

Role of mitochondria in the leishmanicidal effects and toxicity of acyl phloroglucinol derivatives: nemorosone and guttiferone A

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SUMMARY

Nemorosone (Nem) and guttiferone A (GutA) are acyl phloroglucinol derivatives (APD) that are present in different natural products. For both compounds anti-cancer and anti-microbial properties have been reported. In particular, an anti-leishmanial activity of both compounds was demonstrated. The aim of this study was to explore the possible role of mitochondria in the anti-leishmanial activity of Nem and GutA in comparison with their action on mammalian mitochondria. Both APD inhibited the growth of promastigotes of *Leishmania tarentolae* (LtP) with half maximal inhibitory concentration (IC₅₀) values of 0.67 ± 0.17 and 6.2 ± 2.6 μM; while IC₅₀ values for cytotoxicity against peritoneal macrophages from BALB/c mice were of 29.5 ± 3.7 and 9.2 ± 0.9 μM, respectively. Nemorosone strongly inhibited LtP oxygen consumption, caused species-specific inhibition ($P < 0.05$) of succinate:ubiquinone oxidoreductase (complex II) from LtP-mitochondria and significantly increased ($P < 0.05$) the mitochondrial superoxide production. In contrast, GutA caused only a moderate reduction of respiration in LtP and triggered less superoxide radical production in LtP compared with Nem. In addition, GutA inhibited mitochondrial complex III in bovine heart submitochondrial particles, which is possibly involved in its mammalian toxicity. Both compounds demonstrated at low micromolar concentrations an effect on the mitochondrial membrane potential in LtP. The present study suggests that Nem caused its anti-leishmanial action due to specific inhibition of complexes II/III of mitochondrial respiratory chain of *Leishmania* parasites that could be responsible for increased production of reactive oxygen species that triggers parasite death.

Key words: *Leishmania tarentolae*, mitochondria, acyl phloroglucinol derivatives, nemorosone, guttiferone A.

INTRODUCTION

The biological diversity of natural products and their ingredients renewed the interest in the use of natural compounds for medical applications and, more importantly, their role as a basis for drug development. During last decades, some promising advances had been achieved in understanding the chemistry and pharmacology of acyl phloroglucinol derivatives (APD), which are non-polar phenolic compounds. Their hydrophobicity increases with the number of attached prenyl functional groups (Kumar *et al.* 2013; Terrazas *et al.* 2013). Acyl phloroglucinol derivatives are major intermediates in the biosynthetic pathway of xanthenes and occur in plants of the *Clusiaceae* family. In scientific literature, APD have been recognized as interesting due to their biologically active secondary metabolites

(aristophenones, bronianone, clusianone, garcicowin, garcimultiflorone, guttiferones, isogarcinol and xanthochymol) (Protiva *et al.* 2008; Kumar *et al.* 2013; Terrazas *et al.* 2013). They exhibit significant cytotoxic, anti-microbial, anti-oxidant and anti-inflammatory activities (Diaz-Carballo *et al.* 2012; Nunez-Figueroa *et al.* 2012; Kumar *et al.* 2013).

In recent years, two APD, nemorosone (Nem) and guttiferone A (GutA) have been subjected to pharmacological and toxicological studies for testing their anti-cancer (Cuesta-Rubio *et al.* 2002) and anti-microbial properties (Monzote *et al.* 2011). This research has demonstrated the anti-leishmanial activity of APD and their semi-synthetic derivatives (Lenta *et al.* 2007; Monzote *et al.* 2011; Filho *et al.* 2013; Fromentin *et al.* 2013). Recently, the efforts have primarily been directed towards understanding the possible targets and mechanisms of action through which these compounds arrest cancer cells and inhibit the progression of the cell-cycle (Pardo-Andreu *et al.* 2011a, b). Although the potential involvement of mitochondria in the toxicity of GutA (Pardo-Andreu *et al.* 2011a) and

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Nem (Pardo-Andreu *et al.* 2011b) on (mammalian) cancer cells have been addressed, to our knowledge, no molecular studies on anti-microbial, in particular anti-leishmanial mechanisms of action have been reported. Therefore, the aim of this study was to explore the possible role of mitochondria in the anti-leishmanial activity of Nem and GutA in comparison with their action on mammalian mitochondria.

MATERIALS AND METHODS

Chemicals

Diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), glucose, KCN, K_2HPO_4 , KH_2PO_4 , Na_2HPO_4 , NaCl, KCl, HCl, NaN_3 , succinate, sucrose and TRIS were obtained from Merck (Germany). Bovine serum albumin (BSA), cytochrome c^{3+} (cyt c^{3+}), decylubiquinone (dUQ), heat-inactivated fetal bovine serum (HFBS), hemin, 2,6-dichlorophenol-indophenol (DCPIP), 3-carboxy-proxyl (CP*), reduced β -nicotinamide adenine dinucleotide (NADH), penicillin–streptomycin solution, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol–carbocyanine iodide (JC-1) and resazurin were purchased from Sigma-Aldrich (Germany). Pentamidine was obtained from Richet (Buenos Aires, Argentina). Dimethyl sulfoxide (DMSO), desferal (DFO) and triethanolamine (TEA) were from Roth (Germany), Novartis Pharma (Germany) and Fluka (Germany), respectively; while 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-HCl (CMH) and 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH) were from Noxygen (Germany). Yeast extract powder was supplied by Amresco (USA). Idebenone (IQ) was obtained from Takeda (Japan). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma (USA). Decylubiquinol (dUQH₂) was prepared from dUQ as described previously (Gille *et al.* 2001).

