In Vitro Pharmacodynamics and Mechanism of Action Studies of Oxaborole 6-Carboxamides: A New Class of Compounds for the Treatment of African Trypanosomiasis

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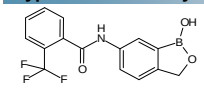
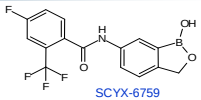
Abstract

Human African Trypanosomiasis (HAT) is a fatal disease caused by *Trypanosoma brucei spp*. There is a need for new treatment for HAT because current treatments are costly, difficult to administer and frequently toxic. We have identified several oxaborole 6-carboxamides that demonstrate potent activity against *T. brucei brucei in vitro* and exhibit efficacy against both the acute and chronic CNS stages of HAT in mouse models. Exposure of *T. b. brucei* to oxaboroles leads to significant changes in shape, reduction in cellular size and detached flagella at the time of death. *In vitro* studies performed to characterize the relationship between killing of *T. brucei* and oxaborole exposure, demonstrate an early (6-9 hrs) onset of trypanocidal activity as shown by the inability of the parasites to generate ATP. Parasite commitment to death *in vitro* occurs with similar kinetics even when compound is washed out 2-3 hours following exposure. For mechanism of action studies, fluorescently tagged oxaborole analogues have been synthesized and incubated with *T. brucei* parasites to identify sub-cellular localization. In addition, representative compounds have been immobilized on agarose matrices for use in affinity capture of parasite target proteins which will be identified by mass spectrometry and data base searches. Collectively these studies which use AN3520 as a example will provide a better understanding of how oxaboroles exert their trypanocidal effects and enable us to develop valuable PK/PD models to ensure appropriate drug delivery for treatment of HAT.

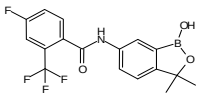
Trypanocidal Activity *In Vitro* – Boron Atom is Essential



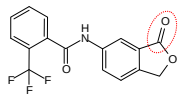
AN3520
T. b. brucei IC_{50} = 0.04 µg/ml
L929 Cytotox IC_{50} = 3.77 µg/ml
Selectivity Index = 94



SCYX-6759
T. b. brucei IC_{50} = 0.075 µg/ml
L929 Cytotox IC_{50} = 9.04 µg/ml
Selectivity Index = 121



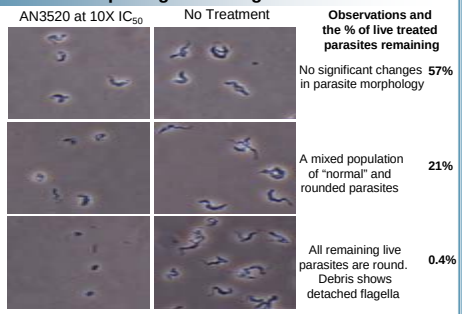
SCYX-7158
T. b. brucei IC_{50} = 0.267 µg/ml
L929 Cytotox IC_{50} > 50 µg/ml
Selectivity Index >187



SCYX-9286
T. b. brucei IC_{50} >50 µg/ml
L929 Cytotox IC_{50} >50 µg/ml
Selectivity Index N/A

Oxaboroles exhibit potent inhibitory activity against *T. b. brucei* blood stage parasites *in vitro* (IC_{50}= 0.04 – 0.267µg/ml) and very low or no activity against a proliferating mammalian cell line, L929 fibroblast (3.77 – >50 µg/ml). A lactone derivative, in which the boron atom is replaced with carbon, does not exhibit trypanocidal activity when tested up to 50 µg/ml against *T. b. brucei in vitro*. The exact mechanism through which oxaboroles act to kill trypanosomes is unknown, but data suggests that the boron atom plays a key role.

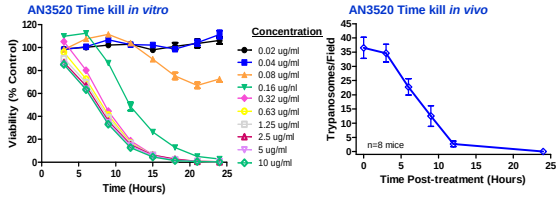
AN3520-Induced Morphological Changes in *T. b. brucei*



T. brucei parasites in the log growth phase were exposed to AN3520 at 10X IC_{50}. A 1 ml aliquot was taken at the indicated times, centrifuged to concentrate the parasites and spotted on slides for microscopic examination. Live parasites were quantitated by hemocytometer.

