



University of Dundee

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Menzies, Stefanie K.; Tulloch, Lindsay B.; Florence, Gordon J.; Smith, Terry K.

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2 TARGET?

3
4 Authors: Stefanie K Menzies, Lindsay B Tulloch, Gordon J Florence and Terry K
5 Smith

6
7 Postal Address: Biomedical Sciences Research Complex, University of St Andrews,
8 St Andrews, Fife, Scotland KY16 9ST

9
10 Running Title: TAO: A Potential Drug Target?

11
12 Corresponding author: Terry K Smith, Biomedical Sciences Research Complex,
13 University of St Andrews, St Andrews, Fife, Scotland KY16 9ST 01334 463412

14 tk1@st-andrews.ac.uk

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Summary

New drugs against *Trypanosoma brucei*, the causative agent of Human African Trypanosomiasis, are urgently needed to replace the highly toxic and largely ineffective therapies currently used. The trypanosome alternative oxidase (TAO) is an essential and unique mitochondrial protein in these parasites and is absent from mammalian mitochondria, making it an attractive drug target. The structure and function of the protein are now well characterized, with several inhibitors reported in the literature which show potential as clinical drug candidates. In this review we provide an update on the functional activity and structural aspects of TAO. We then discuss TAO inhibitors reported to date, problems encountered with *in vivo* testing of these compounds, and discuss the future of TAO as a therapeutic target.

Key Words: Trypanosome Alternative Oxidase, Drug Discovery, Chemotherapy, Human African Trypanosomiasis, Sleeping Sickness, Trypanosoma brucei

48 THE TRYPANOSOME ALTERNATIVE OXIDASE: A POTENTIAL DRUG TARGET?

49

50 INTRODUCTION

51 Up to 70 million people in sub-Saharan Africa are at risk of contracting Human
52 African Trypanosomiasis (HAT) (Simarro *et al.* 2012), also known as African
53 Sleeping Sickness, caused by the kinetoplastid parasite *Trypanosoma brucei*. Two
54 subspecies of the parasite cause disease in humans; *T. brucei gambiense* in West
55 Africa and *T. brucei rhodesiense* in East Africa, both of which are spread by the
56 tsetse fly. Both forms are fatal if untreated and are estimated to cause up to 20,000
57 cases of HAT per year (World Health Organization 2013). *T. brucei* evades the
58 mammalian host immune system by changing their major surface coat proteins,
59 known as variant surface glycoproteins (VSG), prior to each wave of host antibodies
60 raised against the previous VSG type. Due to this sophisticated immune evasion
61 technique known as antigenic variation, a vaccine against the disease is unlikely in
62 the near future. Drugs currently in clinical use are associated with severe adverse
63 effects, difficult administration, and increasing concerns regarding drug resistance.
64 Therefore, new drugs are urgently required (Lüscher *et al.* 2007). The drugs
65 indicated for treatment of the disease (Figure 1) depend upon the subspecies of
66 parasite and stage of the disease.

67

68 Early stage *T. b. gambiense* is treated with pentamidine, a diamidine hypothesized to
69 act as a trypanocidal agent through several mechanisms, including disruption of the
70 nucleus, kinetoplast and mitochondrial membrane potential (Baker *et al.* 2013). Late
71 stage *T. b. gambiense* is treated with a combinational therapy of nifurtimox and
72 eflornithine. Eflornithine is the only drug for HAT with a defined target, the ornithine

73 decarboxylase, but the drug has poor potency against *T. brucei* and combination
74 therapy is required to prevent drug resistance acquired by loss of the drug uptake
75 transporter (Barrett & Croft 2012). Suramin is recommended only for early stage *T.*
76 *b. rhodesiense* due to its inability to penetrate the blood brain barrier. Although the
77 mechanism of uptake by the parasites is known, the trypanocidal mode of action still
78 remains to be determined (Barrett & Croft 2012, Zoltner *et al.* 2016). The arsenical-
79 based drug melarsoprol is recommended for late stage *T. b. rhodesiense* due to its
80 ability to cross the blood brain barrier, however this property creates the often fatal
81 adverse effect of encephalopathy in up to 10% of patients treated with the drug
82 (Kuepfer *et al.* 2012).

83 Differences in the biochemical processes between mammalian and trypanosomatid
84 mitochondria make the mitochondrion an attractive drug target. One main difference
85 between *T. brucei* and mammalian mitochondrial respiration is the presence of the
86 trypanosome alternative oxidase (TAO), an essential non-cytochrome terminal
87 oxidase which has been extensively characterized as a drug target. This review will
88 summarize the structure and function of TAO and discuss the current progress
89 towards the development of inhibitors against this protein.

90

91 STRUCTURE AND FUNCTION OF THE TRYPANOSOME ALTERNATIVE 92 OXIDASE

93 *Function*

94 In 1960, Grant and Sargent first described the glycerol-3-phosphate oxidase (GPO)
95 system as a cyanide-insensitive, oxygen-dependent mechanism of respiration in
96 *Trypanosoma brucei rhodesiense* (Grant & Sargent 1960). The GPO system
97 consists of two enzymes; a mitochondrial FAD⁺-dependent glycerol-3-phosphate

98 dehydrogenase (mG3PDH) and a terminal oxidase they termed the glycerol-3-
99 phosphate oxidase. Clarkson et al (Clarkson *et al.* 1989) proved that ubiquinol links
100 the dehydrogenase and oxidase of the GPO system by acting as an electron carrier,
101 and proposed that glycerol-3-phosphate oxidase was similar to the plant alternative
102 oxidase (AOX) and therefore should be renamed the trypanosome alternative
103 oxidase (TAO). The GPO system is responsible for the cyanide-insensitive oxygen-
104 dependent respiration in bloodstream form *T. brucei*, where the GPO shuttle
105 facilitates the reoxidation of NADH to NAD⁺ required for glycolysis. As shown in
106 Figure 2, the mG3PDH oxidizes glycerol-3-phosphate (Gly-3-P) to dihydroxyacetone
107 phosphate (DHAP), during which four electrons are transferred to ubiquinol. The
108 electrons from ubiquinol are subsequently oxidized by TAO to convert dioxygen into
109 water. Alternative oxidases are found across a broad range of organisms, including
110 plants, nematodes, algae, yeast and *T. brucei*, but, curiously, are not known to be
111 present in the other human-infective trypanosomatids such as *T. cruzi* or *Leishmania*
112 spp.

113 TAO was first identified in *Trypanosoma brucei* by Chaudhuri et al (Chaudhuri *et al.*
114 1995) using antibodies against the alternative oxidase from *Sauromatum guttatum*,
115 which detected a 33 kDa protein in the parasite's mitochondria. This 33 kDa protein
116 was subsequently purified from bloodstream form *T. brucei* mitochondria and
117 confirmed to have ubiquinol oxidase activity. Chaudhuri et al (Chaudhuri *et al.*
118 1998) found that bloodstream form *T. brucei* express TAO 100-fold more than
119 procyclic form, which is believed to be due to the ability of procyclic forms to express
120 Complexes III and IV for ATP production via oxidative phosphorylation. Using areas
121 of high conservation in plant alternative oxidases, primers were designed to amplify
122 TAO from *T. brucei* gDNA (Chaudhuri & Hill 1996). This enabled the identification of

123 the single copy TAO gene (Tb927.10.7090) and subsequent cloning of TAO for
124 recombinant expression in *Escherichia coli* (Chaudhuri & Hill 1996). Recombinant
125 TAO (rTAO) was subsequently used to determine the functional activity, kinetics and
126 inhibitors of the enzyme. Due to the endogenous ubiquinol oxidase activity of *E. coli*
127 by the cytochrome bo and bd complexes, it was necessary to perform these
128 investigations using hemA mutant *E. coli*, which are unable to synthesize the heme
129 necessary for cytochrome assembly. The ability of rTAO to restore respiration in
130 these cells showed the ability of TAO to function as a cyanide-insensitive terminal
131 oxidase. Research of rTAO by the Kita group established protocols for the
132 overproduction, solubilization and purification of rTAO for use in kinetic, structural
133 and inhibitor studies (Fukai *et al.* 1999; Fukai *et al.* 2003; Nihei *et al.* 2003; Yabu *et*
134 *al.* 2003).