Studied APD compounds

Nemorosone and GutA (Fig. 1) were purified from flowers of *Clusia rosea* and fresh fruit of *Garcinia aristata*, respectively (Monzote *et al.* 2011). The purity of both compounds was >98%. Compounds were dissolved in DMSO.

Macrophages

Peritoneal macrophages from mouse (PMM) were isolated at moment of use from normal female BALB/c mice in ice-cold RPMI 1640 medium (SIGMA, St. Louis, Mo, USA) supplemented with antibiotics (100 μ g streptomycin mL^{-1} and 100 U penicillin mL^{-1}).

Leishmania

Leishmania tarentolae promastigotes (LtP) strain P10 from Jena Bioscience were used. Parasites were cultivated at 26 °C in the yeast extract medium (YEM) consisting of 20.7 g L^{-1} yeast extract powder, 1.2 g L^{-1} K_2HPO_4 , 0.2 g L^{-1} KH_2PO_4 , 2.9 g L^{-1} glucose, 5 mg L^{-1} hemin and 50 000 U L^{-1} penicillin – 50 mg L^{-1} streptomycin in 50 mL Saarestedt tubes with gas-permeable caps and agitation in a tube shaker (0.05 s^{-1}).

Influence of APD on cell viability

To analyse the activity of APD in LtP, experiments were carried out to determine the viability of parasites after treatment. *Leishmania tarentolae* promastigotes in YEM were distributed in 96-well plates at 8×10^6 cells mL^{-1} . Compounds were added and five 1:3 serial dilutions were performed. Control rows with YEM (no activity) and with untreated (vehicle) LtP (100% activity) were loaded. Then, 50 μ L of resazurin stock solution was added in each well (20 μ M final conc.) and the plate was incubated at 26 °C during 48 h. Finally, images of the plates were recorded with a Canon EOS 300D camera and reduction levels of resazurin were quantified by the PLReader Software (0.3.8, 2014) using the absorption of the red channel minus the absorption in the green channel in analogy to (Borra *et al.* 2009). In parallel, the cytotoxicity of the compounds in PMM was studied. Macrophages were harvested and distributed to 96-well plates ($1-6 \times 10^5$ PMM mL^{-1}) and incubated at 37 °C under an atmosphere of 5% CO_2 for 2 h. Non-adherent cells were removed and 2 μ L of APD stock solutions were added to 98 μ L of RPMI medium containing 10% HFBS and antibiotics (100 μ g of streptomycin mL^{-1} and 100 U penicillin mL^{-1}). Following, 1:2 serial dilutions were performed and macrophages treated with 2 μ L DMSO were included as controls. The plates were incubated for 72 h at 37 °C and 5% CO_2 . Afterwards the cytotoxicity was determined using the colorimetric assay based on MTT reduction. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solutions were prepared at 5 mg mL^{-1} in saline solution, sterile-filtered immediately before use, and 15 μ L were added to each well. After incubation for additional 4 h, supernatant was eliminated and the accumulated formazan crystals were dissolved by addition of 100 μ L DMSO per well. The optical density was determined using an EMS Reader MF Version 2.4-0, at a wavelength of 560 nm using 630 nm as reference wavelength. In both cases, pentamidine was used as reference drug.

Influence of APD on oxygen consumption

To assay the effect of compounds on oxygen consumption of LtP, a Clark-type oxygen electrode

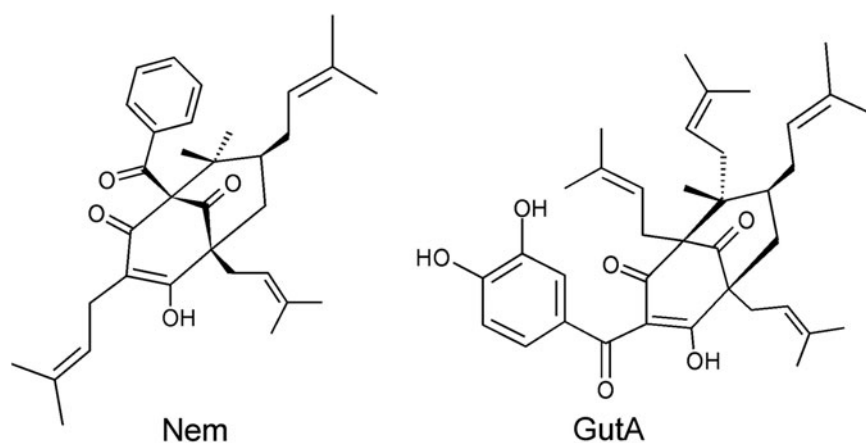


Fig. 1. Chemical structures of studied acyl phloroglucinol derivatives nemorosone (Nem) and guttiferone A (Gut A).

(Hansatech, Germany) and software MCREC were used. *Leishmania tarentolae* promastigotes at 10^8 cells mL^{-1} in YEM were added and treated with increasing concentrations of test compounds between 25 and $400 \mu\text{M}$. Each concentration was assayed in quintuplicates and the results were expressed as percentage of oxygen consumption in comparison with the control LtP treated with the solvent (DMSO).

