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SAR of 4-Alkoxybenzoic Acid Inhibitors of the Trypanosome Alternative Oxidase

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KEYWORDS. Trypanosome alternative oxidase (TAO) inhibitor, Trypanosoma brucei, lipophilic cation, 4-alkoxybenzoic acid derivative.

ABSTRACT: The SAR of 4-hydroxybenzaldehyde inhibitors of the trypanosomal alternative oxidase (TAO), a critical enzyme for the respiration of bloodstream forms trypanosomes, was investigated. Replacing the aldehyde group with a methyl ester resulted in a 10-fold increase in TAO inhibition and activity against T. brucei. Remarkably, two analogues containing the 2-hydroxy-6-methyl scaffold (9e and 16e) displayed single digit nanomolar TAO inhibition, which represents the most potent 4-alkoxybenzoic acid derivatives described to date. 9e was 50-times more potent against TAO and 10-times more active against T. brucei compared to its benzaldehyde analogue 23. The farnesyl derivative 16e was as potent a TAO inhibitor as ascofuranone with IC₅₀ = 3.1 nM. Similar to ascofuranone derivatives, the 2-hydroxy and 6-methyl groups seemed essential for low nanomolar TAO inhibition of acid derivatives, suggesting analogous binding interactions with the TAO active site.

The bloodstream forms (BSF) of African trypanosomes, which cause sleeping sickness in humans and animal trypanosomiasis (nagana) in livestock, rely exclusively on glucose metabolism for energy production. Lacking a functional oxidative phosphorylation pathway, BSF trypanosomes use the trypanosome alternative oxidase (TAO) as terminal oxidase to re-oxidize the NADPH that accumulates during glycolysis. TAO is a non-haem cyanide-insensitive membrane-bound di-iron protein that catalyzes the oxidation of ubiquinol and the four electron reduction of oxygen to water.¹ This enzyme, which is essential for the respiration of BSF trypanosomes² and is conserved among T. brucei subspecies,³ has been validated as a drug target against trypanosomes.⁴ ⁵ ⁶ ⁷

In recent years, different series of TAO inhibitors have been reported that are based on the structure of the natural isoprenoid antibiotic ascofuranone (Figure 1).⁸ ⁹ ¹⁰ ¹¹ Kita’s group reported the pharmacophore of ascofuranone⁸ and, shortly after, the 3-D structures of TAO in the presence and absence of ascofuranone derivatives, allowing a better understanding of inhibitor binding modes.¹² However, to date ascofuranone is the only TAO inhibitor among the ascofuranone-based compounds that has shown in vivo curative activity in different mouse models of trypanosomiasis.⁶ ¹³ The modest success of the ascofuranone-like inhibitors against T. brucei is attributed to poor physicochemical (drug-like) properties of these compounds, as they are highly lipophilic and hardly soluble in water, resulting in poor pharmacokinetic properties. In these inhibitors, a high degree of lipophilicity is required not only for effective binding to TAO⁸ (i.e. a hydrophobic tail is necessary, Figure 1) but probably also to retain the inhibitors in the mitochondrial membrane where TAO is located.¹¹ The requirement for these properties has hampered the search for inhibitors with drug-like properties.

Recently, we have developed different series of TAO inhibitors based on the 4-hydroxybenzoate and 4-alkoxybenzaldehyde chemotypes (Figure 1, A and D). These compounds displayed submicromolar to low nanomolar IC₅₀ values against recombinant TAO (rTAO) and micromolar activities against T. brucei. The conjugation of these inhibitors with a mitochondrion-targeting lipophilic cation tail via a methylene linker yielded very potent trypanocides against wild-type and multidrug-resistant T. brucei strains.¹⁵ ¹⁶ In particular, compound A (R₁ = +PPh₃, IC₅₀ (rTAO) = 0.09 µM) was able to reduce the parasite load of mice infected with T. b. brucei rhodesiense by ip administration at 4×10 mg/kg.¹⁵

In spite of the promising initial in vivo results, the benzoate derivatives A present several drawbacks: 1) a metabolic liability dependent on the substituents of the aromatic head (i.e. limiting the options for SAR development); 2) less than optimum water solubility; 3) a reduced therapeutic window in vivo (e.g. dosage > 4×10 mg/kg/ip was not tolerated, unpublished data). In contrast, the 4-alkoxy inhibitors (e.g. B–D), which are devoid of an ester bond, are expected to be resistant to serum hydrolases. In fact, compound I (Figure 1, D) proved to be stable in mouse
serum although it appeared to bind serum proteins to high degree. This behavior was attributed to the presence of the benzaldehyde group which may form Schiff’s bases with the amino groups of the proteins.

