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Targeting the Trypanosome Alternative Oxidase (TAO) as Promising Chemotherapeutic Approach for African Trypanosomiasis

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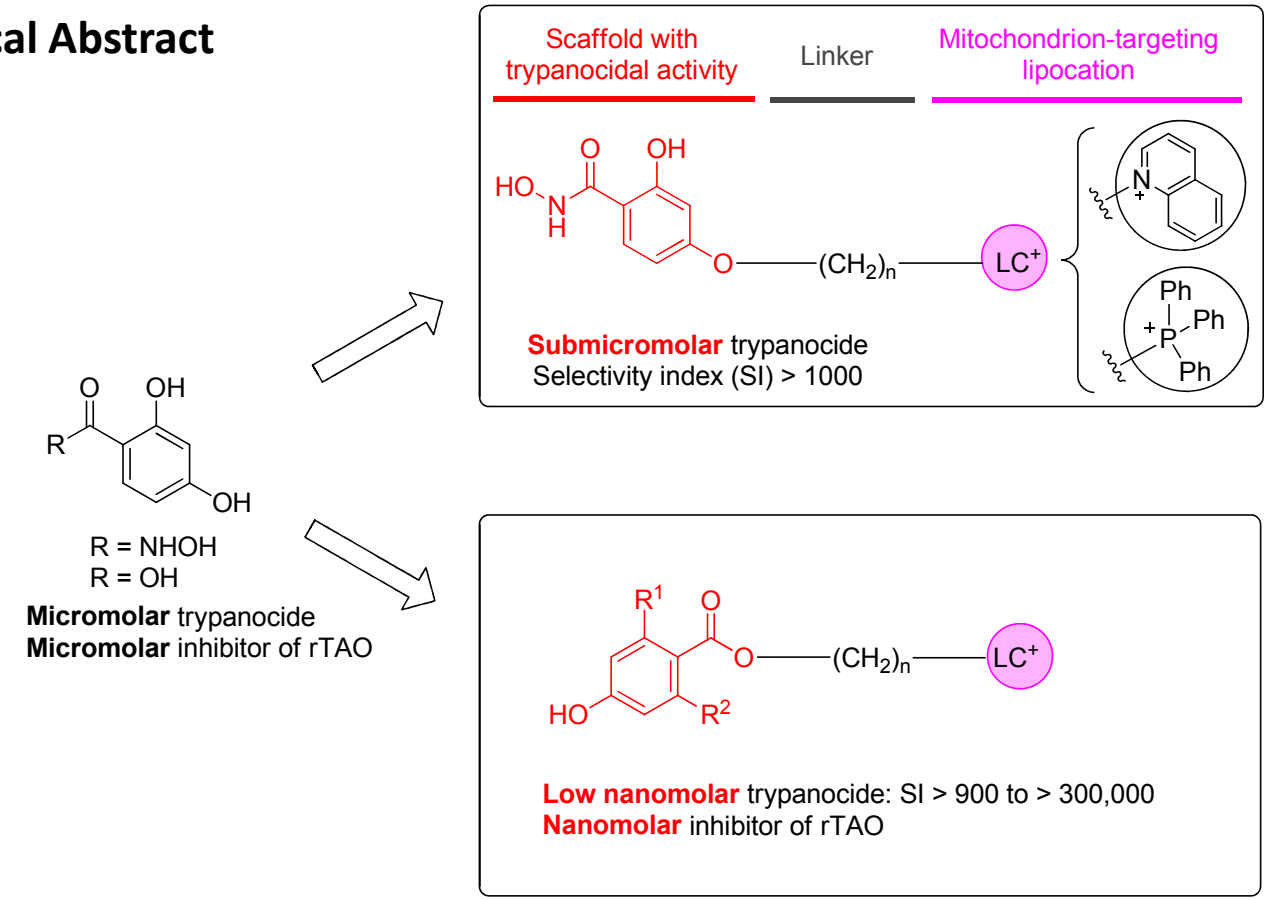
Targeting the trypanosome alternative oxidase (TAO) as promising chemotherapeutic approach for African trypanosomiasis