Influence of APD on mitochondrial complexes

Isolation of the mitochondrial fraction from LtP. To isolate the mitochondrial fraction (LtP-Mit) from LtP, a culture of 2700 mL of LtP was centrifuged for 10 min at 1900 g and 20°C and the supernatant was discarded. The cell pellet was resuspended in buffer (10 mM TRIS-HCl, 0.3 M sucrose, 0.2 mM EDTA, 0.2% BSA, pH 7.4) and washed in two extra centrifugation steps for 10 min at 1900 g and 20°C . The washed cell pellet was incubated in lysis buffer (5 mM TRIS-HCl, pH 7.4) for 10 min at 20°C and subsequently homogenized in a dounce homogenizer. Cell debris was removed by centrifugation for 10 min at 1900 g and 4°C . The supernatant was centrifuged for 20 min at $13\,200 \text{ g}$ and 4°C to sediment the crude mitochondrial fraction. Mitochondrial fractions from LtP were resuspended in 1 mL buffer (250 mM sucrose, 50 mM KH_2PO_4 , 0.2 mM EDTA, pH 7.2) and stored in liquid nitrogen until use.

Isolation of submitochondrial particles from bovine heart. Bovine heart submitochondrial particles (BH-SMP) were obtained from bovine heart mitochondrial suspensions (Nohl and Hegner, 1978). Mitochondrial suspensions were sonicated ten times for 30 s with 1 min interruption at 4°C in buffer (0.25 M sucrose, 10 mM TRIS, 1 mM EDTA, pH 9.0) using a Branson Sonifier set to 40 W energy output. Remaining mitochondria were removed by a centrifugation at 6500 g for 10 min

at 4°C and afterwards BH-SMP were sedimented from the supernatant by centrifugation at $95\,000 \text{ g}$ for 30 min. Pellets were resuspended in buffer (0.25 M sucrose, 10 mM TRIS, 1 mM EDTA, pH 7.4) and stored in liquid nitrogen until use. Protein was assayed by the Biuret method using BSA as standard (Gornall *et al.* 1949).

Inhibition of mitochondrial electron transfer activities. Reduced β -nicotinamide adenine dinucleotide:ubiquinone oxidoreductase (complex I) and succinate:ubiquinone oxidoreductase (complex II) activities were measured in 96-well plates using DCPIP as terminal electron acceptor. In each well, $200 \mu\text{L}$ of pre-mix containing buffer (250 mM sucrose, 20 mM TEA and 1 mM EDTA, pH 7.4), DCPIP ($75 \mu\text{M}$), KCN (1 mM), BSA (4.3 mg mL^{-1} for complex I and 1.25 mg mL^{-1} for complex II), IQ ($62.5 \mu\text{M}$) and LtP-Mit ($170 \mu\text{g protein mL}^{-1}$) or BH-SMP ($10 \mu\text{g protein mL}^{-1}$) were added. In addition, in the first row, $97 \mu\text{L}$ of pre-mix and $3 \mu\text{L}$ of compounds were added. Subsequently, six 1:3 serial dilutions were carried out, transferring always $100 \mu\text{L}$. The reaction was started by adding $50 \mu\text{L}$ of start-mix per well with NADH (1.5 mM final conc.) for complex I or succinate (4 mM final conc.) for complex II, dissolved in the same buffer. After start of the reaction, images of the plates were recorded with a Canon EOS 300D camera in 2 min intervals (20 absorbance measurements). Reduction of DCPIP ($\epsilon_{600 \text{ nm}} = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$) as function of time was quantified by PLReader software using the absorption of the red channel minus the absorption of the green channel. For each individual concentration triplicates were measured and two evaluations were carried out.

To measure the inhibition of ubiquinol:cytochrome c oxidoreductase (complex III) dUQH₂ ($75 \mu\text{M}$) and cyt c^{3+} ($100 \mu\text{M}$) were used as substrates. The reduction of cyt c^{3+} in the presence of dUQH₂ was monitored at 550 nm using 540 nm as reference in buffer (250 mM

sucrose, 50 mM KH_2PO_4 , 0.2 mM EDTA, pH 7.2, 2 mM KCN, 4 mM NaN_3) (Müllebnner *et al.* 2010). Test compounds were added at different concentrations 120 s prior to addition of dUQH₂ starting the enzymatic reaction. In the presence of test compounds, the residual dUQH₂:cyt c³⁺ oxidoreductase activity of BH-SMP (3.2 μg protein mL^{-1}) and LtP-Mit (40 μg protein mL^{-1}) was obtained and expressed in percentage of the non-inhibited rate (100%). For all test compounds the absence of significant reactions with dUQH₂ and cyt c³⁺ was verified in the absence of mitochondrial preparations. All compound concentrations were tested in triplicate and the reduction rates for cyt c³⁺ were calculated using the extinction coefficient from the time trace of the absorption difference at 550 nm minus 540 nm ($\epsilon_{550-540\text{ nm}} = 19\text{ mM}^{-1}\text{ cm}^{-1}$). The specific activities in the absence of xenobiotics were: complex I (NADH:DCPIP) in BH-SMP 2541 $\text{nmol min}^{-1}\text{ mg}^{-1}$ and LtP-Mit 97 $\text{nmol min}^{-1}\text{ mg}^{-1}$, complex II (Succinate:DCPIP) in BH-SMP 2784 $\text{nmol min}^{-1}\text{ mg}^{-1}$ and LtP-Mit 87 $\text{nmol min}^{-1}\text{ mg}^{-1}$ and complex III (dUQH₂:cyt c³⁺) in BH-SMP 4906 $\text{nmol min}^{-1}\text{ mg}^{-1}$ and in LtP-Mit 87 $\text{nmol min}^{-1}\text{ mg}^{-1}$.

