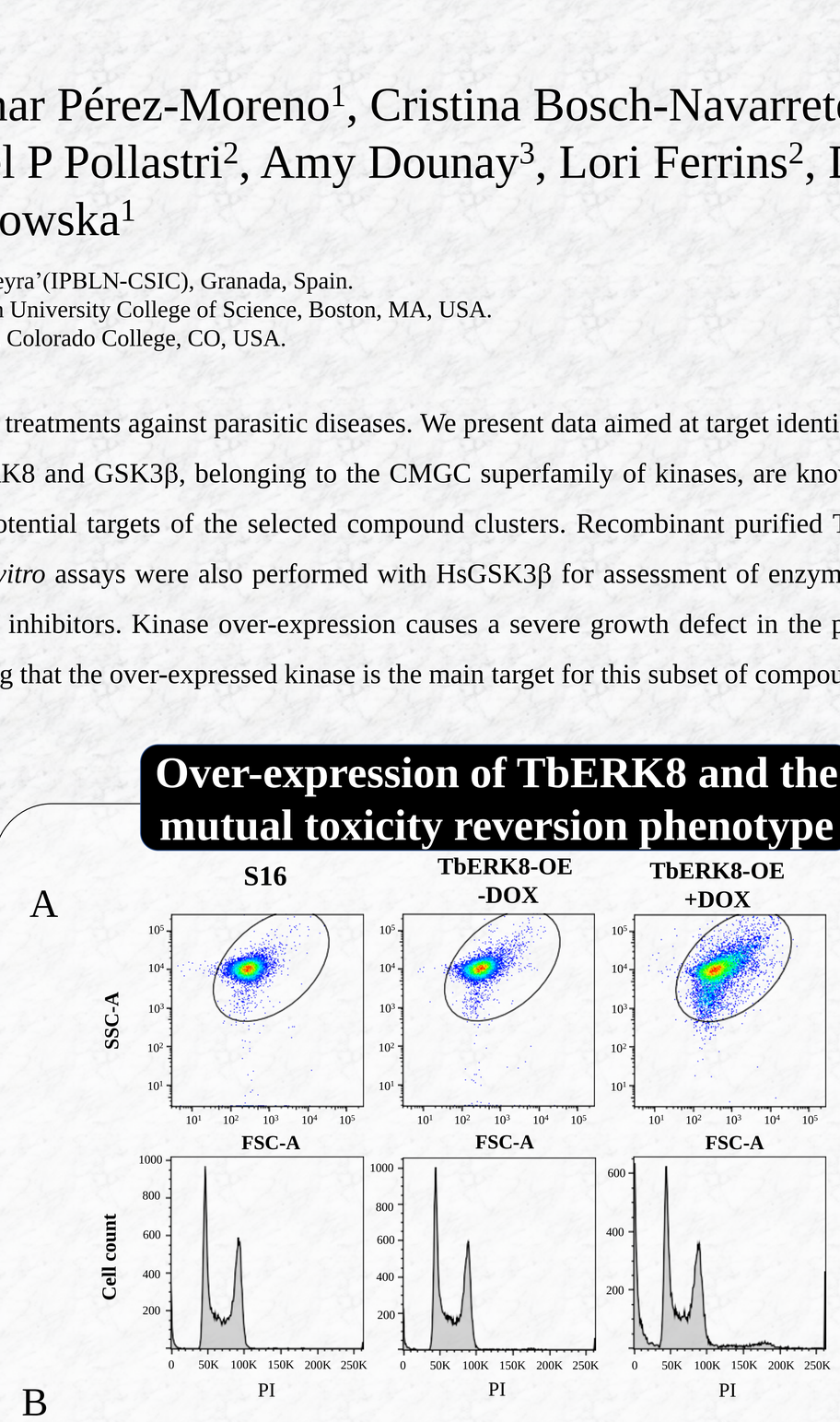
	K_i HsGSK3β (nM)	K_i TcGSK3β (nM)	Fold K_i HsGSK3β/TcGSK3β	SI
6762	478,76	186,40	2,57	2,31
7532	1021,90	129,63	7,88	3,27
7119	102,42	68,49	1,50	2,77
7120	802,89	38,94	20,62	1,53
6447	29,72	4,52	6,58	1,81
6504	448,03	9,81	45,66	0,19
6506	130,30	5,51	23,65	<0,6
AZD5438	13,07	10,04	1,30	36,32
6503	6,59	1,66	3,97	<0,6
6502	7,77	0,95	8,17	<7,94