135 TAO has been implicated in several other cellular activities, such as protection
136 against reactive oxygen species and regulation of surface protein expression. A role
137 of AOX in photosynthetic plants is the rapid turnover of NADPH to protect the
138 photosynthetic machinery from radicals. It is possible that TAO has a related
139 function in *T. brucei*, to protect the rapidly metabolizing cells from damaging radicals.
140 The inhibition of TAO has been shown to induce oxidative damage to proteins and
141 increase production of reactive oxygen species (Fang & Beattie 2003). Similarly,
142 inhibition of the electron transport chain and exposure to hydrogen peroxide causes
143 an upregulation in the expression of TAO (Fang & Beattie 2003). This protection
144 against oxidative damage may explain the ability of TAO to inhibit drug-induced
145 programmed cell death-like phenomena in *T. brucei* (Tsuda *et al.* 2006). Vassella *et*
146 *al.* (Vassella *et al.* 2004) reported the effects of TAO inhibition on the expression
147 levels of the procyclin GPEET, a cell surface protein found in procyclic form *T.*

148 *brucei*. In the presence of the TAO inhibitor salicylhydroxamic acid (SHAM), GPEET
149 levels were heavily reduced, leading the authors to hypothesize that the level of
150 GPEET expression may be linked to the activity levels of TAO. Later studies
151 showed that the expression of TAO influences the expression of GPEET, where a
152 downregulation of both proteins may be important in the adaptation of the parasite to
153 survive within the tsetse fly midgut (Walker *et al.* 2005).

154

155 *Structure*

156 Several structures of the alternative oxidase were proposed (Andersson & Nordlund
157 1999; Berthold *et al.* 2000) prior to the publication of the crystal structure. Initially,
158 hydropathy plots suggested the alternative oxidases contain two conserved
159 transmembrane regions, however later studies by Andersson and Nordlund
160 (Andersson & Nordlund 1999) suggested alternative oxidases are not
161 transmembrane proteins, but rather interfacial inner membrane proteins. This was
162 confirmed with the solving of the crystal structure of TAO (Shiba *et al.* 2013) which is
163 devoid of any transmembrane domains, and instead has a hydrophobic face to
164 partially bury the protein into the membrane (Figure 3). The recent publication of
165 the crystal structure (Shiba *et al.* 2013) should help in the design of improved TAO
166 inhibitors. Sequence analysis of *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*
167 showed that the amino acid sequence of TAO is identical in all three species, and
168 therefore studies on TAO inhibitors and its co-structures can be directly applied from
169 the common laboratory model *T. b. brucei* to the human disease-causing subspecies
170 (Nakamura *et al.* 2010).

171 Early studies on plant alternative oxidases revealed that they were inhibited by metal
172 chelators (Schonbaum *et al.* 1971), and subsequent investigations using electron

173 paramagnetic resonance (EPR) (Berthold *et al.* 2002; Moore *et al.* 2008) and
174 inductively coupled plasma-mass spectrometry (ICP-MS) (Kido *et al.* 2010) showed
175 that the alternative oxidases contained a non-heme diiron catalytic core that was
176 essential for catalytic activity and released during enzyme inactivation. In the crystal
177 structure in its oxidized state, the two Fe (III) ions are coordinated in a distorted
178 square pyramidal geometry to four glutamate residues and a hydroxo-bridge (Figure
179 4). Two conserved histidines located nearby are also likely to be involved in Fe-
180 coordination in the reduced state, as determined through Fourier transform infrared
181 spectroscopy (FTIR) investigations (Marechal *et al.* 2009). Together, the two
182 histidines and two glutamates form part of the two ExxH iron-binding motifs which
183 are common to all AOX proteins and are required for activity (Chaudhuri *et al.* 1998;
184 Ajayi *et al.* 2002).

185

186 *Mechanism of Catalysis*

187 The structure of TAO in complex with ubiquinol has not yet been solved so
188 hypotheses regarding ubiquinol-binding have been made based upon the structure
189 of TAO complexed with ascofuranone analog AF2779OH. Superposition of ubiquinol
190 over AF2779OH indicates that during catalysis a ubiquinol molecule is highly likely to
191 occupy the same position. The molecules gain entry to the diiron active site through
192 a relatively short (~10 Å) hydrophobic channel from the membrane-bound side of
193 TAO (Figure 4). In this position the aromatic head of ubiquinol is less than 4.4 Å
194 from the diiron core and is capable of forming hydrogen bonds with Arg118, Cys119
195 and Tyr220, all of which may be involved directly in catalysis rather than purely
196 substrate binding.

197 A mechanism of catalytic activity has been proposed (Moore *et al.* 2013 and Figure
198 5), which begins with the diiron core in a reduced state (i.e. as Fe(II/II) bridged by a
199 hydroxide). Upon binding of molecular oxygen to the Fe(II/II) diiron core (Figure 5A),
200 one iron passes an electron to an oxygen atom, forming a superoxo intermediate
201 comprising an oxygen radical joined to an Fe(II/III) core. The oxygen radical
202 immediately abstracts a hydrogen atom (proton plus electron) from ubiquinol,
203 yielding a ubisemiquinone and a hydroperoxo intermediate (Figure 5B). The unstable
204 intermediate then undergoes a rearrangement whereby the hydroperoxo loses its
205 proton and electron to the hydroperoxide bridge, which is then released as water
206 (Figure 5C). The Fe(II/III) core then gains an interaction with one of the histidines as
207 determined in FTIR experiments (Marechal *et al.* 2009) and the second atom of the
208 dioxygen forming a peroxodiiron. Homolytic cleavage of the O-O bond (Figure 5D)
209 yields an oxodiiron core, and one of the oxygens abstracts a hydrogen atom (proton
210 plus electron) from Tyr220 generating a tyrosyl radical, as observed by Marechal *et*
211 *al.* (Marechal *et al.* 2009). The tyrosyl can then pick up an electron and proton from
212 the ubisemiquinone, either directly or via Cys119, releasing ubiquinone and returning
213 Tyr220 to its resting state. Moore's model suggests that ubiquinol in a second
214 channel can then provide two electrons and protons to release a second water and
215 reduce the diiron core back to its original Fe(II/II) state bridged by a hydroxide ion
216 through an unknown mechanism (Figure 5E). However, the second ubiquinol
217 channel may not be needed as the release of ubiquinone creates the space for the
218 binding of a second ubiquinol in the same channel in a ping-pong binding fashion.
219 Furthermore, the mechanism of electron and proton transfer could proceed through a
220 similar route as for the first ubiquinol.