Influence of APD on O₂⁻ production

Oxidation of CMH or CPH to stable nitroxyl radicals detectable by electron spin resonance (ESR) spectroscopy was used to measure O₂⁻ production. Measurements were performed in PBS (136 mM NaCl, 1.15 mM KH_2PO_4 , 14 mM Na_2HPO_4 , 2.7 mM KCl, pH 7.4) containing 100 μM DFO and 25 μM DTPA. Before measurements of LtP cell suspensions, cells were washed twice with PBS to remove the YEM. Bovine heart submitochondrial particles (1.08 mg mL^{-1}), LtP-Mit (0.85 mg mL^{-1}) or LtP (5×10^8 cells mL^{-1}) were suspended in the PBS. Samples with LtP cells were supplemented with 15 mM glucose. For samples with mitochondria 12 mM succinate was added as substrate. Subsequently APD stock solutions in DMSO were added. Prior to the ESR measurement 400 μM CPH (for mitochondria) or 400 μM CMH (for cells) were added. For ESR measurements 17 μL of the suspension were aspirated in a gas permeable Teflon tube (ID 0.7 mm). This capillary tube was placed in the resonator of the ESR instrument [Bruker EMX (Germany), split ring MD5] and ten sequential measurements were performed. Following instrument settings were used: microwave frequency 9.682 GHz, modulation frequency 100 kHz, modulation amplitude 1 G, time constant 0.082 s, centre field 3446 G, scan rate 71 G min^{-1} , sweep width 100 G, scan time 84 s and attenuation CPH: 2×10^4 CMH: 7.96×10^3 . From the ESR spectra the middle peak-to-peak intensity was retrieved and concentrations of oxidized CPH and CMH were obtained by comparison with a standard

curve prepared from CP' solutions with defined concentrations.

Influence of APD on mitochondrial membrane potential

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) was used to investigate the changes in mitochondrial membrane potential of LtP. From a stock solution of JC-1 (6.1 mM) in DMSO a diluted solution (15 μM JC-1) in PBS (pH 7.4) and 15 mM glucose was prepared. The cell density of LtP was adjusted to approximately 5×10^7 LtP cells mL^{-1} . *Leishmania tarentolae* promastigotes cultured in YEM were centrifuged in a sterile 50 mL tube for 10 min at 2000 *g* at 20–25 °C. After the centrifugation step, the supernatant was discarded and the cell pellet was resuspended in a corresponding volume of the diluted JC-1 solution. To load cells with JC-1 they had to be incubated for 30 min under dark conditions in the incubator (26 °C). Then a washing step with 10 min centrifugation at 2000 *g* at 20–25 °C was included. The supernatant was removed and the pellet resuspended in a PBS–glucose solution (without JC-1). Subsequently, 200 μL cell suspension loaded with JC-1 were placed in each well of a black 96-well culture plate. In the third row 100 μL extra suspension were added and in the respective wells Nem (200 μM final conc.) or GutA (200 μM final conc.) were placed. Afterwards, a serial dilution (1:3) into the rows below was performed (200–0.82 μM).

Fluorescence measurements (Perkin Elmer Enspire) were started after 4 h of incubation using an excitation wavelength of 488 nm and two emission wavelengths: 530 nm for JC-1 monomers and 590 nm for JC-1 dimers. The fluorescence intensity ratio ($F_{590\text{ nm}}/F_{530\text{ nm}}$) of control cells (including DMSO) were set to 100% and ratios of other samples were expressed in relation to this value. All samples were measured in triplicates.

Statistical analyses

Percentages of residual activity in relation to control experiments (100%) were calculated at each concentration of APD. Resulting activity–concentration plots were used to determine the half maximal inhibitory concentration (IC₅₀) values by a non-linear regression according to a four parameter logistic model (4PL, Hill-Slope model) (Müllebnner *et al.* 2010) using Origin 6.1 (OriginLab Corporation). The results were expressed as mean \pm s.d. In case of PMM, the IC₅₀ value was obtained by fitting a sigmoidal E_{max} model to dose–response curves (Bodley *et al.* 1995). Selectivity indices were calculated by dividing the IC₅₀ values for PMM by the IC₅₀ values for LtP.

RESULTS

Influence of APD on cells viability

Both APD inhibited the growth of LtP and caused partial cytotoxicity in PMM (Table 1). Higher and more selective anti-leishmanial activity ($P < 0.05$) was observed by Nem; whereas GutA was less specific for LtP and showed similar IC_{50} values in PMM ($P > 0.05$). In addition, Nem displayed higher activity and selectivity ($P < 0.05$) compared with pentamidine, a clinically used drug.

Influence of APD on L. tarentolae oxygen consumption

To elucidate the role of mitochondria in the anti-leishmanial activity of Nem and GutA we studied the effect of these APD on the oxygen consumption of LtP cells. Inhibitions of oxygen consumption caused by Nem and GutA with respect to cultures treated with solvent are shown in Fig. 2. The derived IC_{50} values demonstrated that Nem strongly inhibited LtP respiration; while only a moderate reduction of oxygen consumption was observed at GutA concentrations $> 50 \mu M$. The solvent DMSO caused an inhibition lower than 1% with respect to untreated control cultures.

