Aurones Interfere with *Leishmania major* Mitochondrial Fumarate Reductase

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A series of aurones was analyzed for the ability to inhibit respiratory functions of mitochondria of *Leishmania* parasites. The aim of this study was to find a rational explanation for the activity of certain aurones and auronols as novel antiprotozoal compounds of plant origin. In a cell-free assay mitochondrial fumarate reductase from *L. donovani* was inhibited in a concentration-dependent manner. The most active compounds were 4′,6-dihydroxyaurone and 6-methoxyaurone which inhibited parasite enzyme activity at 25 nM by over ninety percent.

Introduction

The human leishmaniases pose increasing health problems in Africa, Asia and Latin America. According to Ashford *et al.* (1992) the World Health Organization estimates that 350 million people live at risk of infection with *Leishmania* parasites. The annual incidence of new infections is 1.5–2 million for cutaneous leishmaniasis (CL), and over 500,000 for visceral leishmaniasis (VL). In the wake of the AIDS pandemic, there is a pronounced increase in VL and HIV co-infections. Both VL and CL are endemic in South European countries with a high number of reactivated cases of VL in immunosuppressed people, particularly those caused by HIV super-infection (Alvar *et al.*, 1997).

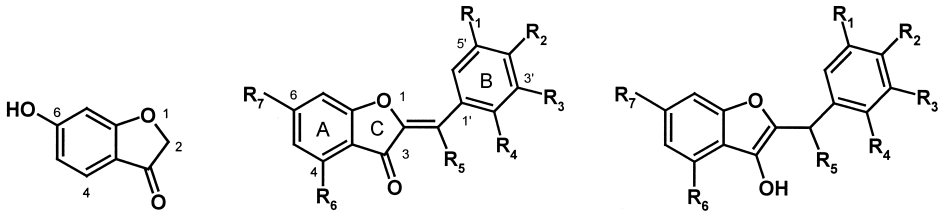
In our previous studies on new antiprotozoal drugs we have found that the natural product group of aurones include potential antileishmanial agents as they inhibit growth of different *Leishmania* species *in vitro* (Kayser *et al.*, 1999; Kayser and Kiderlen, 1999). Certain aurones exhibit a remarkably strong capacity to kill intracellularly persisting amastigotes of *Leishmania donovani* and *L. major* with no significant cytotoxicity for host cells (Kayser *et al.*, 1999). The mechanism(s) by which aurones kill these parasites is unknown. Chalcones have been shown to alter the ultrastructure of the mitochondria in *Leishmania* promastigotes, thereby interfering with their respiratory functions (Chen *et al.*, 1994). As aurones and auronols show chemical and structural similarities with chalcones (Zhai *et al.*, 1995) common molecular targets in the parasites can be hypothesized. The presented data indicate that the antileishmanial activity of certain aurones may also be explained by their interference with respiratory mitochondrial enzymes of the pathogen.

Materials and Methods

Compounds

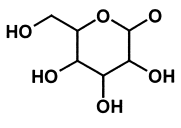
The aurones and auronols (Fig. 1) were kindly provided by Prof. Dr. R. Hänsel (Berlin, Germany). The compounds had been synthesized and their structure and purity (>95%) determined by nuclear magnetic resonance spectroscopy (H/C-NMR) and high-performance liquid chromatography (HPLC) by K. Bley (1973). All drugs were dissolved in dimethyl sulfoxide (DMSO) at 10 mg ml^{-1} and diluted in medium 199 or buffer to the desired concentration immediately before use according to Chen *et al.* (2001).

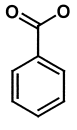
Fig. 1. Chemical structures of the aurones and auronols used in this study.