Exposure to AN3520 leads to loss of the typical slender form to a more round morphology and apparent reduction in overall size. Death of >99% of *T.b. brucei* parasites occurs within 24 hrs following exposure to oxaboroles. Compound induced changes in parasite gross morphology are evident in the first 8-10 hours after compound addition. There is evidence of detached flagella in some of the rounded/dying parasites. The morphological changes are consistent with loss of parasite viability observed through determination of ATP content following exposure to AN3520.

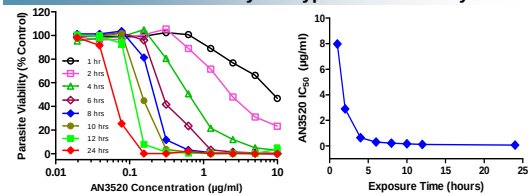
Kinetics of Trypanosome Killing – Time Kill Studies



Time Kill *in vitro*: Trypanocidal speed of action *in vitro* was determined by measurement of ATP content (luciferase assay) as an indicator of parasite viability. Oxaborole-dependent killing of *T. b. brucei* was very rapid and dependent on time and to a lesser extent concentration (up to 0.3 µg/ml). Increased AN3520 above 0.3 µg/ml did not result in faster or stronger killing of the parasites. These data predict that *in vivo* efficacy of oxaborole compounds is likely dictated by time and concentration above MIC values.

Time Kill *in vivo*: Mice (8) were each infected intraperitoneally with 2.5x10^5 *T. b. brucei* (EATRO 110 strain) parasites isolated from an infected rat. A single dose (50 mg/kg) of AN3520 was given orally 72 hours after parasite infection. After treatment, mice were checked for parasitemia at the indicated times by microscopic examination of smears prepared from tail vein blood. Clearance of the parasites was time-dependent with significant reduction by 12 hrs post-treatment. There were no parasites in mouse blood collected 24 hrs post-treatment. These results demonstrate an *in vitro – in vivo* correlation in the pharmacodynamic characteristics of oxaborole compounds.

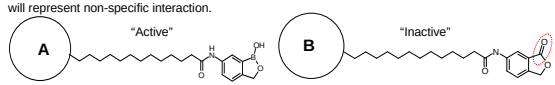
Assessment of Reversibility of Trypanocidal Activity



Reversibility of drug action was determined by exposure of *T. b. brucei* parasites to increasing concentrations of AN3520 for the indicated times. This was followed by washout of compound and incubation in drug-free media for 72 hrs prior to assessment of resazurin reduction as an indicator of parasite viability and determination of IC_{50}s for each time period. Results show that AN3520 treated parasites are committed to death after a short exposure (1-2 hrs) to compound. IC_{50} values drop below 1 µg/ml when compound was washed after only 6 hours of exposure. This oxaborole mediated early "commitment to death" suggests that serum concentrations may not need to be maintained for extended periods *in vivo* to achieve cure or parasite clearance.

Immobilized Oxaborole for Target Identification

An oxaborole compound (A) and its lactone derivative (lacks boron atom, (B)) were immobilized on Sepharose beads via a 14 carbon linker. Immobilization of an oxaborole "active" derivative versus the "inactive" lactone derivative provides a means to identify proteins that are selectively targeted by the trypanocidal specific compounds. Proteins binding to and eluting from the "inactive" affinity tag will represent non-specific interaction.



Incubate immobilized compound with 3-40 mg fresh *T. b. brucei* lysates in a high stringency buffer (1M NaCl)

Wash extensively with 30 bead volumes of the 1M NaCl buffer

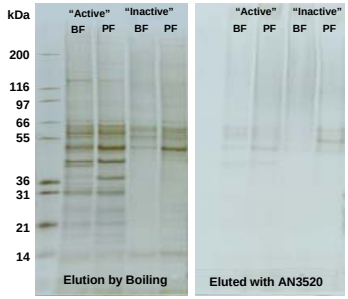
Wash with an additional 30 volumes of 50mM NaCl buffer

Elute proteins with 2 volumes 50mM NaCl buffer supplemented with 1.5mM free compound