Influence of APD on activities of mitochondrial complexes

Since inhibition of oxygen consumption was observed in LtP cells, it was obvious to study the influence of Nem and GutA on the possible targets in the respiratory chain. Both APD are lipophilic and, therefore, competition with the lipophilic electron carrier ubiquinone at its binding sites (complex I – III) is expected. Table 2 shows the effect of both APD on complex I–III activities in LtP-Mit and BH-SMP. Nemorosone caused species-specific inhibition ($P < 0.05$) of succinate:ubiquinone oxidoreductase (complex II) from LtP-Mit and was less active in BH-SMP. However, for NADH:ubiquinone oxidoreductase (complex I) Nem strongly inhibited the activities in BH-SMP but not in LtP-Mit under our conditions ($P < 0.05$). For the ubiquinol:cytochrome c oxidoreductase activity (complex III) similar IC_{50} values ($P > 0.05$) were obtained for both mitochondrial models treated with Nem. In the case of GutA, the data show that it exclusively interfered in the complex III activity of BH-SMP.

Influence of APD on O_2^- production

Inhibition of mitochondrial respiratory complexes can trigger mitochondrial superoxide radical production. The effectivity of xenobiotics in this respect strongly depends on their structure and corresponding binding sites. Therefore, we studied this parameter in BH-SMP, LtP-Mit and LtP cells using

the oxidation of cyclic hydroxylamines (CMH and CPH) in combination with ESR spectroscopy as detection method. In all experiments, oxidation rates of CPH or CMH induced by mitochondrial fractions and cells in the presence of substrates (succinate or glucose) were considered as appropriate control (100%). In mitochondrial fractions without succinate, CPH oxidation rates were slightly higher in comparison with the respective controls possibly due to direct interaction of oxidized cyt c with CPH involving no $O_2^{\cdot-}$. In BH-SMP (Fig. 3), the well-known inhibitor of mammalian mitochondria antimycin A (AA) triggered an enormous increase of CPH oxidation. Likewise, Nem caused a significant increase of CPH oxidation rates; while GutA caused only a non-significant elevation. Using the same concentrations of xenobiotics in LtP (Fig. 4) Nem, GutA and AA resulted in a significant increase in CMH oxidation; however, only Nem caused a more than triplication of CMH oxidation rates. This effect is qualitatively confirmed in LtP-Mit respiring succinate (Fig. 5). In this system, again CPH oxidation rates were more than duplicated in the presence of Nem. In contrast, both GutA and AA caused only non-significant changes in succinate-respiring LtP-Mit.

Influence of APD on mitochondrial membrane potential in LtP

Both Nem and GutA decreased the mitochondrial membrane potential in LtP (Fig. 6), while the effect of GutA was stronger than that of Nem at equal concentrations. In comparison with the positive control with the ionophore valinomycin ($10 \mu M$) which reduced the membrane potential to $42 \pm 0.2\%$, GutA showed an even stronger effect at similar concentrations.

DISCUSSION

A substantial number of leishmanicidal drugs have the mitochondrion as at least one of their targets. These include drugs clinically used or in clinical trials such as: pentamidine, sitamaquine and paromomycin, and anti-leishmanial agents in pre-clinical studies, including licochalcones, phenylphenanones, aurones and others (Monzote *et al.* 2011). The study of the mitochondrion as a likely target for APD is obvious since previous evidences exist that these compounds target mitochondria in cancer cells.

As a *Leishmania* model the LtP, a lizard-infecting species (*Sauroleishmania*) was used which is not pathogenic to humans.

In a previous study, both compounds caused growth inhibition against intracellular amastigotes of *Leishmania amazonensis* (Nem: $IC_{50} = 11.2 \pm 0.6 \mu M$, GutA $IC_{50} = 15.6 \pm 0.1 \mu M$) and *Leishmania*

Table 1. Effect of nemorosone (Nem) and guttiferone A (GutA) on the viability of *L. tarentolae* promastigotes and peritoneal macrophages from BALB/c mice

Products	IC ₅₀ ^a ± s.d. ^b (μM)		Selectivity index ^c
	Promastigotes	Macrophages	
Nem	0.67 ± 0.17	29.5 ± 3.7	44
GutA	6.2 ± 2.6	9.2 ± 0.9	1
Pentamidine ^d	8.0 ± 2.8	11.7 ± 1.7	1

^a IC₅₀: Concentration that caused 50% of mortality.

^b s.d.: Standard deviation.

^c Selectivity index: IC₅₀ in macrophages/IC₅₀ in promastigotes.

^d Pentamidine was included as reference drug.