No.	Compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7
1	6-Hydroxy-benzofuran-3-one	–	–	–	–	–	–	–
	Aurones							
2	6-Hydroxy-2-[phenylmethylene]-3(2*H*)-benzofuranone	H	H	H	H	H	H	OH
3	6-Methoxy-2-[phenylmethylene]-3(2*H*)-benzofuranone	H	H	H	H	H	H	OCH_3
4	3′,4′,6-Trihydroxy-2-[phenylmethylene]-3(2*H*)-benzofuranone	H	OH	OH	H	H	H	OH
5	3′,6-Dihydroxy-2-[phenylmethylene]-3(2*H*)-benzofuranone	H	H	OH	H	H	H	OH
6	2′,6-Dihydroxy-2-[phenylmethylene]-3(2*H*)-benzofuranone	H	OH	H	OH	H	H	OH
7	4′,6-Dihydroxy-2-[phenylmethylene]-3(2*H*)-benzofuranone	H	OH	H	H	H	H	OH
8	6-Hydroxy-3′,4′-dimethoxy-2-[phenylhydroxymethylene]-3(2*H*)-benzofuranone	H	OCH_3	OCH_3	H	H	H	OH
9	4,6,3′-Trihydroxy-4′-methoxy-2-[phenylhydroxymethylene]-3(2*H*)-benzofuranone	H	OCH_3	OH	H	H	OH	OH
10	4,6,4′-Trihydroxy-3′-methoxy-2-[phenylhydroxymethylene]-3(2*H*)-benzofuranone	H	OH	OCH_3	H	H	OH	OH
11	6-Hydroxy-2-[(2,3,4-trihydroxyphenyl)-methylene]-3(2*H*)-benzofuranone-5-β-*D*-glucopyranoside (Bractein)	OCH_3	OH	OCH_3	H	H	OGlc	OH
12	4,6,4′-Triacetyl-3′,5′-dimethoxy-2-[phenylhydroxymethylene]-3(2*H*)-benzofuranone (Bracteintriacetate)	OCH_3	OAc	OCH_3	H	H	OAc	OAc
	Auronols							
13	4,6-Dihydroxy-2-[phenylhydroxymethylene]-3(2*H*)-benzofuran-3-ol	H	H	H	H	OH	OH	OH
14	6-Benzoyl-2-[phenylhydroxymethylene]-3(2*H*)-benzofuran-3-ol	H	H	H	H	OH	H	OBz

Glc: β-D-glucose Bz: benzoyl Ac: acetyl





Parasite culture

The *Leishmania major* strain MHOM/IL/67/LRC-L137, provided by the Kenya Medical Research Institute, Nairobi, was used in this study. Promastigotes were cultured in medium 199 containing 0.02 mg ml^{-1} gentamycin, 25 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), 4 mM L-glutamine, and 10% heat inactivated fetal calf serum at 26 °C.

Mitochondrial NADH-fumarate reductase activity

All biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) unless otherwise stated. Enzyme activity was determined at a final protein concentration of 0.1 mg ml^{-1} in 1 ml cuvettes. All the measurements were carried out in a Perkin Elmer Lamda 40 UV/VIS spectrophotometer. Protein concentrations were determined by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

For the preparation of crude mitochondrial fractions a modified method after Denicola-Seoane (1992) was used which is described in detail by Chen *et al.* (2001). Briefly, after washing twice at 500 × *g*, the parasite pellet was resuspended in 5 mM HCl-buffered tris(hydroxymethyl)-aminomethane (TRIS-HCl), pH 7.4 at room temperature for 10 min to lyse cells by osmotic shock and then homogenized with a Potter-Elvejhem homogenizer. The broken cells were centrifuged at 1000 × *g* for 10 min to remove cellular debris from the supernatant. Then a crude mitochondrial pellet fraction was obtained by centrifugation for 20 min at 13,000 × *g* which was resuspended in phosphate buffer (pH 7.2) at a protein concentration of 10 mg ml^{-1}.

NADH-FRD activity was measured spectrophotometrically as the fumarate-dependent increase of the rate of NADH-oxidation. One unit of fumarate reductase is the amount of protein that oxidizes 1 μmol of NADH per min in the presence of fumarate. Analysis was performed at a final protein concentration of *ca.* 0.1 mg/ ml in 1 ml cuvettes.

Results

Most of the aurones and auronols tested (11 out of 14) showed strong inhibition of *Leishmania major* mitochondrial fumarate reductase (FRD) activity at concentrations of 25 nM or higher. Parasite growth inhibition was recorded in parallel to FRD activity. The aurones were more active than the auronols. Using this well established enzymatic assay, the efficacy of these drugs at 6.25, 12.5, 25.0 and 50.0 nM appeared to be both dose- and structure-dependent. The most efficacious compounds were the aurones **3**, **7** and **10** (Table I) which, at 25 nM, inhibited mitochondrial FRD activity by

Table I. Effect of aurones and auronols on the inhibition of the mitochondrial NADH-fumarate reductase of *L. major* promastigotes. Data are given as percentage (%) ± standard deviation from four experiments. The FRD activity in the medium control (max) was 17.85 ± 2.7 nM min^{-1} mg^{-1}.