221

222 INHIBITORS OF THE TRYPANOSOME ALTERNATIVE OXIDASE

223 The effectiveness of TAO inhibition to kill *T. brucei* has been well debated, with
224 conflicting historical reports in the literature as to whether the inhibition of the GPO
225 system alone is sufficient to kill the cells. As shown in Figure 2, bloodstream form *T.*
226 *brucei* rely solely on glycolysis for ATP production, as opposed to the ATP-producing
227 oxidative phosphorylation used by procyclic forms. In the presence of TAO inhibitors
228 the oxidation of Gly-3-P to DHAP is blocked, causing an accumulation of Gly-3-P in
229 the glycosome, which is converted to glycerol by the ATP-producing glycerol kinase
230 (Yabu *et al.* 2006). This allows the recycling of glycosomal NAD⁺/NADH necessary
231 to continue glycolysis anaerobically.

232 Early reports of *in vivo* testing of TAO inhibitors suggested that although the
233 compounds were able to inhibit the protein *in vitro*, this action alone was not
234 sufficient to clear an infection when tested in animal models, due to anaerobic ATP
235 production by the trypanosomes (Clarkson & Brohn 1976; Grady *et al.* 1993; Yabu *et*
236 *al.* 1998). It was believed that in order to cause cell death the anaerobic production
237 of ATP also needed to be inhibited with the co-administration of glycerol. However,
238 later investigations showed that bloodstream *T. brucei* exposed to TAO inhibitors
239 alone are unable to survive for more than 24 hours using only anaerobic respiration
240 (Helfert *et al.* 2001). Furthermore, subsequent studies of a TAO inhibitor with an
241 optimized dosing regimen but in the absence of glycerol, showed that TAO inhibition
242 alone is sufficient to clear an infection *in vivo* (Yabu *et al.* 2003), indicating that
243 inhibition of TAO is indeed a valid drug target.

244 There are few compounds that have been shown to be inhibitors of TAO. These
245 compounds (Figure 6) all show structural similarity to the TAO substrate ubiquinol
246 and are thought to act as competitive inhibitors, by binding to the ubiquinol binding

247 site.

248

249 *Salicylhydroxamic Acid*

250 The first compounds to be investigated as TAO inhibitors were the aromatic
251 hydroxamates, such a salicylhydroxamic acid (SHAM) (Figure 6). SHAM was known
252 to be a potent inhibitor of the alternative oxidase in plants prior to the discovery of
253 the GPO system in trypanosomes, hence the compound was investigated as a
254 potential inhibitor of TAO. It is thought that hydroxamic acids compete with ubiquinol
255 for binding to TAO, and thus the compounds prevent the translocation of electrons
256 from ubiquinol to oxygen (Pollakis *et al.* 1995). SHAM was found to have moderate
257 ($EC_{50} = 15 \mu\text{M}$) activity against *T. brucei in vitro* and was shown to specifically inhibit
258 all TAO activity at 1 mM (Opperdoes *et al.* 1976), although only a little effect was
259 seen on ATP production. However, when the trypanocidal effect of SHAM was
260 investigated *in vivo*, the compound was unable to clear an infection and was only
261 shown to be trypanocidal when co-administered with glycerol (Clarkson & Brohn
262 1976).

263 SHAM is a poor clinical candidate, due to its low solubility in water (Nihei *et al.*
264 2002), which impairs the compounds from crossing the blood brain barrier, a critical
265 characteristic required for drugs to effectively treat HAT. Numerous attempts were
266 made to improve the potency of hydroxamic acids against TAO, but were unable to
267 match the potency of SHAM when tested *in vivo* (Grady *et al.* 1993). Recently this
268 issue has been revisited, Ott et al (Ott *et al.* 2006) developed novel SHAM analogs
269 to improve its potency and solubility. SHAM analogs such as ACD16 (Figure 6) were
270 designed to include a prenyl side chain, as found in the TAO substrate ubiquinol, and
271 a carbohydrate group to improve solubility, whilst keeping the 2-hydroxybenzoic acid

272 found in SHAM which is essential for TAO inhibition. These modifications lead to the
273 development of three compounds with up to five-fold greater potency than SHAM
274 against rTAO, however *in vitro* testing against *T. b. brucei* growth and respiration
275 revealed none of the modified compounds were more potent than SHAM. There
276 have been no subsequent reports on SHAM as a TAO inhibitor, although recent
277 reports on the efficacy of TAO inhibitors without glycerol (Yabu et al. 2003) may
278 renew interest in attempts to improve upon this compound.

279

280 *3,4-Dihydroxybenzoic Acid*

281 3,4-dihydroxybenzoic acids (Figure 6) were synthesized and tested as alternative
282 inhibitors of TAO, and displayed higher inhibitory activity than SHAM when tested *in*
283 *vitro*, but this high potency was lost when the compounds were tested *in vivo* (Grady
284 *et al.* 1993). To improve the bioavailability of the compounds, a series of *N-n*-alkyl-
285 3,4-dihydroxybenzamides were synthesized to increase solubility and decrease
286 hydrolysis by serum esterases (Grady *et al.* 1993). Structure activity relationships of
287 this series of compounds showed increasing potency and decreasing solubility as
288 the length of the alkyl substituent increases. From this, *N-n*-butyl-3,4-
289 dihydroxybenzamide progressed to *in vivo* studies, and was found to effectively cure
290 mice, but only when administered in conjunction with high doses of glycerol (450
291 mg/kg drug with 15 g/kg glycerol). Similar to SHAM, the high amount of glycerol
292 necessary for a trypanocidal effect of *N-n*-butyl-3,4-dihydroxybenzamide rendered
293 the compound unfavourable as a clinical drug candidate, and no work has been
294 undertaken to identify if an optimized dosing regimen might clear infection *in vivo*
295 without glycerol.

296

297 *Ascofuranone*

298 Ascofuranone (Figure 6), is a biologically active natural product isolated from the
299 fungus *Ascochyta viciae*. Minagawa et al first showed that ascofuranone is a potent
300 inhibitor of mitochondrial respiration of *T. b. brucei*, specifically the glucose- and
301 glycerol-3-phosphate-dependent respiration (Minagawa et al. 1997). Despite its high
302 potency against TAO, ascofuranone was initially found to only be trypanocidal in the
303 presence of glycerol, similar to the other TAO inhibitors. The minimum inhibitory
304 concentration of ascofuranone alone was 250 μ M, whereas in the presence of 4 mM
305 glycerol potency was improved several thousand-fold to 30 nM (Minagawa et al.
306 1997). Initially *in vivo* testing using mouse models found that ascofuranone was only
307 curative when co-administered with a large amount (3 g/kg) of glycerol (Yabu et al.
308 1998). Despite these less than favourable initial results, the dosage of ascofuranone
309 was improved to once again render it a promising clinical drug candidate. Yabu et al
310 (Yabu et al. 2003) trialled the optimal dosage to cure *T. b. brucei* mice without
311 glycerol and determined that 100 mg/kg intraperitoneally for 4 days and 400 mg/kg
312 orally for 8 days completely cleared an infection, with a 50% lethal dose (LD₅₀) of
313 >1.2 g/kg over 8 days. This study also provided evidence of the effects of
314 ascofuranone treatment on TAO, finding that ascofuranone decreased TAO activity
315 by 30% and increased the level of TAO expression within the cells (Yabu et al.
316 2003).

317 Ascofuranone was also shown to inhibit the TAO of *T. vivax*, which causes animal
318 trypanosomiasis (Nagana) in cattle. The *T. vivax* TAO has 76% identical amino acid
319 residues to *T. brucei* TAO (Suzuki et al. 2004) and the recombinant protein was
320 shown to be three-fold more sensitive to ascofuranone. Subsequent *in vivo* testing
321 of ascofuranone in *T. vivax* infected mice found that a single intramuscular dose of

322 50 mg/kg ascofuranone without glycerol was sufficient to clear an infection, which
323 could be reduced still further to 6 mg/kg over 4 days whilst retaining 100% cure rate
324 within 48 hours. The high efficacy of ascofuranone against *T. vivax* may make this
325 compound a suitable drug for use against animal trypanosomiasis.