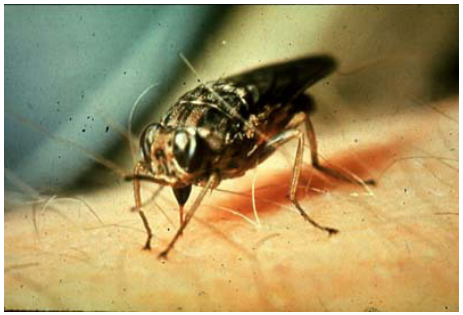
Graphical Abstract



Abstract:
In *Trypanosoma brucei*, a parasite that causes African trypanosomiasis in humans (sleeping sickness) and in livestock (nagana) throughout sub-Saharan Africa, the trypanosome alternative oxidase (TAO) is essential for the respiration of bloodstream form parasites (i.e. the human-infective form). Since TAO has no counterpart in mammalian cells and it is conserved among *T. brucei* subspecies, it has been validated as a promising target for the chemotherapy of African trypanosomiasis.
We present here a successful approach to boost the activity of TAO inhibitors based on the conjugation of the inhibitor with lipophilic cations (LC) that can cross lipid bilayers by non-carrier mediated transport, and thus accumulate specifically into mitochondria, driven by the plasma and mitochondrial transmembrane potentials (negative inside). This design afforded several LC–TAO inhibitor conjugates active in the submicromolar to low nanomolar range against wild type and resistant strains of African trypanosomes (*T. b. brucei*, *T. congolense*), with selectivity over human cells >500.

Keywords: Trypanosome alternative oxidase (TAO) inhibitor; *Trypanosoma brucei*, sleeping sickness, lipocation, mitochondrion

Introduction



African trypanosomiasis

- Population at risk: 65 million in sub-Saharan Africa
- Caused by two subspecies of *Trypanosoma brucei* (*T. b.*) *gambiense* (g-HAT; 98% of reported sleeping sickness cases) and *T. b.* rhodesiense (r-HAT)
- Transmitted by the tsetse fly
- Occurs in two stages: the early stage (stage 1) with non-specific symptoms, often un- or misdiagnosed and the late stage (stage 2) where the parasite crosses the blood-brain barrier, causing serious neurological disorders including sleep cycle disruptions, neurological manifestations, and progressive mental deterioration
- Mortality without treatment: 100 %
- Economic burden: infection of livestock (nagana)

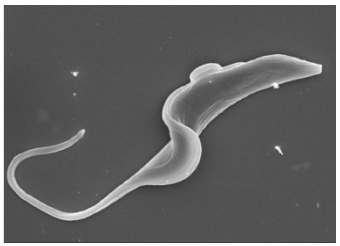
Source: https://www.dndi.org/wp-content/uploads/2017/08/Factsheet_2016_HAT.pdf

Respiration of T. brucei as target for chemotherapy

African trypanosomes adapt their energy metabolism depending on substrate availability:

- **The procyclic** form of the parasite (present in the tsetse fly vector) has a fully functional cytochrome-dependent respiratory chain.

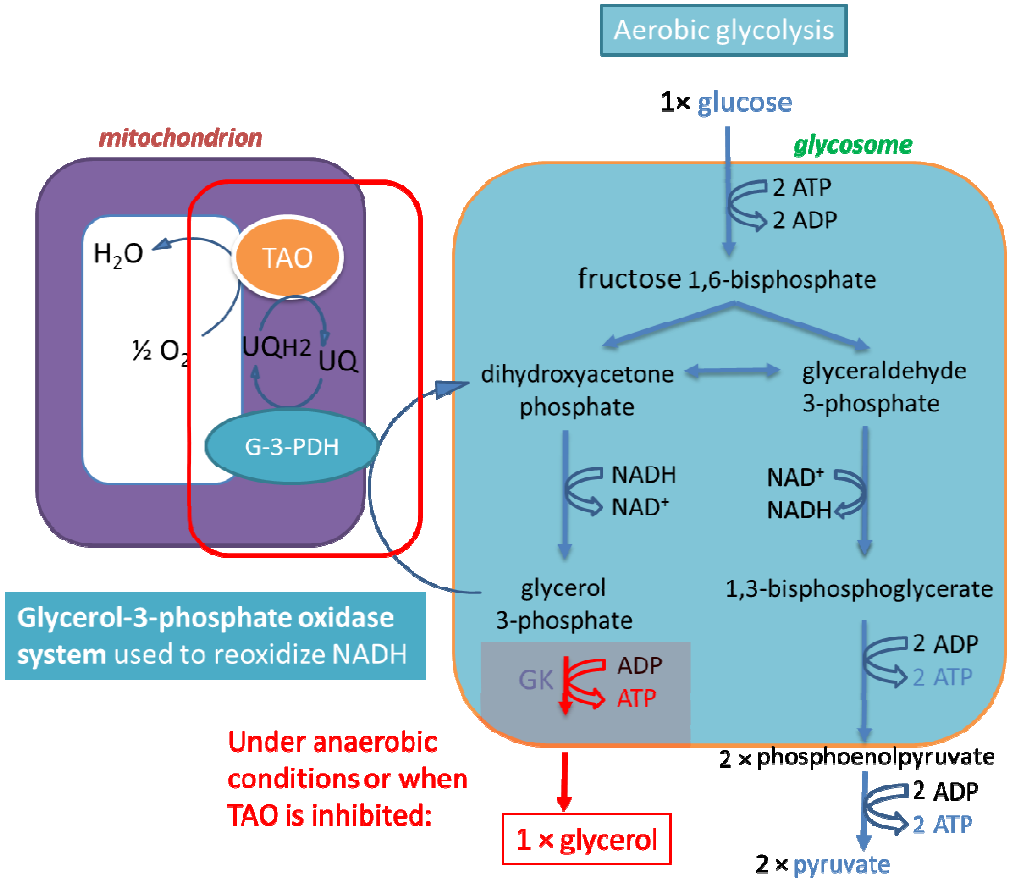
- **Bloodstream** form (BSF) trypanosomes (the human infective form) use the **glycolysis as main source of ATP**
  - ⊘ No cytochrome respiratory pathway
  - ⊘ No oxidative phosphorylation



