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Complex-I inhibitor of NADH-ubiquinone oxidoreductases (NDH-1): Rotenone effects on transplasma membrane electron transport system in *Leishmania donovani* promastigotes

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ABSTRACT

The present study concerns with characterization of rotenone interaction with coupling of some nonpermeable electron acceptors such as (a) alpha lipoic acid (ALA); (b) 1,2- naphthoquinone-4-sulphonic acid (NQSA); (c) ferricyanide ($K_3Fe(CN)_6$), which are mainly reduced via an interaction with the redox sites present in the plasma membrane of *Leishmania donovani* promastigotes. The ALA, NQSA and ferricyanide reduction and part of O_2 reduction is shown to take place on the exoplasmic face of the cell, for it is affected by external p^H and rotenone react with the external surface. Redox enzymes of the transplasma membrane electron transport system orderly transfer electron from one redox carrier to the next with the molecular oxygen as the final electron acceptor. The redox carrier mediates the transfer of electrons from metabolically generated reductant to non permeable electron acceptors and oxygen. Our data explores the effect of rotenone on extracellular reduction with ALA, NQSA, and $K_3Fe(CN)_6$. Simultaneously, the oxygen uptake studies by the effect of rotenone with *Leishmania donovani* promastigotes were observed. Studies showed that strong inhibitory effect by rotenone with respect to the reduction of the electron acceptors ALA, NQSA, ferricyanide from the result. Hence, it can be concluded that the rotenone may be potential (complex-I) inhibitor of controlling the membrane function in *Leishmania donovani* promastigotes.

Keywords: Transplasma membrane; Electron transport system; α-lipoic acid reduction; 1,2- naphthoquinone-4-sulphonic acid reduction; Ferricyanide reduction; rotenone; *Leishmania donovani* promastigotes.

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INTRODUCTION

Leishmania donovani is the causative agent of Leishmaniasis or Kala azar and mainly found in tropical or sub-tropical countries [1-3]. The organism has a digenic life cycle and belongs to kinetoplastida group of parasite. *Leishmania donovani* promastigotes, flagellated vector naturally survives and divides in the alimentary tract of female phlebotomine sandfly. When the sandfly bites, the parasite is rapidly internalized in human lives macrophages, where it overcomes the host defense mechanism and undergoes facile morphogenic transformation of oval, a flagellated amastigote form in the phagolysosomal complex of the macrophages [4]. Rapid proliferation and invasion of neighboring macrophages indicate pathogenesis [5]. The two distinct habitat of this parasite differ primarily in their acid-base properties [6]. The p^H in the midgut of the sand fly is 7-9 and in the phagolysosome of mammalian macrophages in 4.5-5.5 [7]. Adaptation and proliferation in such widely different environmental conditions demand appropriate regulatory mechanism for maintaining a functional cell cytosol. Transplasma membrane electron transport system is critical for maintaining cellular redox balance, viability and the regulation of cell signaling, cell growth, apoptosis, proton pumping and ion channels [8-10]. Electron transport across the plasma membrane has been described in many eukaryotic cells [11,12] such as erythrocytes [13], liver [14], heart [15], transformed liver cells [16], HeLa cells [17], neutrophils [18], yeast [19], plant cell [20] and parasitic protozoa [21,22]. In this present study describes the effect of rotenone interaction with nonpermeable electron acceptors of ALA, NQSA, ferricyanide which are reduced with redox sites present in transplasma membrane electron transport system in *Leishmania donovani* promastigotes. Our data also suggest the transplasma membrane electron transport system of *Leishmania donovani* cell is functionally different in respect to nonpermeable electron acceptor and O_2 reduction activity of mammalian transplasma membrane electron transport system [21-23]. The transplasma membrane electron transport system of *Leishmania donovani* cell contributes to the activity of *Leishmania* species to survive in diverse environments found in mammalian host and the insect vector. The parasite surface membrane is having the cellular component, which interact directly with the host environment. Thus, studying functions of the parasite membrane as transplasma membrane electron transport system is of relevance to understanding of several mechanisms.