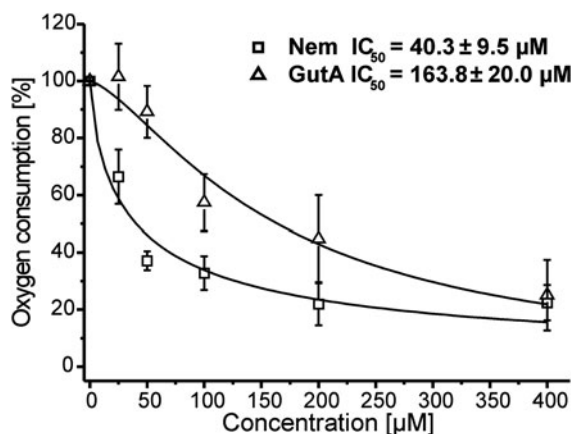


Fig. 2. Influence of nemorosone (Nem) and guttiferone A (GutA) on cellular oxygen consumption of *L. tarentolae* promastigotes. Respiration rates (15.9 ± 3.2 nmol O/min/ 10^8 cells) in the absence of xenobiotics were set to 100% and used as a reference for other measurements. Data represent mean ± s.d. of five independent experiments. For curve fitting and calculation of IC₅₀ values a four parameter logistic model was used.

infantum (Nem: IC₅₀ = 32.9 ± 5.4 μM, GutA IC₅₀ = 13.5 ± 0 μM), causal pathogens of cutaneous and visceral leishmaniasis, respectively (Monzote *et al.* 2011). Although in the present study different parasite species and models have been used, in most cases Nem demonstrated a stronger inhibitory activity and higher selectivity for *Leishmania* (Table 1) compared with GutA. As LtP showed also sensitivity to both studied APD, experiments were carry out to explore the role of mitochondria in the leishmanicidal and toxicity effects caused by Nem and GutA. In addition, LtP have been employed in several pharmacological investigations, such as drug screening (George *et al.* 2006), amplification of genes involved in the resistance to certain anti-leishmanial drugs such as amphotericin B (Singh *et al.* 2001) and in the purification and characterization of proteins used as potential anti-leishmanial targets (Fritsche *et al.* 2007).

Since mitochondria are the principal site for generation of cellular ATP, which is vital for cell

function, mitochondrial dysfunction could trigger death. As in most eukaryotes, the *Leishmania* mitochondrion is also responsible for cellular respiration, which occurs via the electron transport chain (ETC) containing four complexes located in the inner mitochondrial membrane (Roy *et al.* 2008). Mitochondrial functions can be affected by the compounds directly or indirectly. To assess mitochondrial involvement in the anti-leishmanial effects of APD we compared the influence of APD on mitochondrial functions in mammalian mitochondria (BH-SMP) and leishmanial mitochondria (LtP-Mit). Inhibition of oxygen consumption was observed in LtP with both compounds. However, only Nem produced significant inhibition of oxygen consumption at concentrations (IC₅₀ = 40.3 ± 9.5 μM, Fig. 2) which could be relevant for anti-leishmanial effects. GutA with an IC₅₀ value of 163.8 ± 20.0 μM was much less effective in respiration inhibition.

For Nem higher concentrations were required to inhibit oxygen consumption than to inhibit viability, which suggests a cumulative effect. Due to the different time scales of viability (72 h) and oxygen consumption assays (0.5 h) both assays reflect different phases of Nem and GutA action on *Leishmania*. While the viability assay gives information about the final outcome of the experiments, oxygen consumption inhibition is an indicator of direct targets in the mitochondrial ETC. Typical consequences of such processes can be elevated formation of reactive oxygen species (ROS) and/or shortage of cellular ATP (by ETC inhibition or uncoupling) which could gradually contribute to cell death. Depending on the cellular ATP and ROS status already minor inhibition of ETC in the long run could be sufficient to compromise viability. In addition, the possibility that Nem targets other cellular functions in *Leishmania* parasites cannot be excluded, since many known anti-leishmanial compounds act via multiple mechanisms.

Likewise the assays on individual electron transfer complexes (Table 2) gave information about a direct effect of APD on mitochondria. In our assays Nem displayed a versatile activity on mitochondrial

Table 2. Effect of nemorosone (Nem) and guttiferone A (GutA) on electron transport chain complexes from LtP-Mit and BH-SMP

Mitochondrial complexes	IC ₅₀ ± s.d. (μM) ^{ab}			
	Nem		GutA	
	LtP-Mit ^c	BH-SMP ^d	LtP-Mit ^c	BH-SMP ^d
Complex I	>100	2.0 ± 0.8*	>100	>100
Complex II	36.5 ± 7.2*	>100	>100	>100
Complex III	39.6 ± 1.9	30.4 ± 4	>100	2.0 ± 0.2*

^a IC₅₀: Concentration that caused 50% of inhibition.

^b s.d.: Standard deviation.

^c LtP-Mit: Mitochondrial fraction from *L. tarentolae* promastigotes.

^d BH-SMP: Submitochondrial particles from bovine heart.

* Depicts statistically significant differences ($P < 0.05$, Student's *t*-test) between LtP-Mit and BH-SMP.