➡ Respiration of BSF trypanosomes is dependent on a cyanide-insensitive **alternative terminal oxidase (TAO)**

Respiration of T. brucei as target for chemotherapy

- As BSF trypanosomes have no functional respiratory chain, the mitochondrial glycerol-3-phosphate oxidase system is used to re-oxidize NADH produced during glycolysis. Specifically, this system oxidizes glycerol-3-phosphate (G3P) using an electron transport system in the inner mitochondrial membrane consisting of G3P dehydrogenase, ubiquinone, and TAO. Thus, aerobic respiration leads to the net production of 2 moles of ATP and 2 moles of pyruvate per glucose molecule.



- **Under anaerobic conditions**, or in the presence of a TAO inhibitor, G3P accumulates inside the glycosome, and it is disposed off by conversion to glycerol by a reverse action of glycerol kinase (GK). This leads to the net production of 1 mole of ATP and equimolar amounts of pyruvate and glycerol.

- However, BSF trypanosomes do not survive for long time periods **under anaerobic conditions:** when glycerol accumulates in the cell, **mass action induces glycerol kinase to convert glycerol to G3P and the glycolysis stops.**

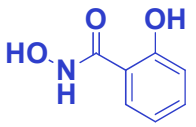
Adapted from: Chaudhuri, M. et al. *Trend Parasitol.* **2006**, *22*, 484-491

TAO is a validated target of trypanosomes:

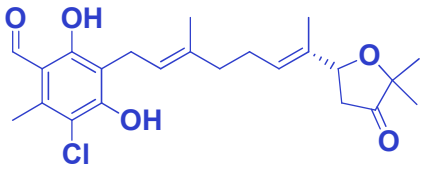
- TAO is **essential** for viability of BSF trypanosomes
- TAO is **expressed in all subspecies**
- TAO is unique (**absent in mammals**)
- TAO is **sensitive to specific inhibitors** such as salicylhydroxamic acid (SHAM)

or ascofuranone

- TAO inhibitors are **active in mouse models** of *T. brucei* infection (e.g. SHAM, ascofuranone)

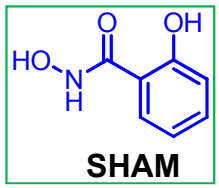


SHAM



Ascofuranone

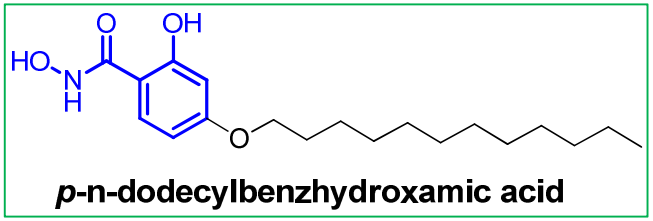
Examples of early TAO inhibitors active against T. brucei



SHAM

- K_i = 21 µM
- EC_{50} = 39 µM
- Trypanocidal without glycerol
- Glycerol needed to see a therapeutic effect in vivo

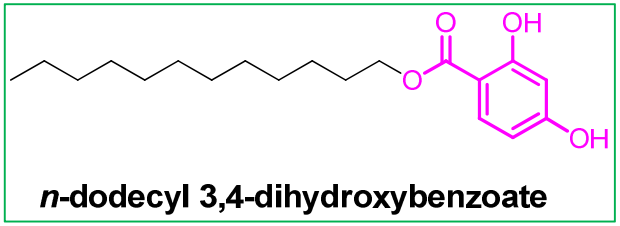
(Clarkson et al. *Mol. Biochem. Parasitol.* **1981**, 3, 271-291)



***p*-n-dodecylbenzhydroxamic acid**

- K_i = 1.1 µM
- EC_{50} = 1.5 µM (with glycerol)
- Trypanocidal in vitro when combined with glycerol
- Inactive in vivo → poor water solubility

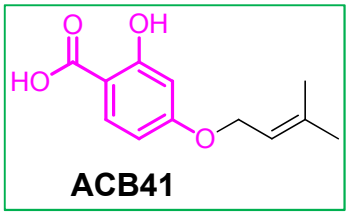
(Grady et al. *Mol. Biochem. Parasitol.* **1986**, *19*, 231-240)