326 Kinetic analysis of ascofuranone inhibition of rTAO indicated a competitive
327 mechanism of inhibition against ubiquinol (Nihei *et al.* 2003). Recent studies of
328 ascofuranone have revealed the mechanism of inhibition, interaction with TAO and
329 the pharmacophore responsible for the inhibitory activity of ascofuranone (Saimoto
330 *et al.* 2013). The length of the linker chain between the aromatic ring and furanone
331 ring was shown to be important for its inhibitory activity, where the potency of
332 inhibitor with a propyl linker was a thousand-fold lower compared to nonyl and decyl
333 linkers. This is likely due to the interactions between the prenyl tail and membrane
334 lipid bilayers, where hydrophobicity of the inhibitor is influenced by the length of the
335 prenyl tail, which is important to access the membrane-associated TAO (Mogi *et al.*
336 2009; Saimoto *et al.* 2013). Attempts to improve the potency and selectivity of
337 ascofuranone-like analogs have been reported, such as the prenylphenol LL-Z1272
338 series by Mogi *et al.* (Mogi *et al.* 2009) (Figure 6), although no results from *in vivo*
339 testing have been reported to date.

340

341 *Aurachin D*

342 Recently the natural product Aurachin D (Figure 6), a ubiquinol oxidase inhibitor
343 isolated from the bacterium *Stigmatella aurantiaca* strain Sg a15, was shown to have
344 inhibitory activity against *T. b. gambiense* (Li *et al.* 2013). Aurachin D is a mimic of
345 ubiquinol, with a quinolone core and prenyl chain. Li *et al.* (Li *et al.* 2013) found that
346 Aurachin D inhibited *T. b. gambiense* with an IC₅₀ of 1 μM, with a selectivity index

347 greater than 35. Various analogs of Aurachin D were synthesized and tested for
348 trypanocidal activity, but none were improved compared to the natural product and
349 hence the compound has not been taken forward into animal models.

350

351 CONCLUSIONS AND FUTURE PERSPECTIVES

352 Although drugs against TAO have been studied for over 40 years, there are still no
353 drug candidates approaching clinical trials. The search for an effective TAO inhibitor
354 has been hampered until recently by the difficulty in obtaining a crystal structure of
355 the relatively unstable purified protein, and the historical conflicting reports on
356 whether inhibition of TAO alone is sufficient to kill *T. brucei in vivo*. However, recent
357 evidence renews the idea of TAO as a valid drug target. Although there are few
358 inhibitors of TAO reported in the literature, it is hoped that the publication of the
359 crystal structure of TAO will significantly improve the design of novel, potent
360 inhibitors against the enzyme. Further work is also still required to confirm the
361 mechanism of electron transfer by TAO and that ubiquinol is the true native co-
362 factor.

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370 References

371 **Ajayi, W., Chaudhuri, M. and Hill, G.C.** 2002. "Site-Directed Mutagenesis Reveals
372 the Essentiality of the Conserved Residues in the Putative Diiron Active Site of the
373 Trypanosome Alternative Oxidase." *Journal of Biological Chemistry* 277 (10): 8187–
374 93. doi:10.1074/jbc.M111477200.

375

376 **Andersson, M.E. and Nordlund, P.** 1999. "A Revised Model of the Active Site of
377 Alternative Oxidase." *FEBS Letters* 449: 17–22. doi:S0014-5793(99)00376-2 [pii].

378

379 **Baker, N., de Koning, H.P., Mäser, P. and Horn, D.** 2013. "Drug Resistance in
380 African Trypanosomiasis: The Melarsoprol and Pentamidine Story." *Trends in*
381 *Parasitology* 29 (3): 110–18. doi:10.1016/j.pt.2012.12.005.

382

383 **Barrett, M.P., and Croft, S.L.** 2012. "Management of Trypanosomiasis and
384 Leishmaniasis." *British Medical Bulletin* 104 (1): 175–96. doi:10.1093/bmb/lds031.

385

386 **Berthold, D.A., Andersson, M.E. and Nordlund, P.** 2000. "New Insight into the
387 Structure and Function of the Alternative Oxidase." *Biochimica et Biophysica Acta*
388 1460 (2–3): 241–54. doi:10.1016/S0005-2728(00)00149-3.

389

390 **Berthold, D.A., Voevodskaya, N., Stenmark, P., Gräslund, A. and Nordlund, P.**
391 2002. "EPR Studies of the Mitochondrial Alternative Oxidase: Evidence for a Diiron
392 Carboxylate Center." *Journal of Biological Chemistry* 277 (46): 43608–14.
393 doi:10.1074/jbc.M206724200.

394

395 **Bringaud, F., Barrett, M.P. and Zilberstein, D.** 2012. "Multiple Roles of Proline
396 Transport and Metabolism in Trypanosomatids." *Frontiers in Bioscience* 17: 349–74.
397

398 **Chaudhuri, M., Wilfred A. and Hill, G.C..** 1998. "Biochemical and Molecular
399 Properties of the Trypanosoma Brucei Alternative Oxidase." *Molecular and*
400 *Biochemical Parasitology* 95: 53–68.
401

402 **Chaudhuri, M., Wilfred, A., Temple, S. and Hill, G.C.** 1995. "Identification and
403 Partial Purification of a Stage-Specific 33 kDa Mitochondrial Protein as the
404 Alternative Oxidase of the Trypanosoma Brucei Brucei Bloodstream
405 Trypomastigotes." *The Journal of Eukaryotic Microbiology* 42 (5): 467–72.
406

407 **Chaudhuri, M., and Hill, G.C.** 1996. "Cloning, Sequencing and Functional Activity of
408 the Trypanosoma Brucei Brucei Alternative Oxidase." *Molecular and Biochemical*
409 *Parasitology* 83: 125–29.
410

411 **Clarkson, A.B. and Brohn, F.H.** 1976. "Trypanosomiasis: An Approach to
412 Chemotherapy by the Inhibition of Carbohydrate Catabolism." *Science (New York,*
413 *N.Y.)* 194 (4261): 204–6. doi:10.1126/science.986688.
414

415 **Clarkson, A.B., Bienensb, E.J., Pollakisz, G. and Gradyll, W.** 1989. "Respiration
416 of Bloodstream Forms of the Parasite Trypanosoma Brucei Brucei Is Dependent on
417 a Plant-like Alternative Oxidase." *The Journal of Biological Chemistry* 264 (30):
418 17770–76.
419

420 **Fang, J., and Beattie, D.S.** 2003. "Alternative Oxidase Present in Procyclic
421 Trypanosoma Brucei May Act to Lower the Mitochondrial Production of Superoxide."
422 *Archives of Biochemistry and Biophysics* 414: 294–302. doi:10.1016/S0003-
423 9861(03)00196-6.

424

425 **Fukai, Y., Amino, H., Hirawake, H., Yabu, Y., Ohta, N., Minagawa, N., Sakajo, S.,**
426 **Yoshimoto, A., Nagai, K., Takamiya, S., Kojima, S. and Kita, K.** 1999. "Functional
427 Expression of the Ascofuranone-Sensitive Trypanosoma Brucei Brucei Alternative
428 Oxidase in the Cytoplasmic Membrane of Escherichia Coli." *Comparative*
429 *Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology*
430 124 (2): 141–48. doi:10.1016/S0742-8413(99)00040-7.

