# Biological activities of fluorescent Trypanosome Alternative Oxidase inhibitor conjugates incorporating a cationic julolidine-based viscosity sensor.

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## Introduction

- ✓ African trypanosomiasis caused by species of Trypanosoma brucei is a neglected tropical diseases of public health concern
- ✓ a severe challenge to agriculture within endemic regions.
- ✓ T. brucei possess certain unique metabolic features that can be exploited for the purpose of effective drug development[1].
- ✓ In earlier studies, we reported the development and trypanocidal efficacies of two classes of inhibitors based on 4-hydroxybenzoate and 4-alkoxybenzaldehyde scaffolds[2].
- ✓ These compounds were designed to target the distinctive and critical enzyme of trypanosome respiration, the Trypanosome Alternative Oxidase (TAO), which is situated in the mitochondrion of the parasite.
- ✓ The inhibitors were targeted to the mitochondrial matrix by coupling to lipophilic cations (LC; triphenylphosphonium or quinolinium salts).
- ✓ To enable optimal interaction with the enzyme's active site, the coupling used a long and flexible C14 linker.
- ✓ The inhibitors were optimised by systematically exploring substitutions on the central phenyl ring
- ✓ Molecular rotors (MR) are fluorescent probes known to form twisted intramolecular charge-transfer (TICT) complexes in the excited state, the fluorescence quantum yield of which is dependent on the nearby milieu.
- ✓ (E)-4-(2-(8-hydroxy-julolidine-9-yl)vinyl-1-methylpyridine-1-ium bromide (HJVPI) was selected as the fluorescent MR in this work because of its suitable photophysical and biological properties (i.e. red emitting fluorescence, good membrane permeability, mitochondrial selectivity, low cytotoxicity, and increase in fluorescence in the presence of glycerol
- ✓ The use of HJVPI should enable us establish the localization of the inhibitor in the target site during TAO inhibition - which results in excess glycerol production in the parasite.

## Aim

➢ In the present work, we aim to explore the biological activity of fluorescent TAO inhibitor probes (1a, 2a–d) that incorporate a julolidine-based molecular rotor with a cationic pyridinium salt as mitochondrial targeting moiety. We also aim to establish the localization of the inhibitor in the target site.

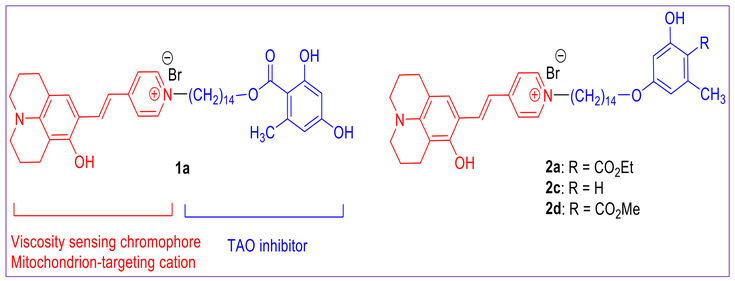



**Figure 1. Structures of fluorescent TAO inhibitor conjugates 1a, 2a, 2c, and 2d bearing a molecular rotor as viscosity sensor (in red) and a TAO inhibitor pharmacophore (in blue)**

## Method

- ❖ Resazurin assay was used to determine the susceptibilities of bloodstream trypomastigotes of *T. b. brucei*
- ❖ Cytotoxicity of the test compounds was carried out using Human Embryonic Kidney cells.
- ❖ Ubiquinol oxidase activity was carried out on recombinant TAO lacking the mitochondrial targeting signal (ΔMTS).
- ❖ ΔMTS TAO activity was determined using a V-630 Jasco UV-Vis spectrophotometer by measuring the absorbance change of the substrate- ubiquinol ($\varepsilon_{278}$ = 15,000 $M^{-1}$ $cm^{-1}$) at 278 nm over a period of 2 min in a 1 cm cuvette.
- ❖ Mitochondrial Staining of *T. brucei* was performed using MitoTracker™ Green FM and Hoechst 33342 Staining Dye Solution, and viewed under DeltaVision microscope (GE Healthcare) using softWoRx software and processed using ImageJ software .
- ❖ Fluorescence microscopy in human cells was performed using MC3T3-E1 preosteoblast (ECACC 99072810) cell line.