MATERIALS AND METHODS

All biochemical's unless otherwise mentioned were from Sigma (St. Louis, MO, USA). Panmade was purchased from Paines and Byrne (Greenford, Middlesex, UK).

Cell culture

Leishmania donovani promastigotes strain MHOM/IN/1978/UR6, a clinical isolate from a confirmed Kala-azar patient [24], was grown at 24°C on blood agar medium, p^H 7.5 [25]. The cells were washed at 500 X g thrice in cold Tris-sucrose-salt solution (250mM sucrose, 50mM NaCl, 20mM KCl, 1mM ethylene diamine tetracetic acid (EDTA), 20mM Tris, p^H 7.2) and kept in

it at 4°C until use. Viability of the harvested cells was monitored microscopically by trypan blue exclusion method [26].

Protein estimation

The amount of *Leishmania donovani* cell protein was determined by the biuret method in the presence of 0.2% deoxycholate [27]. Bovine serum albumin (BSA) was used as standard 1mg of whole cell protein corresponds to 1.4 X 10^8 cells.

Measurement of ferricyanide reduction by *Leishmania donovani* cells (LDC)

Ferrocyanide quantitation was performed using 1,10-phenanthroline complex as described by Avron and Shavit [28]. The incubation mixture contained acetate buffer (potassium acetate 20mM, sodium chloride 120mM, magnesium chloride 5mM), p^H 6.4, 3mg LDC, 5µmol D-glucose and 3µmol $K_3Fe(CN)_6$ in a final volume of 1ml. The incubation was carried out at 24°C. The reaction was terminated by the addition of 0.1ml 30%(w/v) trichloroacetic acid (TCA) followed by centrifugation at 10,000 X g for 15min, ferrocyanide in supernatant was measured by 1.5ml of 1,10-phenanthroline reagent containing 1.5mM sodium acetate, 0.1mM citric acid, 0.75µM ferric chloride and 12.6µM 1,10-phenanthroline at 510nm. The blanks were carried out with all reagents except LDC.

ALA, NQSA (1,2-napthoquinone-4-sulphonic acid) reduction assay

ALA, NQSA reduction by *Leishmania donovani* promastigote cells was assayed as the formation of ferrocyanide as a result of the reduction of ferricyanide by dihydrolipoic acid, and hydroquinone of NQSA. Ferrocyanide was estimated according to the method of Avron and Shavit [28]. The reaction mixture consisted of 3mg LDC, acetate buffer (potassium acetate 20mM, sodium chloride 120mM, magnesium acetate 5mM), p^H 6.4, 5µmol D-glucose, 1mM ALA, or 0.4mM NQSA in a final volume of 3ml. The reaction mixtures were incubated for 10min at 24°C. After incubation, the reaction mixtures were kept in ice and then centrifuged at 4°C at 10,000 X g for 10min. Then, 1.4ml of the supernatant was removed and added to 0.1 ml of 75mM $K_3Fe(CN)_6$, followed by 1.5ml 1,10-phenanthroline reagent, the absorbance was recorded at 510nm. The blanks were carried out with all reagents except LDC. Two molecules of ferricyanide have been considered to react with one molecule of dihydrolipoic acid or one molecule of hydroquinone of NQSA.

Exposure of *Leishmania donovani* cells (LDC) to rotenone

The liquid media used for growth experiments was a semisynthetic medium developed by Kar et al. [29]. The cultured cells of *Leishmania donovani* promastigotes cells in 100ml conical flask were incubated for 96hrs with rotenone of increasing concentrations supplied as a solution in DMF (5µl/ml medium) with proper vehicle control (DMF). After incubation, the numbers of elongated motile promastigotes were counted.

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Measurement of oxygen uptake

Oxygen uptake of the *Leishmania donovani* promastigotes cells was determined with late log growth phase cells (72hrs) and protein estimation, cells was suspended in PBS (p^H 6.4) containing 10mM glucose to give a cell density of 4mg/ml. Oxygen consumption was measured at 25°C with a DW1 Hansatech oxygraph plus (Hansatech Instruments Ltd., Norfolk, UK) [30]. Respiration of 2mg cells in a 1ml final volume was measured and effect of rotenone was evaluated. Rotenone at 0.01 mM concentration was given as a solution in dimethyl formamide (DMF;5µl/ml,PBS). Appropriate vehicle control (DMF) was used for each experiment. Respiration rates are expressed as nanomoles of oxygen consumed per ml/min.