***n*-dodecyl 3,4-dihydroxybenzoate**

- IC_{90} = 0.9 µM
- MIC = 1 -10 µM (10 mM glycerol)
- Reduces parasitaemia in mice when combined with glycerol

(Grady et al. *Mol. Biochem. Parasitol.* **1986**, *21*, 55-63)



ACB41

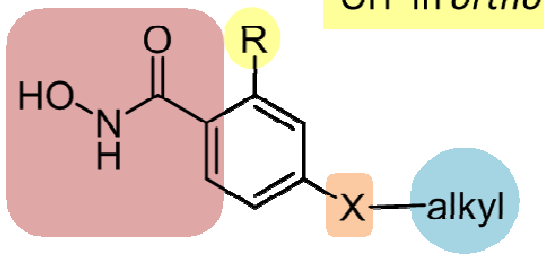
- K_i = 5 µM
- EC_{50} = 16.5 µM
- Trypanocidal without glycerol

(Ott et al. *Acta Trop.* **2006**, 172-184)

TAO inhibition: SAR of benzhydroxamic and benzoic acids reported in the literature

Hydroxamate or carboxylic acid conjugated to Ar ring

OH in *ortho* of the carbonyl group



- Lipophilic alkyl subst. is best
- n-$C_{14}H_{29}$ is best
- increasing the length of alkyl chain is not linear
- limited solubility for long alkyl chains

X = O > CONH

Grady et al. *Mol. Biochem. Parasitol.* **1986**, *19*, 231-240
Grady et al. *Mol. Biochem. Parasitol.* **1986**, *21*, 55-63

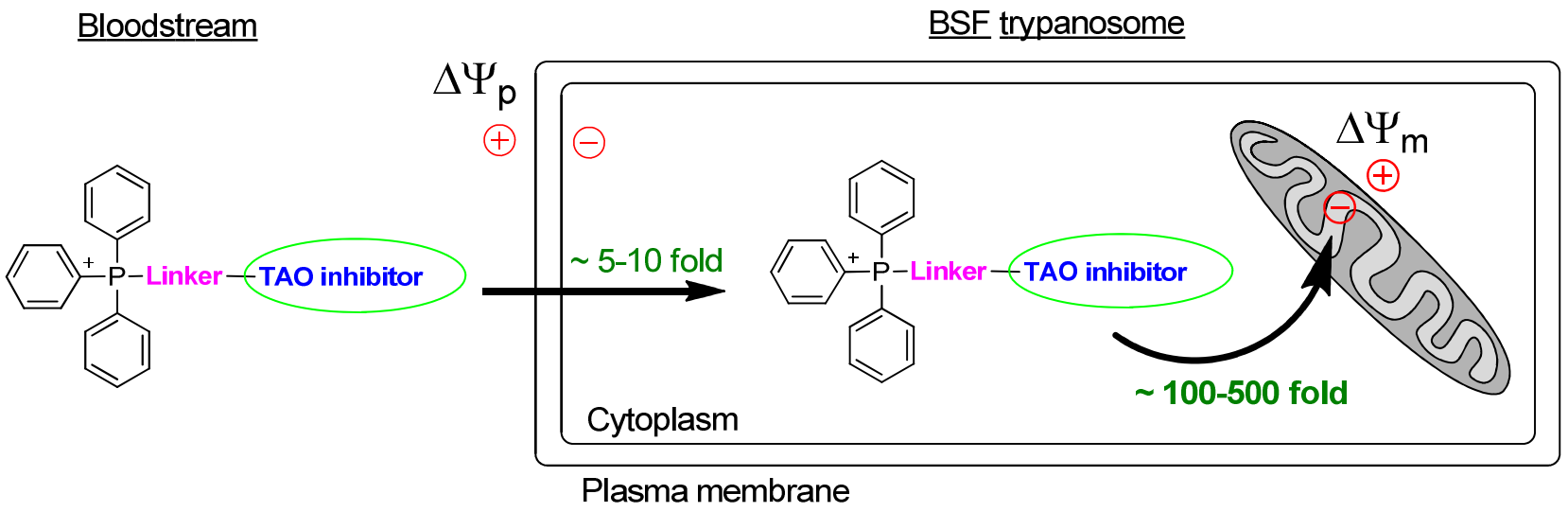
Drawbacks of these inhibitors:

- Low potency of inhibition of TAO and *T. brucei*
- Many compounds require glycerol (i.e. inhibit anaerobic pathway) to be trypanocidal
- Limited solubility