Run samples on SDS-PAGE gel and visualize by silver or coomassie blue stain

For large scale pull-down experiments, procyclic stage (tissue culture) or blood stage (rat blood) *T.b. brucei* parasites were used to generate lysates. Eluted samples from affinity pull-downs were separated on gels, trypsin digested, subjected to MALDI/TOF/TOF analysis and protein identified by searching the *T. b. brucei* database http://www.genedb.org/genedb/tryp/index.jsp

Affinity Capture of Oxaborole Interacting *T. brucei* Proteins



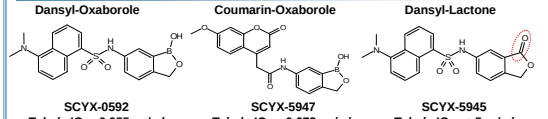
Protein Samples:
BF - **B**lood stream **F**orm *T. brucei* from rats
PF - **P**rocyclic **F**orm *T. brucei* from culture

PF& **BF** parasites show similar sensitivity to oxaboroles *in vitro*. PF parasites grow to much higher density than BF stages and therefore provide a better source of protein for this work.

MALDI-TOF/Database Searches
- DEAD/H RNA helicase
- Three hypothetical proteins
- 60S ribosomal protein L4
- 60S ribosomal protein L2
- Lysophospholipase
- Glycosomal aldolase
- Tubulins – alpha & beta

Conclusions
1. "Active" affinity ligand interacts with more proteins than the "inactive" resin
2. Tubulin was present in boron and non-boron containing ligand
3. Most of the captured "targets" represent those that are generally abundant in *T. brucei* lysates
4. Lysophospholipase is not essential in *T. brucei*
5. Inhibiting glycosomal aldolase would be expected to show differential effects on BF vs PF – procyclic and blood stream form parasites are equally sensitive to oxaboroles – data not shown.

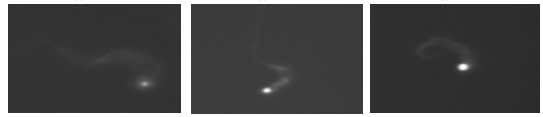
Intracellular Localization of Oxaboroles

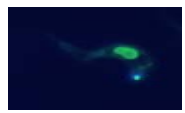


SCYX-0592
T. b. b. IC_{50}: 0.055 µg/ml

SCYX-5947
T. b. b. IC_{50}: 0.072 µg/ml

SCYX-5945
T. b. b. IC_{50}: >5ug/ml





Overlay with DyeCycle Orange (Green)

Test compounds were modified to incorporate a dansyl or coumarin fluorophore to determine the intracellular destination in live *T. b. brucei* parasites after incubation for 3 hrs. It appears that compounds concentrate in the posterior portion of the parasite, proximal to the kinetoplast/flagellar pocket region. Faint and diffuse fluorescence is also observed throughout the parasite. Dansyl or coumarin fluorophores alone did not accumulate in *T. b. brucei*, indicating that the test compounds are responsible for the distribution pattern. The live cell DNA stain DyeCycle Orange was used to determine the location of the kinetoplast and assess potential co-localization with fluorescent test compounds.

Boron containing compounds and the lactone derivative localized to the same region suggesting that localization per se is not responsible for activity of the compounds. Lack of co-localization of fluorescently tagged oxaboroles and DyeCycle orange suggest that the kinetoplast is unlikely to be the target of these compounds. The flagellar pocket is predicted to be the site of accumulation, but it's role in oxaborole MOA is unclear at this point.

Summary

- Oxaborole carboxamides are potent and selective inhibitors of *T. brucei*. The boron atom is essential for trypanocidal activity.
- Microscopic analysis shows morphological changes of trypanosomes within several hours following exposure to oxaboroles *in vitro*.
- Time kill studies demonstrate fast killing of *T. brucei* by oxaboroles *in vitro* and *in vivo*. *T. brucei* parasites do not recover from oxaborole effects following a transient exposure to compounds.
- Agarose immobilized ligands interact with several proteins in *T. brucei* lysates. Non-trypanocidal lactone interacts with fewer proteins than the active oxaborole.
- Fluorescently tagged analogues of AN3520 localize to the flagellar pocket region of *T. brucei*. Both active and inactive analogues accumulate in the same region.