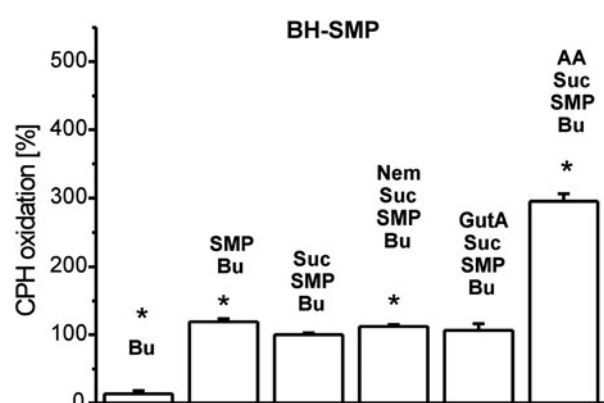


Fig. 3. Superoxide radical production in submitochondrial particles from bovine heart (BH-SMP) measured by oxidation of CPH (400 μM) and detection by ESR spectroscopy. The CPH oxidation rate in the presence of buffer (Bu), succinate (Suc, 12 mM) and SMP (1.08 mg mL⁻¹) was set to 100% and other rates were expressed in relation to this reference. Xenobiotics present were nemorosone (Nem, 100 μM), guttiferone A (GutA, 100 μM) and antimycin A (AA, 5 μM). Data represent mean ± s.d. of four independent experiments. *Statistical significant differences in comparison with the Suc/SMP/Bu sample were identified on the level $P < 0.05$ by Student's *t*-test.

complexes. A specific inhibition of complex II from LtP-Mit was observed, with IC₅₀ values in same range ($P > 0.05$) as IC₅₀ values for oxygen consumption. Although complexes I and III are the main producers of ROS in mammalian cells (Murphy, 2009), complex II may also act as a source of ROS (Ishii *et al.* 2007). Since *Leishmania* parasites have a limited electron transport between complexes I–III, succinate might be a primary electron donor for energy production (Santhamma and Bhaduri, 1995). Notably, recent reports described specific inhibition of complex II as good target in *Leishmania*, including benzophenone-derived bisphosphonium salts (Luque-Ortega *et al.* 2010), phenyl-phenalenones

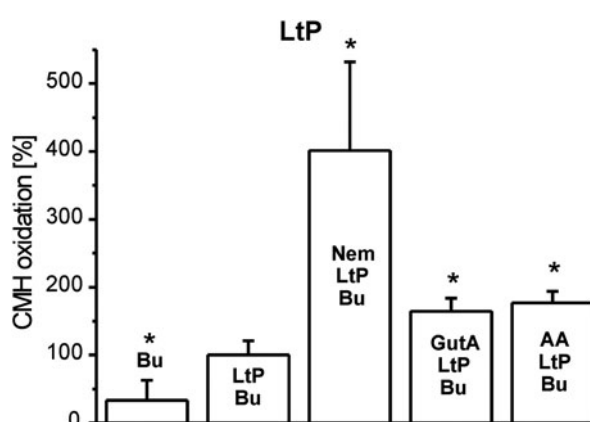


Fig. 4. Superoxide radical production in *L. tarentolae* promastigotes (LtP) measured by oxidation of CMH (400 μM) and detection by ESR spectroscopy. The CMH oxidation rate in the presence of PBS containing 15 mM glucose (Bu), and LtP (5 × 10⁸ cells mL⁻¹) was set to 100% and other rates were expressed in relation to this reference. Xenobiotics present were nemorosone (Nem, 100 μM), guttiferone A (GutA, 100 μM) and antimycin A (AA, 5 μM). Data represent mean ± s.d. of four independent experiments. *Statistical significant differences in comparison with the LtP/Bu sample were identified on the level $P < 0.05$ by Student's *t*-test.

(Luque-Ortega *et al.* 2004) and thenoyltrifluoroacetone (Mehta and Shaha, 2004). In parallel, inhibition of complex III was observed, although a similar activity was also observed in BH-SMP. Based on these data, complex II inhibition by Nem in LtP could be responsible for the observed inhibition of oxygen consumption in LtP.

A complex I assay was also performed for LtP-Mit and BH-SMP, which revealed a strong inhibition by Nem in mammalian mitochondria. However, since complex I in LtP is considerably different from mammalian complex I (not rotenone sensitive, not significantly linked to oxygen consumption) this difference in inhibition could also have principal reasons. In contrast, GutA did not interfere with

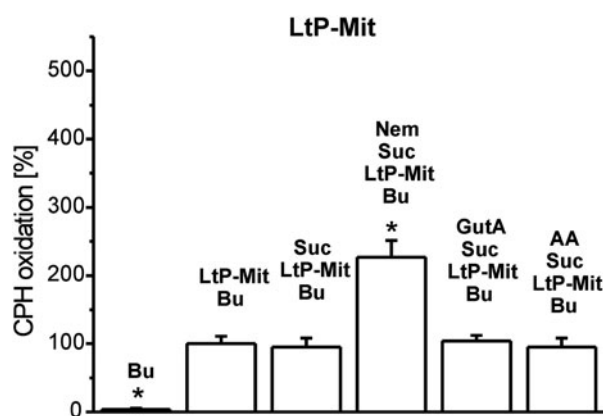


Fig. 5. Superoxide radical production in mitochondria from *L. tarentolae* promastigotes (LtP-Mit) measured by oxidation of CPH (400 μM) and detection by ESR spectroscopy. The CPH oxidation rate in the presence of buffer (Bu), succinate (Suc, 12 mM) and LtP-Mit (4.04 mg mL⁻¹) was set to 100% and other rates were expressed in relation to this reference. Xenobiotics present were nemorosone (Nem, 100 μM), guttiferone A (GutA, 100 μM) and antimycin A (AA, 5 μM). Data represent mean \pm S.D. of four independent experiments. *Statistical significant differences in comparison with the Suc/LtP-Mit/Bu sample were identified on the level $P < 0.05$ by Student's *t*-test.