RESULTS

Concentration of electron acceptors

TABLE 1: Comparison of K_m, V_{max} and pH optimum values of nonpermeable electron acceptors reduced by *Leishmania donovani* cells[a]

Electron acceptors	K_m (mM)	V_{max} (nmoles/min/mg/protein)	pH optimum
ALA	0.66	8.38	6.4
NQSA	4.00	3.90	6.2
$K_3Fe(CN)_6$	1.00	1.84	6.2

Methods for the reduction of various electron acceptors are given in materials and methods.
[a] Activities were measured on 3-day-old cell culture using four aliquots of cells were used in all experiments.

ALA, NQSA and $K_3Fe(CN)_6$ reduction by *Leishmania donovani* cell shows typical saturation kinetics with increasing ALA, NQSA and $K_3Fe(CN)_6$ concentrations. A double reciprocal plot of the data yielded a K_m value for ALA 0.66mM, NQSA 4mM and for $K_3Fe(CN)_6$ is 1mM respectively (Table 1).

p^H optimum for assays

ALA reduction by *Leishmania* cells gives optimum rate at p^H 6.4, where as NQSA or $K_3Fe(CN)_6$ reduction by *Leishmania* cells gives optimum rates at p^H 6.2 (Table 1).

Reduction of electron acceptors

Leishmania cells can reduce disulfide electron acceptors ALA and also reduce naphthoquinone electron acceptor NQSA and iron co-ordinate complex $K_3Fe(CN)_6$. The concentrations of tested compounds, which give a maximum reduction rate with *Leishmania* cells. The tested compounds that are active and have standard redox potential at p^H 7.0 ranging from +360 mV to – 290 mV (Table 2).

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TABLE 2Comparison of the rates of nonpermeable electron acceptors reduced by *Leishmania donovani* cells[a]

Electron acceptors	Concentration (mM)	Rate of reduction (nmol/min/mg protein)	Redox potential E^{o}pH (7.0) (mV)
$K_3Fe(CN)_6$	5	1.09 ± 0.162	+ 360
NQSA	2	1.03 ± 0.082	+ 187
ALA	1	2.27 ± 1.172	– 290

[a] Activities were measured on one cell culture using four aliquots of cells computed ± standard deviation. 3-day-old cell cultures were used in all experiments. Methods for the reduction of various electron acceptors are given in materials and methods.

Effect of rotenone on *Leishmania donovani* cell

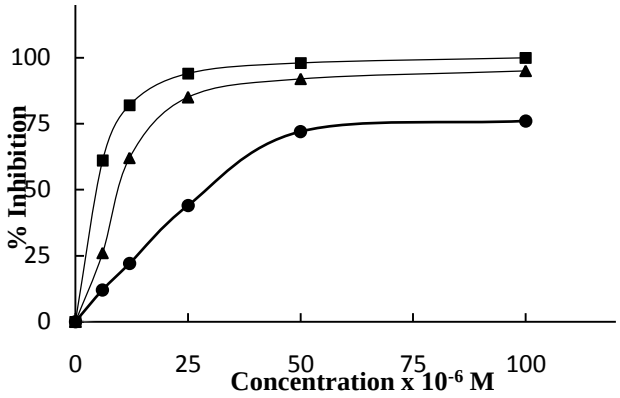

FIGURE 1 Concentration–response curve describing the ALA (●), NQSA (▲) and $K_3Fe(CN)_6$ (■) reduction inhibition in *Leishmania donovani* cell by rotenone. Assay was made as described in materials and methods. Each point represents the mean of four experiments.