## Results

**Table 1. Biological activity of fluorescent TAO inhibitor conjugates 1a and 2a-d in vitro activity against *T . brucei* wild-type (WT) and drug-resistant lines, cytotoxicity against mammalian cells, and inhibition of TAO.**

| | *T. b. brucei* $EC_{50}$ (μM) | | | | | Cytotoxicity $CC_{50}$ (μM) | | rTAO $IC_{50}$ (μM)[g] |
|---|---|---|---|---|---|---|---|---|
| Cmpd | S427 (WT)[a] | B48[b] | RF[c] | AQP1-3 KO[d] | RF[c] | HEK[e] | SI[f] | |
| **1a** | 1.01 ± 0.04 | 1.19 ± 0.05 | 1.17 | 1.048 ± 0.050 | 1.03 | >100 | >100 | 0.36 ± 0.07 |
| **2a** | 0.80 ± 0.05 | 0.85 ± 0.05 | 1.07 | 0.663 ± 0.054 | 0.83 | 23.3 ± 0.1 | 29.1 | 0.0016 ± 0.0002 |
| **2c** | 0.50 ± 0.07 | 0.63 ± 0.05 | 1.25 | 0.43 ± 0.05 | 0.85 | 22.2 ± 2.2 | 44.1 | 0.145 ± 0.015 |
| **2d** | 0.54 ± 0.02 | 0.59 ± 0.01 | 1.10 | 0.44 ± 0.04 | 0.81 | 23.6 ± 1.5 | 43.6 | 0.042 ± 0.02 |
| Pentamidine | 0.0049 ± 0.0004 | 0.50 ± 0.05 | 102 | 0.034 ± 0.03 | 6.9 | | | |
| Phenylarsine oxide | | | | | | 0.46 ± 0.04 | | |

[a]Trypomastigotes of *T. b. brucei* s427 (n =3). [b]*T. brucei* cell lines from which both aquaporins AQP2 and AQP3 have been knocked out. [c]Resistance factor relative to WT. [d]T. brucei cell line from which all aquaporins were knocked out. [e]Cytotoxicity on human embryonic kidney cells (n = 3). [f]Selectivity index (SI) = CC50/EC50 (*T. brucei WT*). [g]Purified recombinant trypanosome alternative oxidase (ΔMTS-TAO) from *T. b. brucei* (n = 3). [h]Not tested. *, P<0.05 and **, P<0.01 from WT control $EC_{50}$ by Student's unpaired t-test; n=3.

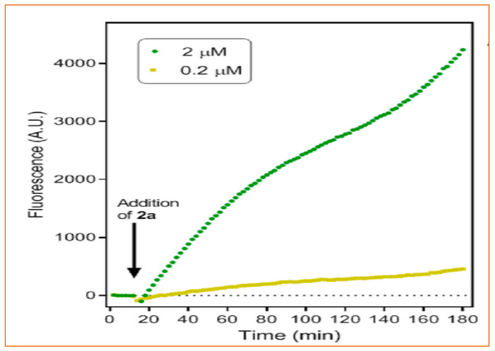



**Fig. 2. Real-time monitoring of fluorescence in *T. b. brucei* BSF. Fluorescence of *T. b. bruceis* 427WT incubated with 2a, added to the indicated concentrations at 14 min into the recording.**

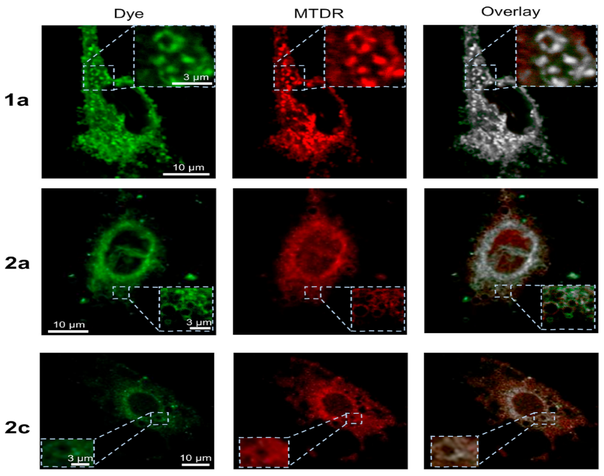



**Fig. 4. Colocalization experiments between compounds 1a, 2a and 2c (green channel) and MTDR as mitochondrial tracker (red channel) in the preosteoblast cell line The overlay images show colocalized pixels in white. Insets represent zoomed sections of the overall images.MC3T3-E1using single-photon excitation fluorescence microscopy.**