431

432 **Fukai, Y., Coichi N., Keisuke K., Yoshisada Y. and Suzuki, T.** 2003.
433 "Overproduction of Highly Active Trypanosome Alternative Oxidase in Escherichia
434 Coli Heme-Deficient Mutant." *Parasitology International* 52: 237–41.
435 doi:10.1016/S1383-5769.

436

437 **Grady, R.W., Bienen, E.J., Dieck, H.A., Saric, M. and Clarkson, A.B.** 1993. "N-N-
438 Alkyl-3,4-Dihydroxybenzamides as Inhibitors of the Trypanosome Alternative
439 Oxidase: Activity in Vitro and in Vivo." *Antimicrobial Agents and Chemotherapy* 37
440 (5): 1082–85. doi:10.1128/AAC.37.5.1082.

441

442 **Grant, P.T. and Sargent, J.R.** 1960. "Properties of L-Alpha-Glycerophosphate
443 Oxidase and Its Role in the Respiration of Trypanosoma Rhodesiense." *The*
444 *Biochemical Journal* 76: 229–37.

445

446 **Helfert, S., Estévez, A.M., Bakker, B., Michels, P. and Clayton, C.** 2001. "Roles of
447 Triosephosphate Isomerase and Aerobic Metabolism in Trypanosoma Brucei." *The*
448 *Biochemical Journal* 357 (Pt 1): 117–25. doi:10.1042/0264-6021:3570117.

449

450 **Kido, Y., Shiba, T., Inaoka, D.K., Sakamoto, K., Nara, T., Aoki, T., Honma, T.,**
451 **Tanaka, A., Inoue, M., Matsuoka, S., Moore, A., Harada, S. and Kita, K.** 2010.
452 "Crystallization and Preliminary Crystallographic Analysis of Cyanide-Insensitive
453 Alternative Oxidase from Trypanosoma Brucei Brucei." *Acta Crystallographica.*
454 *Section F, Structural Biology and Crystallization Communications* 66 (Pt 3).
455 International Union of Crystallography: 275–78. doi:10.1107/S1744309109054062.

456

457 **Kuepfer, I., Schmid, C., Allan, M., Edielu, A., Haary, E.P., Kakembo, A., Kibona,**
458 **S., Blum, J. and Burri, C.** 2012. "Safety and Efficacy of the 10-Day Melarsoprol
459 Schedule for the Treatment of Second Stage Rhodesiense Sleeping Sickness."
460 *PLoS Neglected Tropical Diseases* 6 (8). doi:10.1371/journal.pntd.0001695.

461

462 **Li, X.W., Herrmann, J., Zang, Y., Grellier, P., Prado, S., Muller, R. and Nay, B.**
463 2013. "Synthesis and Biological Activities of the Respirator Chain Inhibitor Aurachin
464 D and New Ring versus Chain Analogues." *Beilstein Journal of Organic Chemistry* 9
465 (Figure 1): 1551–58. doi:10.3762/bjoc.9.176.

466

467 **Lüscher, A., de Koning, H.P. and Mäser, P.** 2007. "Chemotherapeutic Strategies
468 against Trypanosoma Brucei: Drug Targets vs. Drug Targeting." *Current*
469 *Pharmaceutical Design* 13 (April): 555–67. doi:10.2174/138161207780162809.

470

471 **Marechal, A., Kido, Y., Kita, K., Moore, A.L. and Rich, P.R.** 2009. “Three Redox
472 States of Trypanosoma Brucei Alternative Oxidase Identified by Infrared
473 Spectroscopy and Electrochemistry.” *The Journal of Biological Chemistry* 284 (46):
474 31827–33. doi:10.1074/jbc.M109.059980.

475

476 **Minagawa, N., Yabu, Y., Kita, K., Nagai, K., Ohta, N., Meguro, K., Sakajo, S. and**
477 **Yoshimoto, A.** 1997. “An Antibiotic, Ascofuranone, Specifically Inhibits Respiration
478 and in Vitro Growth of Long Slender Bloodstream Forms of Trypanosoma Brucei
479 Brucei.” *Molecular and Biochemical Parasitology* 81 (2): 127–36. doi:10.1016/0166-
480 6851(96)02665-5.

481

482 **Mogi, T., Ui, H., Shiomi, K., Omura, S., Miyoshi, H. and Kita, K.** 2009. “Antibiotics
483 LL-Z1272 Identified as Novel Inhibitors Discriminating Bacterial and Mitochondrial
484 Quinol Oxidases.” *Biochimica et Biophysica Acta - Bioenergetics* 1787 (2): 129–33.
485 doi:10.1016/j.bbabbio.2008.11.016.

486

487 **Moore, A.L., Carré, J.E., Affourtit, C., Albury, M.S., Crichton, P.G., Kita, K. and**
488 **Heathcote, P.** 2008. “Compelling EPR Evidence That the Alternative Oxidase Is a
489 Diiron Carboxylate Protein.” Article. *Biochimica et Biophysica Acta* 1777 (4): 327—
490 330. doi:10.1016/j.bbabbio.2008.01.004.

491

492 **Moore, A.L., Shiba, T., Young, L., Harada, S., Kita, K. and Ito, K.** 2013. “Unraveling
493 the Heater: New Insights into the Structure of the Alternative Oxidase.” *Annual*
494 *Review of Plant Biology* 64 (January): 637–63. doi:10.1146/annurev-arplant-042811-

495 105432.

496

497 **Nakamura, K., Fujioka, S., Fukumoto, S., Inoue, N., Sakamoto, K., Hirata, H.,**
498 **Kido, Y., Yabu, Y., Suzuki, T., Watanabe, Y.I., Saimoto, H., Akiyama, H. and Kita,**
499 **K.** 2010. "Trypanosome Alternative Oxidase, a Potential Therapeutic Target for
500 Sleeping Sickness, Is Conserved among Trypanosoma Brucei Subspecies."
501 *Parasitology International* 59 (4). Elsevier Ireland Ltd: 560–64.
502 doi:10.1016/j.parint.2010.07.006.

503

504 **Nihei, C., Fukai, Y., Kawai, K., Osanai, A., Yabu, Y., Suzuki, T., Ohta, N.,**
505 **Minagawa, N., Nagai, K. and Kita, K.** 2003. "Purification of Active Recombinant
506 Trypanosome Alternative Oxidase." *FEBS Letters* 538 (1–3): 35–40.
507 doi:10.1016/S0014-5793(03)00120-0.

508

509 **Nihei, C., Fukai, Y. and Kita, K.** 2002. "Trypanosome Alternative Oxidase as a
510 Target of Chemotherapy." *Biochimica et Biophysica Acta - Molecular Basis of*
511 *Disease* 1587 (2–3): 234–39. doi:10.1016/S0925-4439(02)00086-8.

512

513 **Opperdoes, F.R., Borst, P. and Fonck, K.** 1976. "The Potential Use of Inhibitors of
514 Glycerol-3-Phosphate Oxidase for Chemotherapy of African Trypanosomiasis."
515 *FEBS Letters* 62 (2): 169–72. doi:10.1016/0014-5793(76)80045-2.

516

517 **Ott, R., Chibale, K., Anderson, S., Chipeleme, A., Chaudhuri, M., Guerrah, A.,**
518 **Colowick, N. and Hill, G.C.** 2006. "Novel Inhibitors of the Trypanosome Alternative
519 Oxidase Inhibit Trypanosoma Brucei Brucei Growth and Respiration." *Acta Tropica*

520 100 (3): 172–84. doi:10.1016/j.actatropica.2006.10.005.