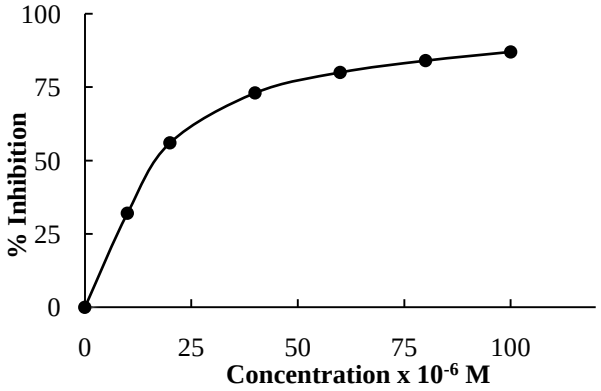

FIGURE 2 Concentration–response curve describing the inhibition of *Leishmania donovani cell* growth by rotenone. The culture was exposed for 96hrs with the rotenone. Each point represents the mean of three experiments.

The effect of electron transport inhibitor rotenone on transplasma membrane electron transport system has illustrated in the (Table-3) that rotenone, an inhibitor of energy coupling NADH dehydrogenase (Complex 1) [31] at 0.1mM rotenone concentration brought about

almost complete inhibition for ferricyanide reduction, while at same concentration of rotenone for ALA and NQSA reduction was 82% and 95% respectively. IC_{50} value of rotenone on $K_3Fe(CN)_6$ reduction was found to be 7.5µM respectively, where as, IC_{50} value of capsaicin on ALA, NQSA reduction was found to be 37µM and 12µM (Fig. 1). Rotenone was inhibited the growth of LDC maximally 87% at 100µM concentration and IC_{50} value of growth inhibition by rotenone was found to be 20µM (Fig. 2). It is evident from the (Table- 4) that the electron acceptors except $K_3Fe(CN)_6$ at certain concentration as in ALA and NQSA showed substantial oxygen uptake inhibition. The observation showed that the O_2 uptake was 62% inhibited by complex I inhibitor rotenone.

DISCUSSION

The emergency of many diverse roles for transplasma membrane electron transport system might, in part, may be due to the numerous redox enzymes present within the membrane [8,9]. The high capacity of *Leishmania donovani* cell to transfer electrons from intracellular donors to extracellular electron acceptors like ALA, NQSA, $K_3Fe(CN)_6$ and oxygen is poorly understood [21,22]. The present study describes the evidence for the presence of a numerous ectoredox enzymes to donate electron to impermeable electron acceptors with different redox potential. The reagents of this type will be important in determining the position and orientation of redox carriers in transplasma membrane electron transport system. In ALA, disulfide bridge is intra molecularly linked with an aliphatic chain is reduced after accepting electrons from transplasma membrane electron transport system, due to higher affinity and reaction rate. The p^H optimum for ALA is differ from the p^H optimum of NQSA and $K_3Fe(CN)_6$ (Table 1 and Table 2).

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TABLE 3 Effect of rotenone on extracellular reduction of ALA, NQSA and $K_3Fe(CN)_6$ by *Leishmania donovani* Cells

Addition	Rate of electron acceptor reduction in nmol/min/mg protein[a]								
	Concentration[c] (mM)	Rate of ALA reduction	Relative rate	Concentration[c] (mM)	Rate of NQSA reduction	Relative rate	Concentration[c] (mM)	Rate of $K_3Fe(CN)_6$ reduction	Relative rate
None[b]	–	1.22 ± 0.24	100	–	0.76 ± 0.14	100	–	1.48 ± 0.28	100
Rotenone	0.1	0.32 ± 0.04	18	0.1	0.16 ± 0.03	05	0.1	0.25 ± 0.03	01

[a] ALA, NQSA and $K_3Fe(CN)_6$ reduction was assayed according to procedure as given in Materials and Methods. Electron transport inhibitor rotenone was added to LDC, 10min before the addition of electron acceptors. Incubation time with ALA, NQSA and $K_3Fe(CN)_6$ was 10min. Control experiments, which received an equal volume of solvent given along with the electron transport inhibitor rotenone, had no effect on electron acceptor reduction in LDC. The values represent the average of four experiments.

[b] Control incubation contained LDC, D–glucose and electron acceptor as described in materials and methods.