Figure 1. Structure of different TAO inhibitors chemotypes: ascofuranone, 4-hydroxybenzoates (A), 15, 16 4-alkoxybenzoic acids (B), 17 and 4-alkoxybenzaldehydes (C, D). The main interactions of ascofuranone-like inhibitors with the TAO active site are shown in green color. The predicted main interactions of 4-alkoxybenzaldehyde D with the amino acids of the TAO active site are highlighted in red color. HB = hydrogen bond.

In the current work, we further investigated the SAR of this promising TAO inhibitor scaffold by replacing the aldehyde group of D with a carboxylic acid (or ester), modulating the nature of the lipophilic tail (e.g. alkyl, geranyl, farnesyl) and of the substituent on the aromatic ring (Figure 2). In the designed compounds, the carboxylic acid function is expected to be in close proximity to the diiron center of TAO whereas the oxygen atom in 4-position is expected to engage in hydrogen bond (HB) interactions with polar amino acids from the TAO binding site (e.g. Arg118) similarly to 1 (Figure 1). The presence of a free acid group in the molecule should enhance the water solubility of the molecules.

Figure 2. Structural modifications of 4-alkoxybenzoic acid derivatives studied in this work.

Results and discussion. The inhibitors with a lipocation alkyl tail (6a, 7a-e, 10b, 10c, and 10e) were synthesized in two steps starting from the corresponding 4-hydroxybenzoates 2a-e (Scheme 1). Alkylation of 2a with 1,10-dibromocane and NaHCO3 gave the bromoalkoxy compound 4a with low yield (22%). Better yields of 5a-e (43–67%) were obtained using the Mitsunobu protocol18 with 14-bromotetradecan-1-ol. Nucleophilic substitution of these bromo derivatives with triphenylphosphine or quinoline yielded the phosphonium (6a, 7a-e) and quinolinium salts (10b, 10c, 10e). Smooth hydrolysis of the ester function with potassium carbonate in MeOH/H2O at 50 ºC yielded the carboxylic acids 8a and 9a-e with excellent yields. For the quinolinium salts, the hydrolysis was performed with 48% aqueous HBr to yield the acid derivatives 11b-e (Scheme 1).

Scheme 1. Synthesis of TAO inhibitors with lipocation alkyl tail

Reagents and conditions. (i) NaHCO3, CH3CN, 65 ºC; (ii) DIAD, Ph3P, THF, 0 ºC to rt; (iii) Ph3P, CH3CN, 80 ºC; (iv) K2CO3 (3 eq.), MeOH/H2O (5/1) or EtOH/H2O (5/1, for 7e), 50 ºC; (v) Quinoline, CH3CN, 80 ºC; (vi) HBr 48%, H2O, 60 ºC.
Scheme 2. Synthesis of TAO inhibitors with geranyl and farnesyl tail

![Synthesis Scheme]

Reagents and conditions. (i) 1) SeO\(_2\), BuOOH, salicylic acid (cat.), CH\(_2\)Cl\(_2\), 30 min, rt; 2) geranyl acetate or farnesyl acetate, 45 h, rt; (ii) CBr\(_2\)Cl\(_2\), Ph\(_3\)P, CH\(_2\)Cl\(_2\); (iii) DIAD, Ph\(_2\)P, THF, 0 °C to rt; (iv) 1) 1M KOH, MeOH, 60 °C, 2) 1M HCl; (v) K\(_2\)CO\(_3\), MeOH, rt (for 17a) or K\(_2\)CO\(_3\), EtOH, 35 °C (for 17e).

The compounds were assayed as inhibitors of the ubiquinol oxidase activity of purified ΔMTS-ΔTAO as described.\(^{15}\)

Cationic TAO inhibitors: The replacement of the aldehyde group of 23 by a methyl ester (7a) led to a 15-fold increase in TAO inhibition potency resulting in a 7-fold improvement in activity against T. brucei (Table 1). In this methyl ester series, replacement of the 2-OH group by a fluoro group (7b) or methyl substituent (7c) resulted in 50- and >300-fold decreased inhibition compared with 7a, respectively (2-OH > 2-F >> 2-Cl). These data confirmed the need for a hydrogen bond-forming substituent at this position for tight binding to TAO. As noted earlier with the 4-hydroxybenzoate series,\(^{15}\), a methylene linker of less than C14 was detrimental to TAO inhibition (compare 6a/7a). Switching the 4-alkoxy tail (7a) to the 3-position (7d) of the aromatic ring hardly influenced TAO inhibition (0.015 and 0.012 µM, respectively), which correlated with similar trypanocidal activities for both compounds (0.018 and 0.028 µM, respectively).