521

522 **Pollakis, G., Grady, R.W., Dieck, H.A. and Clarkson, A.B.** 1995. “Competition
523 between Inhibitors of the Trypanosome Alternative Oxidase (TAO) and Reduced
524 Coenzyme Q9.” *Biochemical Pharmacology* 50 (8): 1207–10. doi:10.1016/0006-
525 2952(95)00259-3.

526

527 **Saimoto, H., Kido, Y., Haga, Y., Sakamoto, K. and Kita, K.** 2013. “Pharmacophore
528 Identification of Ascofuranone, Potent Inhibitor of Cyanide-Insensitive Alternative
529 Oxidase of *Trypanosoma Brucei*.” *Journal of Biochemistry* 153 (3): 267–73.
530 doi:10.1093/jb/mvs135.

531

532 **Schonbaum, G.R., Bonner, W.D., Storey, B.T. and Bahr, J.T.** 1971. “Specific
533 Inhibition of the Cyanide-Insensitive Respiratory Pathway in Plant Mitochondria by
534 Hydroxamic Acids.” *Plant Physiology* 47 (1): 124–28. doi:10.1104/pp.47.1.124.

535

536 **Shiba, T., Kido, Y., Sakamoto, K., Ken, D., Tsuge, C. and Tatsumi, R.** 2013.
537 “Structure of the Trypanosome Cyanide-Insensitive Alternative Oxidase.”
538 *Proceedings of the National Academy of Sciences* 110 (12): 4580–85.
539 doi:10.1073/pnas.1218386110/-
540 /DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1218386110.

541

542 **Simarro, P.P., Cecchi, G., Franco, J.R., Paone, M., Diarra, A., Ruiz-Postigo, J.A.,**
543 **Fèvre, E.M., Mattioli, R.C. and Jannin, J.G.** 2012. “Estimating and Mapping the
544 Population at Risk of Sleeping Sickness.” *PLoS Neglected Tropical Diseases* 6 (10):

545 e1859. doi:10.1371/journal.pntd.0001859.

546

547 **Suzuki, T., Nihei, C.I., Yabu, Y., Hashimoto, T., Suzuki, M., Yoshida, A., Nagai,**

548 **K., Hosokawa, T., Minagawa, N., Suzuki, S., Kita, K. and Ohta, N.** 2004.

549 “Molecular Cloning and Characterization of *Trypanosoma Vivax* Alternative Oxidase

550 (AOX) Gene, a Target of the Trypanocide Ascofuranone.” *Parasitology International*

551 53 (3): 235–45. doi:10.1016/j.parint.2004.02.001.

552

553 **Tsuda, A., Witola, W.H., Konnai, S., Ohashi, K. and Onuma, M.** 2006. “The Effect

554 of TAO Expression on PCD-like Phenomenon Development and Drug Resistance in

555 *Trypanosoma Brucei*.” *Parasitology International* 55 (2): 135–42.

556 doi:10.1016/j.parint.2006.01.001.

557

558 **Vassella, E., Probst, M., Schneider, A., Studer, E., Renggli, C.K., and Roditi, I.**

559 2004. “Expression of a Major Surface Protein of *Trypanosoma Brucei* Insect Forms

560 Is Controlled by the Activity of Mitochondrial Enzymes.” *Molecular Biology of the Cell*

561 15 (9): 3986–93. doi:10.1091/mbc.E04-04-0341.

562

563 **Walker, R., Saha, L., Hill, G.C. and Chaudhuri, M.** 2005. “The Effect of over-

564 Expression of the Alternative Oxidase in the Procyclic Forms of *Trypanosoma*

565 *Brucei*.” *Molecular and Biochemical Parasitology* 139 (2): 153–62.

566 doi:10.1016/j.molbiopara.2004.11.003.

567

568 **World Health Organization.** 2013. “Control and Surveillance of Human African

569 Trypanosomiasis.” *World Health Organization Technical Report Series*, no. 984: 1–

570 237.

571

572 **World Health Organization.** 2016. "Human African Trypanosomiasis."

573 <http://apps.who.int/gho/data/node.main.A1635?lang=en>.

574

575 **Yabu, Y., Minagawa, N., Kita, K., Nagai, K., Honma, M., Sakajo, S., Koide, T.,**

576 **Ohta, N. and Yoshimoto, A.** 1998. "Oral and Intraperitoneal Treatment of

577 *Trypanosoma Brucei Brucei* with a Combination of Ascofuranone and Glycerol in

578 Mice." *Parasitology International* 47 (2): 131–37. doi:10.1016/S1383-5769(98)00011-

579 7.

580

581 **Yabu, Y., Suzuki, T., Nihei, C.I., Minagawa, N., Hosokawa, T., Nagai, K., Kita, K.**

582 **and Ohta, N.** 2006. "Chemotherapeutic Efficacy of Ascofuranone in *Trypanosoma*

583 *Vivax*-Infected Mice without Glycerol." *Parasitology International* 55 (1): 39–43.

584 doi:10.1016/j.parint.2005.09.003.

585

586 **Yabu, Y., Yoshida, A., Suzuki, T., Nihei, C.I., Kawai, K., Minagawa, N.,**

587 **Hosokawa, T., Nagai, K., Kita, K. and Ohta, N.** 2003. "The Efficacy of

588 Ascofuranone in a Consecutive Treatment on *Trypanosoma Brucei Brucei* in Mice."

589 *Parasitology International* 52 (2): 155–64.

590

591 **Zoltner, M., Horn, D., de Koning, H.P., and Field, M.C.** 2016. "Exploiting the Achilles' Heel of

592 Membrane Trafficking in Trypanosomes." *Current Opinion in Microbiology* 34. Elsevier Ltd:

593 97–103. doi:10.1016/j.mib.2016.08.005.

594

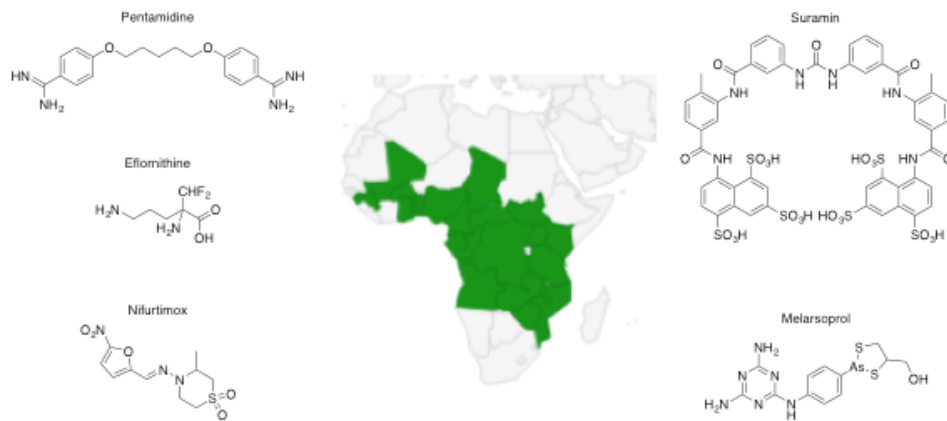


Figure 1. Distribution of countries endemic for *Trypanosoma brucei* according to WHO and the currently used clinical drugs