[c] Value represents the concentration required for maximum inhibition or stimulation.

TABLE 4 Studies on effect of rotenone on oxygen uptake by *Leishmania donovani* cells

Addition	Concentration (mM)	Oxygen uptake rate[a] (nmol/min/mg protein)	Relative rate
None	-	18 ± 1.78	100
ALA	1.0	8.28 ± 0.90	46
NQSA	0.4	5.22 ± 0.52	29
$K_3Fe(CN)_6$	3.0	18 ± 1.70	100
Rotenone	0.01	9.00 ± 0.91	38

[a] Oxygen uptake was assayed according to the procedure discussed in materials and methods. The incubation time with effectors was 10min. Control experiment that received an equal volume of solvent given along with the effectors had no effect on oxygen uptake in *Leishmania donovani* cell.

Effect of rotenone with electron acceptors ALA, NQSA, $K_3Fe(CN)_6$ reduction assay and O_2 uptake described in (Table 3 and Table 4). The major effect under normal conditions appears to be due to oxygen inhibition of the redox sites leading to ALA, NQSA, $K_3Fe(CN)_6$ reduction. Thus, $K_3Fe(CN)_6$ did not produce oxygen uptake inhibition in *Leishmania donovani* cell, where as ALA, NQSA inhibited the oxygen uptake (Table-4). We have observed that, the incubation of *Leishmania donovani* cell in presence of $K_3Fe(CN)_6$ resulted in increased uptake of ^{14}C-deoxy-D-glucose (data not presented). It is tempting to speculate that increased influx of D-glucose has generated higher level of NADH/NADPH and consequently higher rate of O_2 uptake, which compensated oxygen uptake inhibition by $K_3Fe(CN)_6$ and the rotenone was found to be a potent inhibitor ALA, NQSA, $K_3Fe(CN)_6$ reductase activity and O_2 uptake was partially inhibited. The rotenone is the most widely used inhibitor of mitochondrial and bacterial electron transport because of the selectively inhibit the oxidation of NADH-linked substrates [31,32]. The binding site of rotenone responsible for the inhibition of NADH oxidation in complex I of mitochondrial electron transport chain is localized in the O_2 site of the dehydrogenises and not between the NADH & flavoprotein [33].

The rotenone, complex-I inhibitor of NADH-ubiquinone oxidoreductases (NDH-1) present in the respiratory chain of various species of bacteria and mitochondria, can be divided into two groups [31-36]. One group of enzymes that bear the energy-coupling site is designated as NDH-1 and the other group that does not as NDH-2. In response of rotenone to the three electron acceptors they can be divided into highly sensitive NQSA, $K_3Fe(CN)_6$ reductase group and less sensitive ALA (Fig. 1, Table. 1). The sensitivity to ALA, NQSA, $K_3Fe(CN)_6$ reduction, inhibition in *Leishmania donovani* cell by rotenone, supports the notion of the presence of an energy-coupling site (NDH-1) in transplasma membrane electron transport system. This finding correlates the inhibition of plasma membrane NADH oxidase activity (NDH-1) with inhibition of growth by rotenone (Fig.2). The rotenone caused a dose-dependent decline in *Leishmania donovani* cell growth that closely paralleled its inhibition of reductase activities. The observation supports the idea that, the transmembrane electron transport depends on the production of an cytosolic reducing agent, such as NADH, the oxidation liberates electron which travel to the outside of the cell via trans PMET chain [21,22].

These results suggest that, the transplasma membrane electron transport helps to maintain a stable redox environment required for *Leishmania donovani* cell viability. The experimental studies illustrate the presence of different redox sites for three different electron acceptors. The possibility that, there is more than one site of electron egress to the electron acceptor cannot be ruled out. However, the redox chain appears to be branched at several points, which incorporates NADH linked energy coupling site as judged by the rotenone inhibition. Electron transfer in the transplasma membrane electron transport system requires an energized state of the membrane. Resistance to normal mammalian cell inhibition by rotenone forms the basis for transplasma membrane electron transport as an antileishmanial drug target.

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