Results and discussion

Mitochondrion targeting with lipocations

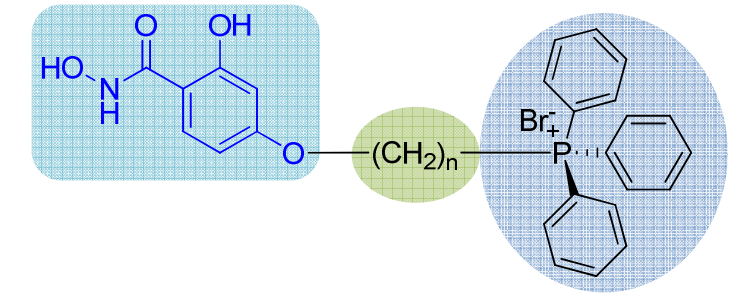
Way **to improve the potency** of the early TAO inhibitors?
TARGETING USING A LIPOPHILIC CATION
→ accumulate in the mitochondrion driven by the plasma and transmembrane potentials



Results and discussion

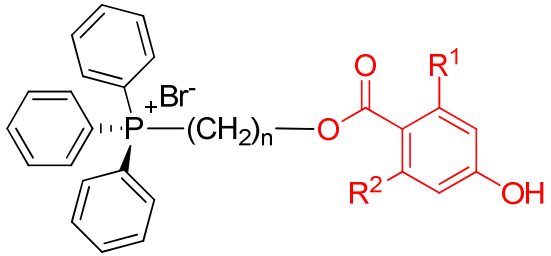
1. *Design and general structure of the SHAM, 2,4-dihydroxybenzaldehyde (DHBZ), and 2,4-dihydroxybenzoic acid (DHBA) conjugates*

SHAM-TPP conjugates:

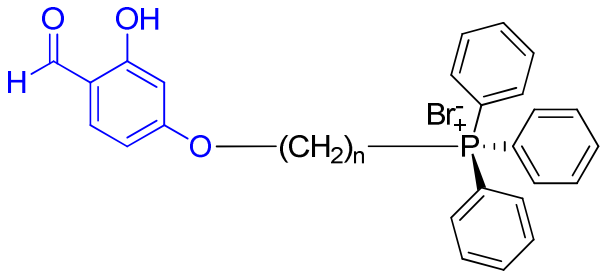


Scaffold with trypanocidal activity | Linker | Mitochondrion-targeting lipocation

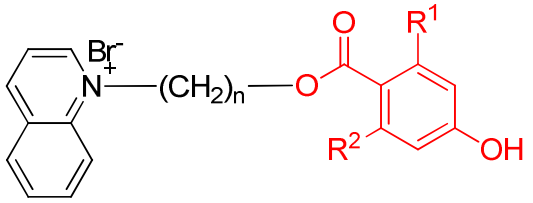
2,4-DHBA-TPP conjugates:



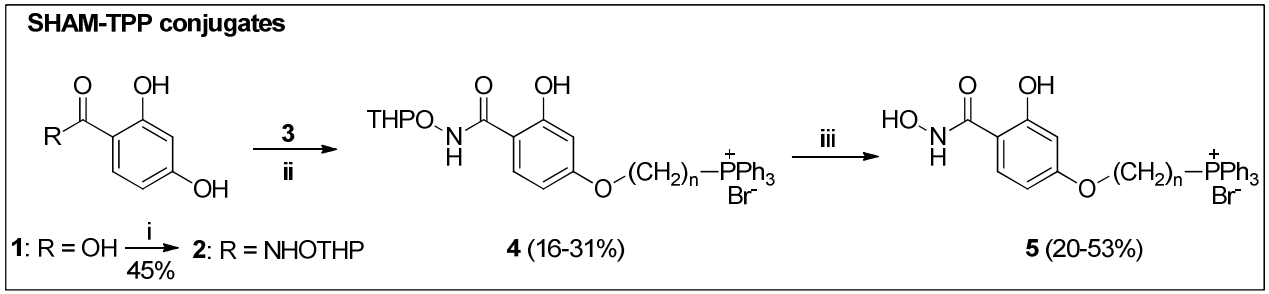
2,4-DHBZ-TPP conjugates:

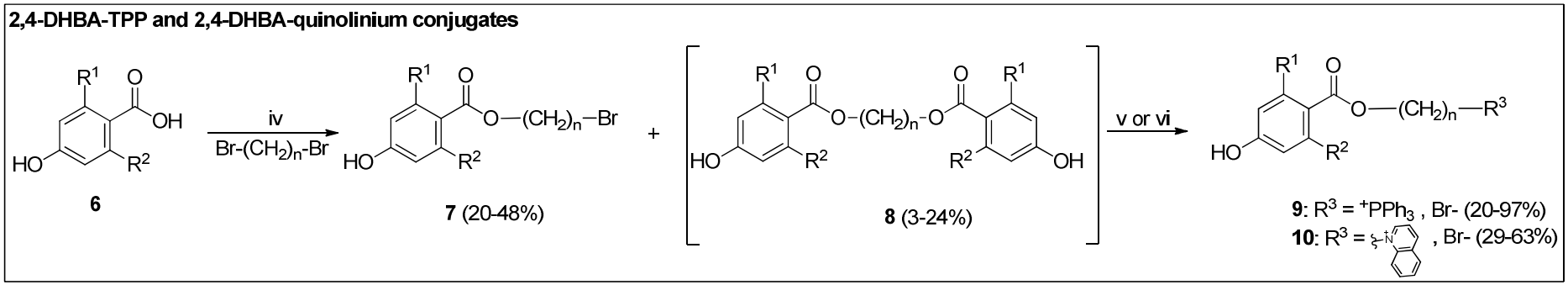


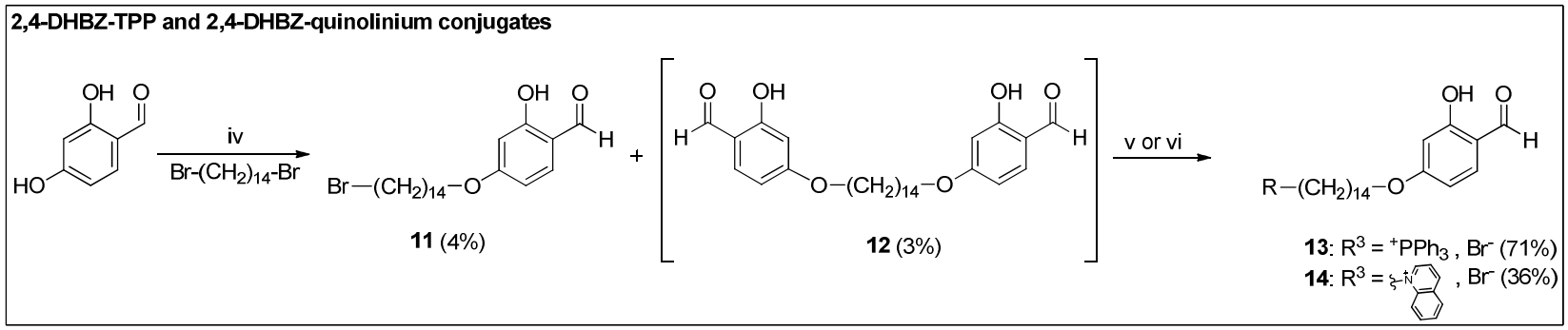
2,4-DHBA-quinolinium conjugates:



2. Synthesis of the inhibitors

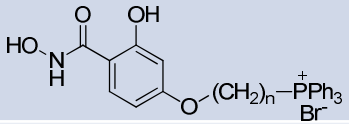
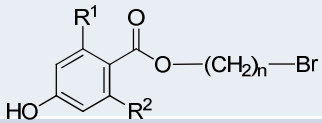
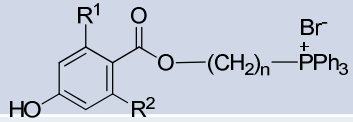
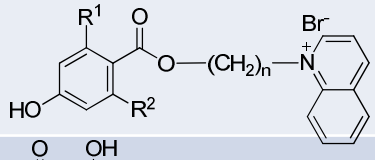
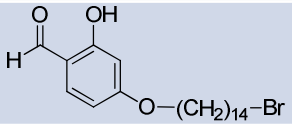
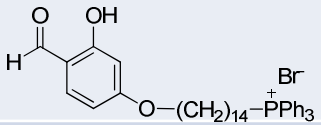
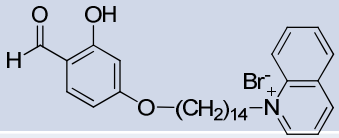






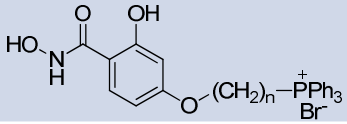
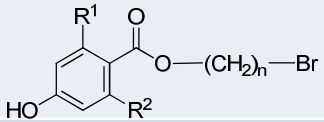
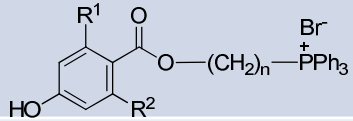
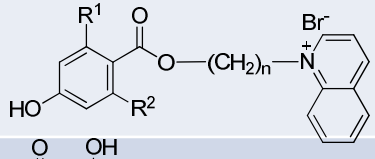
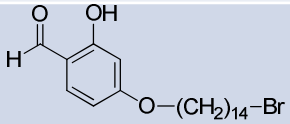
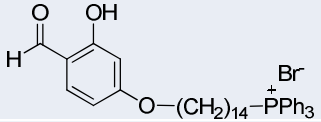
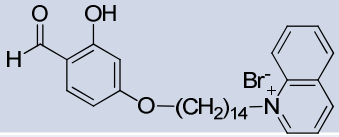
Reagents and conditions: (i) $THPONH_2$, EDC, NMM, HOBt, DMF, MWI, 120 ºC, 30 min; (ii) Br-$(CH_2)_n$-$PPh_3^+Br^-$ (**3**), $NaHCO_3$, NaI, CH_3CN, 65 ºC, 3 days; (iii) TsOH (cat.), MeOH, rt; (iv) $NaHCO_3$, CH_3CN or DMF, Δ; (v) Ph_3P, CH_3CN, 80 ºC, 10 days; (vi) quinoline, CH_3CN, 80 ºC, 10 days.