Interestingly, TAO inhibition was limited by the size of the ester substituent at C1: an ethyl ester substituent was detrimental with a 60-fold drop in inhibitor potency compared to the free acid (compare 7e/9e, 15e/16e). The same trend was observed for the geranyl series (Table 2) with higher IC\(_{50}\) values for the ethyl ester 19e (>5 µM) compared to the free acid 18e (2.4 µM). Intriguingly, replacement of the methyl ester (7a and 7b) or aldehyde group (23) at C1 by a carboxylic acid led to a drastic loss of inhibition (IC\(_{50}\) > 5 µM for 9a and 9b). In contrast, the 2-hydroxy-6-methyl acid analogue 9e inhibited TAO in the low nanomolar range (IC\(_{50}\) = 4.2 nM) indicating that the presence of the 6-methyl substituent in 9e is playing a key role in the binding of inhibitors with an acid functional group, similar to the ascofuranone-based inhibitors.\(^{3}\) The triphenylphosphonium cation was more favorable than the 1-quinolinium cation for upper inhibition of TAO (compare 7e/10e, 7b/10b). In this series of quinolinium salts, an aldehyde group at C1 (24, IC\(_{50}\) = 1.23 µM) was superior to an ester or acid group (IC\(_{50}\) > 5 µM).

Non-cationic inhibitors with methylene, geranyl, and farnesyl tail: the acid derivative 15e with a farnesyl lipophilic tail was the most potent inhibitor of these series (IC\(_{50}\) = 3.1 nM), superior to the geranyl counterpart 18e and the rest of geranyl acid derivatives (18a–e) which were single digit micromolar inhibitors (Table 2). Remarkably, one extra isoprenyl unit (15e) was responsible for an increase of inhibition by 3 orders of magnitude within this series. In contrast, the benzoate derivatives (15e, 17a, 17c, 19b, 19e) were much less potent TAO inhibitors (IC\(_{50}\) > 5 µM), with the exception of 5a and 19c (IC\(_{50}\) = 2 µM). These inhibition values are in the same range as previously reported TAO inhibitors bearing a 4-alkoxy isoprenoid chain such as B (ACB41, K\(_i\) = 5µM)\(^{17}\) or C (IC\(_{50}\) = 1 µM).\(^{12,17}\) We decided to study whether a geranyl tail would have the same (weak) effect on the inhibitory potency of the benzoate series derived from scaffold A. The benzoate derivative 20a with the geranyl tail at C1 was synthesized by reaction of 1 equivalent of 2,4-dihydroxybenzoic acid and 1 equivalent of 14 in the presence of potassium carbonate in anhydrous acetone at 60 °C. The product of dialkylation 21a was also isolated by silica chromatography (Scheme 3).


Reagents and conditions. (i) K\(_2\)CO\(_3\), acetone, 60 °C, 2 h.

Compound 20a inhibited TAO in the low nanomolar range (IC\(_{50}\) = 9.1 nM), with a potency similar to that of its 14-bromotetradecane benzoate analogue (10.8 nM).\(^{13}\) This results indicates that the geranyl tail is a valid biososteric replacement of the methylene tail for the 4-hydroxybenzoate inhibitor series (A) but it is less effective for the 4-alkoxybenzoic acid derivatives. Not surprisingly, the disubstituted derivative 21a with the 4-OH position blocked by the 4-alkoxygeranyl group inhibited TAO poorly (>5 µM). The 4-hydroxybenzoate inhibitors are expected to bind TAO with the 4-OH group close to the dirion catalytic center, which is not possible with this disubstituted compound.