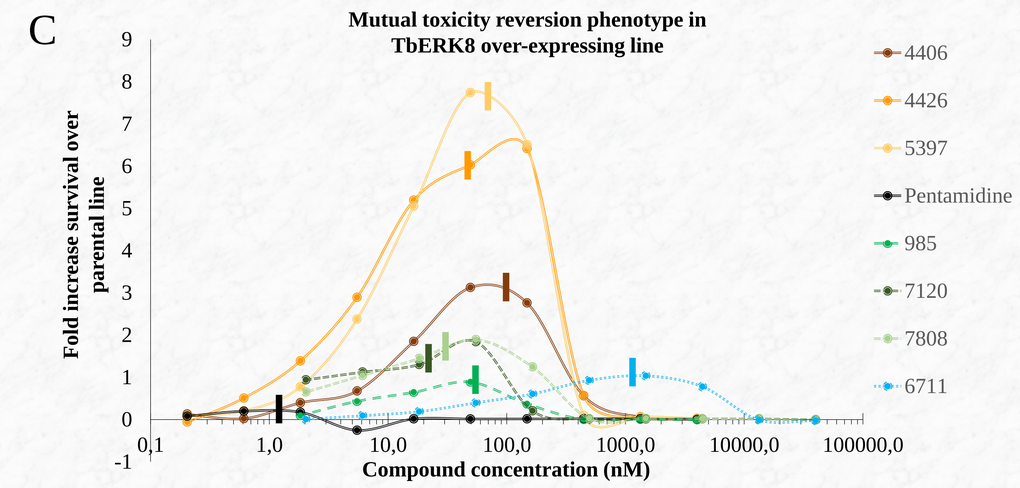
B C



Certain AZD5438 derivatives exhibit selective inhibition of TcGSK3β versus the human enzyme (A) yet a correlation analysis indicates that the inhibition profiles are mostly similar for both enzymes (B). Enzyme inhibition does not significantly correlate with antitrypanosomal activity, suggesting additional targets for this cluster (C).
A) K_i values of AZD5438 derivatives for both human and *T. cruzi* GSK3β SI: selectivity index in culture (CC50 L6/EC50 *T. cruzi*) **B)** Correlation between *in vitro* inhibition for HsGSKβ and TcGSKβ **C)** Correlation of trypanocidal activity and *in vitro* inhibition of AZD5438 derivatives.

Over-expression of TbERK8 and the mutual toxicity reversion phenotype





A subset of pyrazolo[1,5-b]pyridazine derivatives revert the toxicity associated to ERK8 over-expression in *T. brucei*. This phenotype is dose dependent and reaches a maximum at concentrations close to the parental S16 EC50 values. The mutual toxicity reversion phenotype is evident in the cell cycle analysis, where inhibitors revert the cell cycle defects observed in the over-expressing cell line TbERK8-OE.
Flow cytometry analysis (SSC vs FCS in upper panels; histogram cell count vs propidium iodide in lower panels) for **A)** parental line (S16 –single marker-), TbERK8-OE -DOX, and TbERK8-OE +DOX. Induction was performed for 72 hours and **B)** TbERK8-OE +DOX cells exposed to different compounds during induction. **C)** Fold survival over parental line at different doses for the indicated compounds. The vertical lines denote the S16 EC50 value for each compound. Pentamidine is shown as a negative control of the reversion phenotype.

Conclusions

- The compound clusters analysed mostly inhibit kinetoplastid ERK8 and GSK3β except in the case of the pyrazolo[3,4-c] pyridazine series, that is inactive against TbERK8.
- The correlation between trypanocidal activity and *in vitro* inhibition of GSK3β suggests that the main target of the pyrazolo[3,4-c] pyridazines series is GSK3β.
- Selective inhibition of TcGSK3β versus HsGSK3β by certain AZD5438 derivatives provides a starting point for the design of compounds specifically targeting the trypanosomal enzyme.
- The toxicity reversion observed with certain pyrazolo[1,5-b]pyridazine derivatives in parasites overexpressing TbERK8 strongly suggests that this enzyme has a major role in the mode of action of this class of compounds.

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