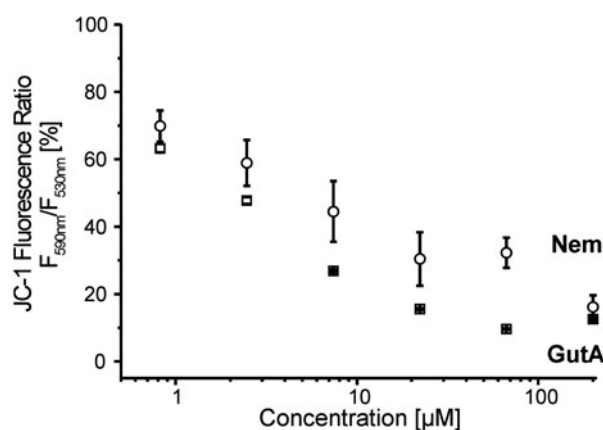


Fig. 6. Mitochondrial membrane potential in *L. tarentolae* promastigotes (LtP) measured by JC-1 in the presence of increasing concentrations of nemorosone (Nem) and guttiferone A (GutA). LtP (5×10^7 cells mL⁻¹) were incubated in PBS containing 15 mM glucose and 15 μM JC-1 for 4 h at 26 °C. Control LtP and LtP with the highest amount of vehicle (DMSO 0.75%) showed $100.0 \pm 0.9\%$ and $102.0 \pm 0.5\%$ relative membrane potential, respectively. The positive control in the presence of 10 μM valinomycin was $42.0 \pm 0.2\%$. Data represent mean \pm S.D. of three independent experiments.

mitochondrial complexes, except a strong inhibition of complex III of BH-SMP. In this context, it is noteworthy that effects of GutA on rat liver mitochondria have been reported, which demonstrated a dissipation of mitochondrial membrane potential, depleted ATP and increased ROS levels (Pardo-Andreu *et al.* 2011a).

Mitochondria have been recognized to produce most ROS via electron transfer in the respiratory chain. Mitochondrial ROS production could be an indirect evidence of impaired mitochondria (Halliwell and Gutteridge, 1990). In our study, in BH-SMP (Fig. 3), LtP (Fig. 4) and LtP-Mit (Fig. 5) Nem significantly increased the mitochondrial superoxide production above control levels at 100 μM , while strongest effects of Nem were observed in LtP and LtP-Mit. Although GutA also triggered superoxide radical production in LtP, it was much less effective than Nem. In liver mitochondria a decrease of H₂O₂ release at low micromolar concentrations of Nem was observed, which, however, was attributed to its uncoupling effect at lower concentrations (Reis *et al.* 2014).

As a parameter susceptible to both direct and indirect effects (lipid peroxidation, permeability transition pore formation) on mitochondria the mitochondrial membrane potential in LtP was studied by the JC-1 method (Fig. 6). Our data demonstrated that at concentrations relevant for inhibiting leishmanial viability, both compounds decreased the membrane potential; however, GutA is stronger than Nem over a wide concentration range. This is also supported by the structural differences: GutA structure provides three dissociable OH groups for H⁺ shuttling over the inner mitochondrial membrane while Nem has only one OH group. In addition, non-protonophoric mechanisms for uncoupling by GutA in mammalian mitochondria were proposed (Pardo-Andreu *et al.* 2011a).

Thus, the mammalian toxicity of GutA could be related to rather specific inhibition of complex III, while the observed anti-leishmanial effects of GutA could have extra-mitochondrial reasons or are related to uncoupling of leishmanial mitochondria but rather not to inhibition of the ETC or to excessive O₂⁻ formation. In this case, the mitochondrial dysfunction observed could be only an indirect consequence. Anti-leishmanial effects of Nem are supported by inhibition of mitochondrial complexes II and III, strongly increased O₂⁻ formation (at higher concentrations) and disruption of the membrane potential (already at lower concentrations). In mammalian mitochondria obviously rather complex I is a major target in the ETC, which differs, however, structurally and functionally from leishmanial complex I equivalent.

Also toxicological effects of other APD explained by mitochondrial dysfunction have been reported. In this context, garcinol (Pan *et al.* 2001), diphenylacetaldehyde (Almeida *et al.* 1999), xanthochymol, guttiferone E and guttiferone H (Protiva *et al.* 2008) were mentioned. Despite Nem and GutA sharing some structural core elements, the mechanism of action involved in their anti-leishmanial activity seems to be different. This was also observed with other APD, the 7-epi-nemorosone and plukenetione

A, which caused anti-retroviral activities by different mechanism of action (Diaz-Carballo *et al.* 2010). In scientific literature, other cellular targets have been suggested for APD, including kinases (Diaz-Carballo *et al.* 2012), G₀/G₁ phase of cell cycle (Popolo *et al.* 2011) and microtubule inhibition (Roux *et al.* 2000).

In summary, the present work represents the first investigation into the mode of anti-leishmanial activity of Nem and GutA. We suggest that Nem caused its anti-leishmanial action due to inhibition of complexes II and III of mitochondrial ETC of *Leishmania* parasites that could cause an increase in ROS production and triggers parasites death. In this sense, inhibition of mammalian mitochondrial complex I by Nem could also explain the cytotoxicity seen in mammalian cells. However, in case of GutA, mitochondrial complex III is involved in mammalian toxicity caused by this compound, but obviously mitochondria are not the primary anti-leishmanial site of action.

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