The trypanocidal activity of the new compounds was assessed against wild-type and multidrug-resistant strains of T. b. brucei.
In the cationic series, the triphenylphosphonium conjugates (7b, 7c, 7e) were 6- to 48-times more potent than the quinolinium salts (10b, 10c, 10e; Table 1) in agreement with previous results. However, the benzoates quinolinium salts 10b and 10e were 5- to 10-fold more potent than the benzaldehyde hit 1 (EC50 = 1.75 µM). Replacing the aldehyde group (1, R3 = H) by a methyl ester function (7a, R3 = OMe) resulted in a 7-fold increase in trypanocidal activity against T. b. brucei. In this benzoate series, replacement of the 2-OH with a 2-Me (7c) had no influence on the activity whereas a 2-F (7b) or 2-OH, 6-Me (7e) substituents (7e) gave slightly less potent compounds (2.5-fold). The influence on trypanocidal activity of a free acid group in R1 depended on the nature of the substituents R1 and R2: the benzoate derivatives 7b (R2 = F, R3 = H) and 7e (R2 = Me, R3 = OH) were 1.4- to 4-times less active than the free acids 9b and 9e, respectively. In contrast, the benzoates 7a (R1 = OH, R2 = H), 7c and 10c (R1 = Me, R2 = H) were 5-, 10-, and 66-times more active than the free acids 9a, 9c and 11c, respectively.

Table 1. Biological evaluation of 4-alkoxybenzoate-lipocatin conjugates against rTAO (IC50, µM), T. b. brucei (EC50, µM), and human cells (CC50, µM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>rTAOα</th>
<th>T. b. brucei WTb</th>
<th>HEK cells</th>
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<tr>
<td>22c</td>
<td>H</td>
<td>H</td>
<td>0.073 ± 0.011</td>
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<td>5a</td>
<td>H</td>
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<td>2.3 ± 0.2</td>
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<tr>
<td>5c</td>
<td>Me</td>
<td>OEt</td>
<td>&gt;5</td>
<td>8.9 ± 1.2</td>
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<td>5d</td>
<td>F</td>
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<td>&gt;5</td>
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<tr>
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Footnotes and control drugs: see Table 1.

Little difference in activity was observed between WT and the multidrug resistant cell line B48 with resistance factors (RF) close to 1 (Tables S1), indicating they do not utilise the known drug transporters TbAT1 and HAPT.1,2 In contrast, several inhibitors (5d, 5e, 8a, 9c, 9e, 10b, 20a) were more effective against the T. b. brucei cell line from which all three aquaporins were deleted (AQ1P-3 triple KO). These trypanosomes are particularly sensitive to TAO inhibitors because they cannot dispose of the glycerol produced in large quantity under anaerobic conditions (i.e. when TAO is inhibited). Accumulation of glycerol is toxic to the cells as a result of the inhibition of the glycerol kinase and depletion of ATP production.14,24 The same compounds were also significantly more effective against T. b. brucei WT (RF < 1) when co-incubated with 5 mM glycerol, which inhibits the anaerobic ATP production pathway (Tables S1). Analogous results were obtained with the benzaldehyde inhibitors 1, 22, and 23,13 supporting the view that TAO is highly likely to be the main target of 5d, 5e, 8a, 9c, 9e, 10b, and 20a. In contrast, compounds 7b, 7c, 7e, 9a, and 9b appeared to be less dependent on TAO inhibition as shown by the RF > 2 against the AQ1P-3 knockout line and against T. b. brucei WT+glycerol indicating that these compounds probably have
multi-target activity. Cytotoxicity against HEK cells was low (> 20 µM) for the non-cationic inhibitors with a geranyl (17a, 17c, 20a), farnesyl (15e) and methylene (5d, 5e) tail, and the cationic inhibitors with a free acid group (8a, 9a-c) (CC50 > 4 µM, SI from 11 to 227). In contrast, the inhibitor 9e was more cytotoxic (CC50 = 0.83 µM) although this still yielded selectivity indexes of 64 and 230 towards T. brucei WT and AQP1-3 knockout strains, respectively. Other inhibitors with a lipocation tail displayed variable cytotoxicity against human cells (CC50 < 1 µM for 6a, 7a-d; CC50 > 20 µM for 11e).

In summary, cationic 4-alkoxy benzoic acid derivatives are more potent TAO inhibitors than their benzaldehyde counterparts. The 2,4-dihydroxy-6-methyl carboxylic acid scaffold, in particular, gave the most potent TAO inhibitors (9e, 16e). Most of the 4-alkoxybenzoate-lipocation conjugates were also effective nanomolar range trypanocides. However, higher toxicity against human cells was observed resulting in lower selectivity indexes compared with previous series.

ASSOCIATED CONTENT
Supporting Information
Synthesis of compounds 4–21. Biology experimental protocols. Table S1. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. *These authors contributed equally.

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ABBREVIATIONS
BSF trypanosome, bloodstream form trypanosome; DIAD, diisopropylazodicarboxylate; MTS, mitochondrion-targeting sequence; HB, hydrogen bond; RF, resistance factor; TAO, trypanosome alternative oxidase

REFERENCES


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### Table 1

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