Compound	NADH-fumarate reductase activity [% of max] 6.25 nM	12.5 nM	25.0 nM	50.0 nM
1	5.6 ± 5.4	6.6 ± 4.6	13.8 ± 3.9	24.9 ± 3.8
2	5.7 ± 4.4	5.9 ± 4.8	8,9 ± 2.5	10,8 ± 5.6
3	63.3 ± 3.5	94.3 ± 1.8	97.4 ± 1.4	99.1 ± 1.3
4	12.9 ± 1.9	13.9 ± 2.5	90.4 ± 5.9	79.3 ± 3.6
5	10.2 ± 5.7	36.2 ± 3.6	88.4 ± 1.5	95.6 ± 0.7
6	17.0 ± 3.8	51.9 ± 2.9	91.7 ± 1.8	97.6 ± 0.5
7	12.8 ± 5.3	60.1 ± 4.0	95.4 ± 1.3	97.5 ± 0.6
8	14.8 ± 3.6	48.7 ± 4.2	83.6 ± 1.6	97.8 ± 0.4
9	18.6 ± 2.5	60.6 ± 3.4	91.4 ± 1.2	96.5 ± 0.8
10	39.5 ± 2.5	85.2 ± 1.5	96.3 ± 0.8	96.4 ± 0.5
11	12.6 ± 4.4	21.1 ± 4.5	18.0 ± 3.5	33.2 ± 3.8
12	13.6 ± 3.8	36.4 ± 2.5	85.5 ± 1.8	98.8 ± 0.3
13	37.8 ± 3.7	56.4 ± 3.8	79.8 ± 3.4	93.2 ± 1.0
14	49.6 ± 2.9	72.9 ± 1.8	83.6 ± 1.3	91.5 ± 0.7
Licochalcone A	24.7 ± 2.8	46.6 ± 3.9	75.7 ± 3.7	96.6 ± 1.8

97.4, 95.4 and 96.3%, respectively (untreated control = 0%). At the highest concentration (50 mM) further aurones, *e.g.* **6**, **8**, **9**, and **12** and the auronol **14** revealed similar inhibitory efficacy (97.6, 97.8, 96.5, 96.4 and 83,6%, respectively). However, at concentrations below 25 mM, only the aurones **3** and **10** and the auronol **14** remained active (Table I). Interestingly, the parent compound **1** did not have any appreciable effect on respiratory mitochondrial function (<15%) at any concentration.

Discussion

The presented data show strong inhibitory activity of certain aurones and auronols on *L. major* mitochondrial fumarate reductase. The efficacy of the tested aurones and auronols appeared to be both dose- and structure-dependent, albeit the limited number of compounds tested allows only a preliminary evaluation of structure-activity relationships. The differences in activity at concentrations of 25 μg ml^{-1} and below may result from a number of parameters, including the number of chemical group substituents, the oxygenation pattern of the aromatic rings, and structural rigidity. In view of the inactive parent structure 6-hydroxy-benzofuran-3*(2H)*-one (**1**), the presence of a phenyl (B-) ring seems to be essential for this class of compounds to effectively inhibit FRD activity. However, as illustrated by the quasi inactive compounds **2** and **11**, a second aromatic ring by itself is not sufficient. Instead, antiparasitic activity seems to depend on the number and type of oxygen substituents at the B-ring as well. In comparison to the parent compound **1** highly active compounds like **3**, **7**, and **10** possess one or two additional oxygen substituents at C-4 and C-4′, indicative of a lipophilic nature. This high activity was documented by FRD inhibition rates of 97.4, 95.4, and 96.3%, respectively, at compound concentrations of 25 nM in comparison to **1** (13.8%) or untreated control (0%). Looking at moderately active aurones, a further increase in oxygen substituents at the aromatic rings as revealed by compounds **6**, **8**, **9**, and **12** might be responsible for reduced inhibitory activity at lower concentration (<25 nM). On the other hand, differences in activity were minimal for most test compounds (11 out of 14) at the highest concentration (50 nM) which nearly completely inhibited l FRD activity throughout.

In conclusion, this study shows that a novel class of naturally occurring compounds structurally related to the chalcones, inhibit mitochondrial functions which may be the cause for their published growth inhibitory activity on *L. major* parasites. The precise mechanism of this activity and the reason for its selectivity for mitochondria of the protozoan pathogen is still unknown. Nevertheless, these findings may open a new outlook for the chemotherapy of leishmaniasis at a time when drugs for clinical use are seriously lacking.

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