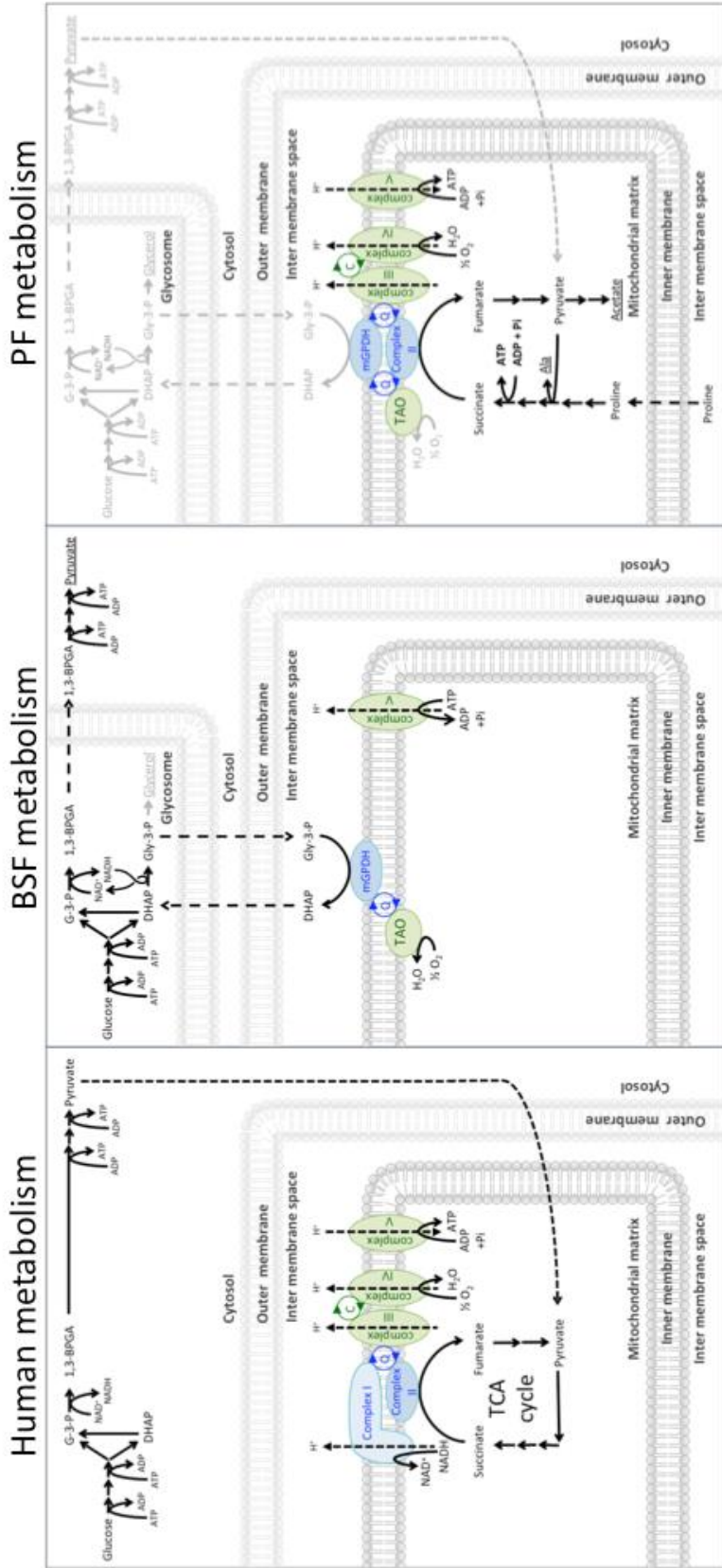


Figure 2. Carbon source metabolism. Human cells glycolytically metabolise glucose to pyruvate in the cytosol. Pyruvate is taken into the mitochondrial matrix where it is further completely metabolised to CO₂ and water through the TCA cycle and electron transport chain (shown in green). Entry points to the electron transport chain are shown in blue. The malate-aspartate shuttle (not shown) maintains cytosolic NAD(H) redox.

BSF *T. brucei* metabolise glucose to 1,3-BPGA in the cytosol. A high rate of glycolysis means that sufficient ATP is produced through this route alone and the parasite can secrete the pyruvate produced as waste rather than spend energy consuming it further. The GPO system (mGPDH and TAO) is required to maintain glycolytic NAD(H) redox. If the GPO system is inhibited BSF *T. brucei* will convert Gly-3-P to the secreted end product glycerol to maintain glycolytic NAD(H) redox.

PF *T. brucei* are able to metabolise glucose, however, in the insect midgut glucose is so low in abundance that the main carbon source is proline. Proline is converted to acetate and alanine in a TCA-like, non-cyclical fashion. ATP is generated through oxidative phosphorylation and TAO, present at low levels, is hardly used.