3. Inhibition of recombinant TAO enzyme

Compound series (conjugates)	Structure	*rTAO* IC_{50} (μM)
5 (SHAM-TPP)		> 5
7		0.007 – 0.45
9 (2,4-DHBA-TPP)		0.030 – 1.46
10 (2,4-DHBA-quinolinium)		0.030 – 1.36
11		0.073
13 (2,4-DHBZ-TPP)		0.22
14 (2,4-DHBZ-quinolinium)		1.23
SHAM Ascofuranone		5.93 0.002

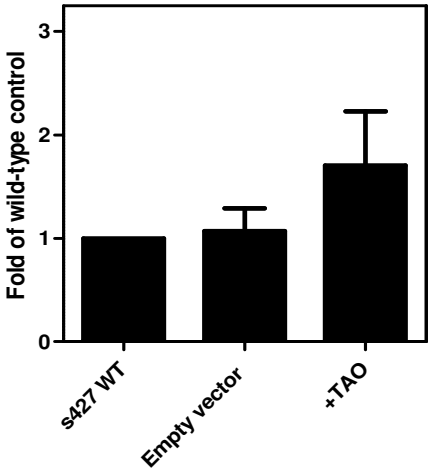
In general, the addition of a mitochondrion-targeting lipocation barely affected the inhibitory potency against rTAO, showing that the lipocation does not participate in the interaction with the binding pocket (or, at the very least, does not interfere with binding to TAO) when a C14 linker is used.

4. In vitro activity against T. b. brucei and T. congolense

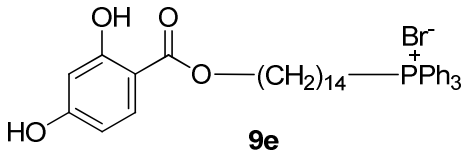
Compound series (conjugates)	Structure	*T. b. brucei* s427 (WT) EC_{50} (µM) Selectivity index (SI)	*T. congolense* EC_{50} (µM) (SI)	Cytotoxicity Human cells CC_{50} (µM)
5 (SHAM-TPP)		0.14 – 0.4 (SI > 1000)	27 – 46 (SI > 8)	>200
7		14.4 – 45.7	>50	>200
9 (2,4-DHBA-TPP)		0.0012 –0.073 (SI > 500)	0.03 – 3.9 (SI: 5 to > 3000)	>200
10 (2,4-DHBA-quinolinium)		0.14 – 0.33 (SI > 600)	3.0 – 7.3 (SI > 34)	>200
11		17.6	42.6	>200
13 (2,4-DHBZ-TPP)		0.133 (SI > 1500)	0.27 (SI > 740)	>200
14 (2,4-DHBZ-quinolinium)		1.75 (SI > 114)	2.1 (SI > 95)	>200
SHAM Pentamidine Diminazene Phenylarsine oxide		38.7 0.003 0.065 0.001	0.20	0.29

5. Mechanism of action studies

Most of these TAO inhibitors were significantly more effective in the presence of 5 mM glycerol, and against aquaglyceroporin-null trypanosomes which have glycerol efflux defects, consistent with TAO being the principal target of these inhibitors in the parasite cell.



EC_{50} values (μM) against *T. b. brucei* line overexpressing TAO



9e

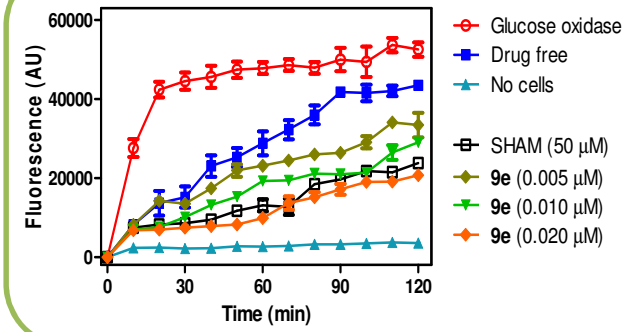
9e (2.6-fold) and SHAM (1.6-fold) were significantly less effective against TAO-overexpressing cell line vs WT

Figure 2. Expression of TAO in *T. b. brucei* trypomastigotes.
Relative levels of TAO expression were determined by qPCR in wild-type Lister 427, in the same cell line transfected with the 'empty vector' pHD1336 (no insert) and with the TAO open reading frame in pHD1336. Average and SEM of 3 determinations.