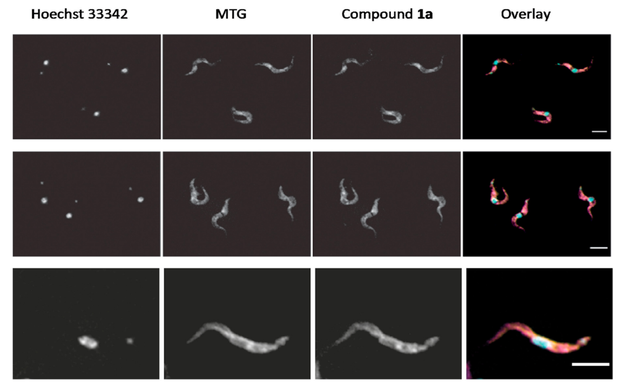



**Fig. 3. Mitochondrial localization of 1a in *T. brucei.* The first column is Hoechst 33342 (stains DNA), the second Mitotracker Green, third compound 1a and the last one the overlay(Hoechst in cyan, Mitotracker in yellow and 1a (40mM, 1 h) in magenta). All scale bars indicate 5mm.**

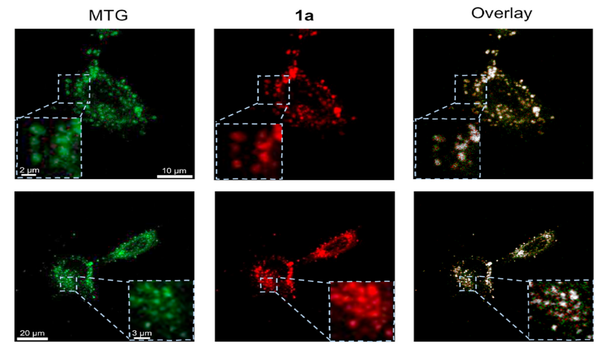
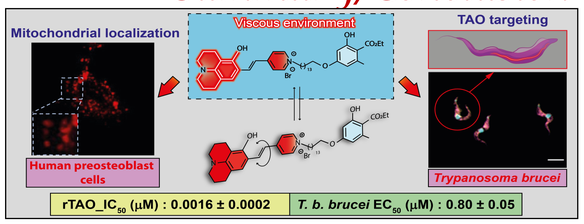



**Fig. 5. Two representative images of colocalization experiments between1a(red channel) and MTG as mitochondrial tracker (green channel) in the preosteoblast cell line MC3T3-E1 using 850-nm two-photon excitation fluorescence microscopy**

## Summary/Conclusion




**Reference:** **1.** Cueto-Díaz, E.J., Ebiloma, G.U., Alfayez, I.A., Ungogo, M.A., Lemgruber, L., González-García, M.C., Giron, M.D., Salto, R., Fueyo-González, F.J., Shiba, T. and González-Vera, J.A., 2021. Synthesis, biological, and photophysical studies of molecular rotor-based fluorescent inhibitors of the trypanosome alternative oxidase. *European Journal of Medicinal Chemistry*, *220*, p.113470. **2.** Ebiloma, G.U., Ayuga, T.D., Balogun, E.O., Gil, L.A., Donachie, A., Kaiser, M., Herraiz, T., Inaoka, D.K., Shiba, T., Harada, S. and Kita, K., 2018. Inhibition of trypanosome alternative oxidase without its N-terminal mitochondrial targeting signal (ΔMTS-TAO) by cationic and non-cationic 4-hydroxybenzoate and 4-alkoxybenzaldehyde derivatives active against T. brucei and T. congolense. *European journal of medicinal chemistry*, *150*, pp.385-402.