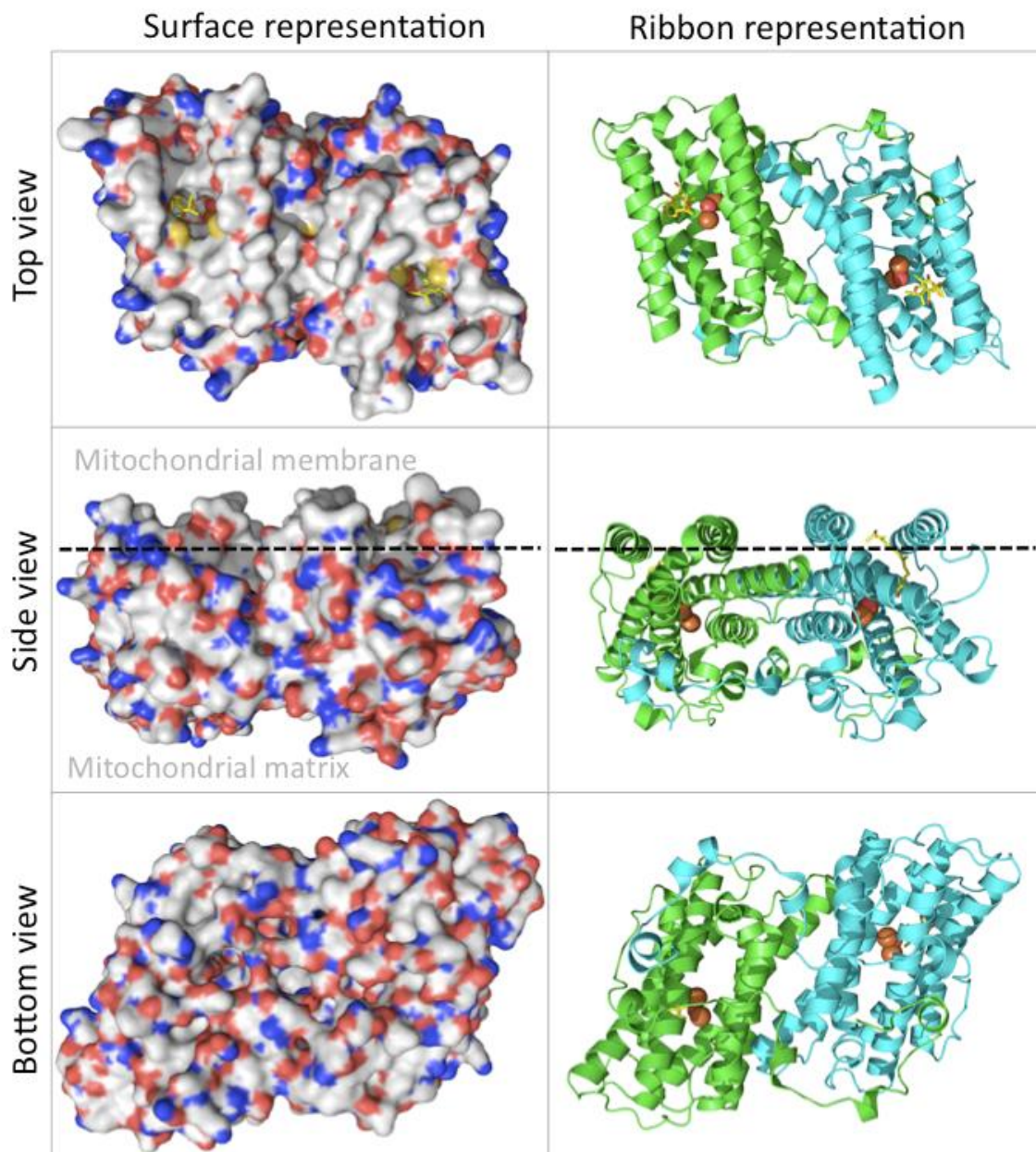


Figure 3. The TAO dimer in complex with diiron/hydroxo core (shown as spheres) and inhibitor AF2779OH (shown in yellow carbon stick). For surface representation, hydrophobic areas are grey and hydrophilic areas are blue/red. The upper face of the dimer is highly hydrophobic allowing TAO to burry itself within a single layer of the inner mitochondrial membrane. The approximate position of the membrane/matrix interface is represented by a dashed line. The diiron catalytic core is burried deep within the protein structure and a channel from the membrane to the core allows access of ubiquinol substrate (or analogous inhibitor AF2779OH).

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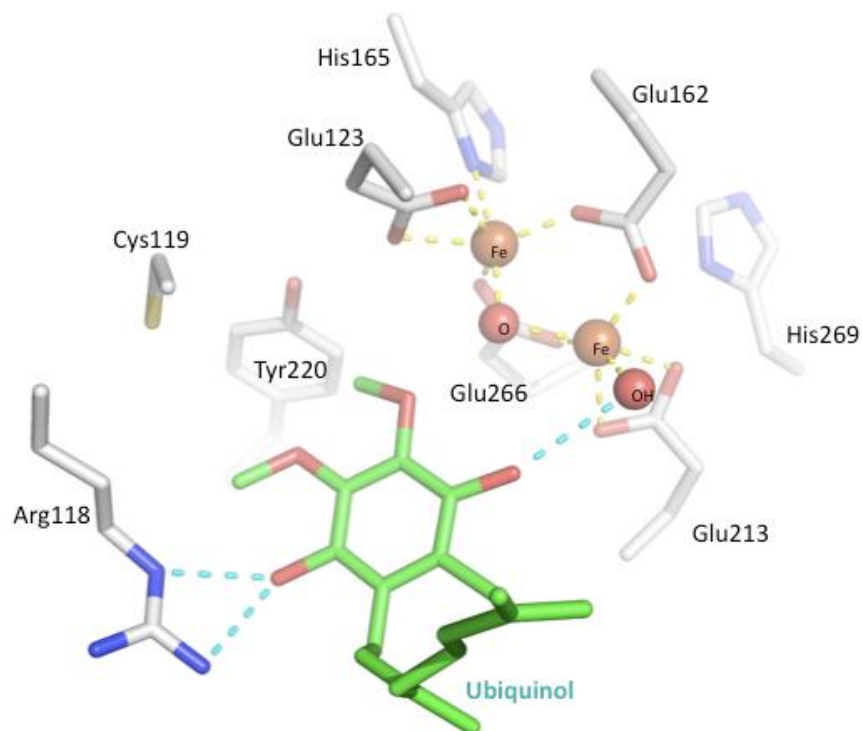


Figure 4. The TAO active site with ubiquinol (green) superposed in the place of inhibitor AF2779OH. The diiron core is in the Fe(III/III) oxodiiron state (see mechanism part in the main body text), coordinated by four glutamates, two histidines, an oxygen and a hydroxyl (yellow dashed lines).

For the first ubiquinol oxidation, the coordinated OH would abstract a hydrogen atom (proton plus electron) from ubiquinol (blue dashed line) and leave as water, reducing the core to Fe(II/III) and oxidising ubiquinol to semiubiquinol. The oxygen bridge will take a hydrogen from Tyr220 to form a hydroxo bridge and the resulting tyrosyl radical will take a hydrogen from semiubiquinol either directly or via Cys119 and return to its native state, leaving the core in a reduced diferrous Fe(II/II) state bridged by a single hydroxyl. With the reaction complete, ubiquinone will leave allowing molecular oxygen and a second ubiquinol to enter.

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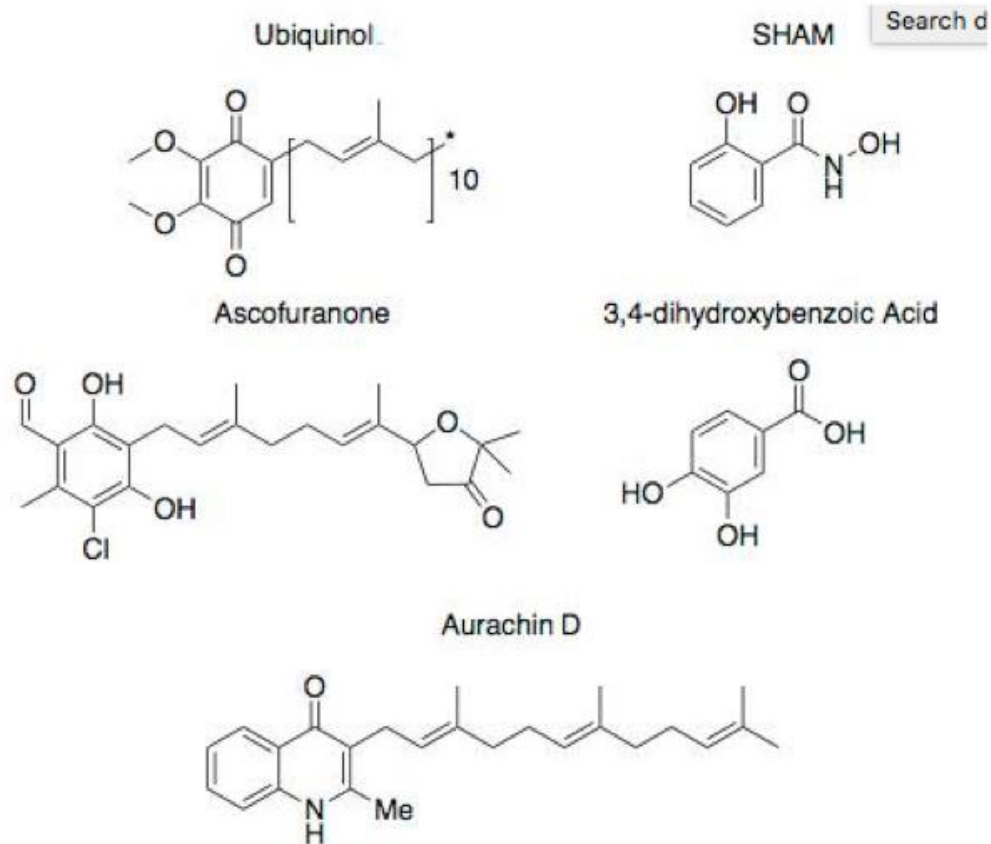


Figure 5. Chemical structures of TAO substrate ubiquinol and the TAO inhibitors salicylhydroxamic acid (SHAM), ascofuranone, 3,4-dihydroxybenzoic acid and Aurachin D.

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