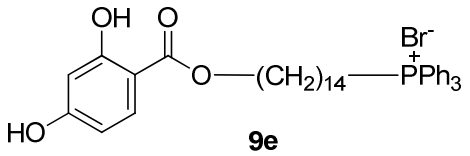
Fueyo González et al. *J. Med. Chem.* **2017**, *60*, 1509-1522

5. Mechanism of action studies

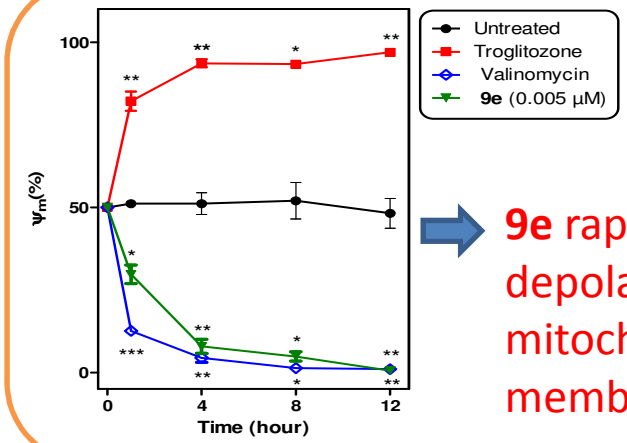
Effect of **9e** on **oxygen consumption**, **mitochondrial membrane potential** and **cell cycle** in *T. b. brucei* WT



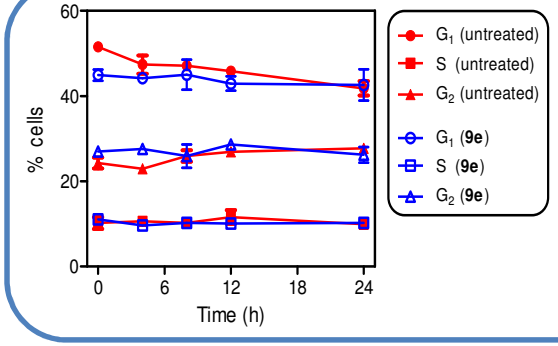
9e affects the respiration of BSF trypanosomes.
9e and SHAM dose-dependently reduced the rate of oxygen consumption by trypanosomes.



9e



9e rapidly depolarized the mitochondrial membrane



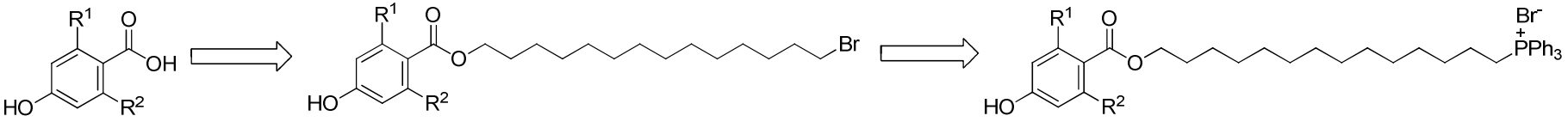
9e did not inhibit progression through the cell cycle.

Fueyo González et al. *J. Med. Chem.* **2017**, *60*, 1509-1522

Conclusions

We have successfully developed a class of potent and selective new hits active against human (*T. brucei* spp.) and veterinary (*T. congolense*) African trypanosomes, and established their probable mode of action via TAO inhibition. This was accomplished by efficiently targeting the compounds to the trypanosome's mitochondrion, thereby increasing the potency of the original small molecule inhibitors against *T. brucei* by up to 3 orders of magnitude.

- ❑ Attaching a Lipophilic Cation to a TAO inhibitor using a 14-methylene linker:
  - ✓ nanomolar trypanocidal activity
  - ✓ not detrimental to inhibition of TAO
- ❑ The 2,4-DHBA-TPP conjugates are the most potent and selective against *T. brucei*
- ❑ Metabolic stability in serum depends on R^1 and R^2 → candidates for in vivo studies have been selected.



6

IC_{50} = 112 - 143 μM (rTAO)
EC_{50} > 15 μM (*T.b.b.*)

7

IC_{50} = 0.007 - 0.030 μM (rTAO)
EC_{50} = 14.5 μM (*T.b.b.*)
SI > 13

9

IC_{50} = 0.030 - 0.088 μM (rTAO)
EC_{50} = 0.0006 - 0.0024 μM (*T.b.b.*)
SI > 83,000

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