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TRICYCLIC DRUGS REDUCE PROTON MOTIVE FORCE IN *LEISHMANIA DONOVANI* PROMASTIGOTES

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Abstract—Tricyclic compounds have been suggested as potential anti-leishmanial drugs. We have studied the effect of tricyclic drugs on several cellular functions in *L. donovani* promastigotes. Imipramine inhibits proline transport and reduces ΔpH and cellular ATP at relatively high concentrations (IC_{50} = 50–80 μM). High concentrations of imipramine are also required to kill *L. donovani* promastigotes (LD_{50} > 50 μM). The presence of a chlorine atom in the side ring of either imipramine or promazine results in a three-fold increase in both IC_{50} and LD_{50} values. Tricyclic compounds in which the nitrogen in the middle ring was substituted with a carbon atom (amitryptyline and chlorprothixene) are most effective in causing cell death and in decreasing proline transport and ΔpH ($IC_{50} \simeq 5$ μM), whereas depletion of cellular ATP requires a higher drug concentration (IC_{50} = 12 μM). Transchlorprothixene has IC_{50} values for proline transport, ΔpH and cellular ATP that are similar to those of amitriptyline, whereas the *cis* isomer is less active. Imipramine, chlomipramine and chlorpromazine decrease the membrane potential in promastigotes. There is a direct correlation between inhibition of membrane transport of proline and the size of the membrane potential at various concentrations of the drugs. Taken together, the multiple effects of the tricyclic drugs on cellular functions in *Leishmania* suggest that the drugs cause cellular death by non-specific mechanisms, probably involving a general increase in membrane permeability.

Tricyclic drugs, antidepressants and antipsychotics are toxic to *Leishmania* [1, 2]. Compounds of both groups kill *L. donovani* and *L. major* amastigotes within macrophages as well as extracellular promastigotes *in vitro* [1–3]. The mode of action of tricyclic drugs against *Leishmania* is unknown. Evans *et al.* [3] suggested that these compounds are non-specifically toxic to *Leishmania*. Some of these compounds however, demonstrate a high selectivity: they kill parasites at a relatively low concentration, but their toxicity to host macrophages is relatively low. Neal and Allen [4] have recently shown that amitriptylin, an analog of imipramine, and chlorprothixene, a derivative of promazine, are highly toxic to *L. donovani*. Furthermore, amitriptylin demonstrated a therapeutic index of at least 100 [4] suggesting that this group of compounds has a potential for use against *Leishmania*.

It was previously suggested that antidepressants are toxic because they inhibit membrane functions which are essential for the survival of *Leishmania* within its hosts. Evidence for this hypothesis arose from experiments which demonstrated that imipramine and clomipramine inhibit transport of L-proline in promastigotes of *L. donovani* [1]. Proline is actively accumulated in *L. donovani* promastigotes and the transport is driven by the proton electrochemical gradient across the plasma membrane [5]. This gradient is created by proton pumps on the plasma membrane of *L. donovani* promastigotes [6, 7]. It was recently demonstrated that these proton pumps play an important role in the regulation of proton motive force and the intracellular homeostasis of pH [8, 9] and of other ions [10]. Effects of tricyclic drugs on membrane functions have also been previously described in yeast. Eilam [11, 12] demonstrated in *Saccharomyces cerevisiae* that trifluoperazine and other phenothizines cause hyperpolarization of the cell membrane, increase calcium influx, and enhance potassium efflux. These effects are due to the inhibition by these drugs of the plasma membrane H^+-ATPases in this organism [12].

The aim of this work is to examine the effect of tricyclic compounds on the proton motive force in *L. donovani* promastigotes. We find that both antidepressant and antipsychotic compounds have a similar effect on $\Delta\mu_{H+}$ and cellular ATP levels, which are probably due to a general increase in membrane permeability.

MATERIALS AND METHODS

Materials. L-[^{3}H]proline and [^{3}H]tetraphenylphosphonium were purchased from Amersham (Bucks, U.K.); acridine orange, imipramine, and chlorpromazine from the Sigma Chemical Co. (Poole, U.K.) amitriptyline was a gift from Glaxo Group Research Ltd (U.K.); Chlorprothixene was a gift from H. Lundbeck & Co. (Denmark).

Parasites. A cloned line of *L. donovani* strain 1-S promastigotes [13] was used in all experiments. The parasites were grown in medium 199 supplemented with 10% fetal calf serum.

Determination of pH_i. Intracellular pH was calculated from quenching of the fluorescence of acridine orange as previously described [8], except that the cell concentration used was 2×10^7 cell/mL.

Determination of ATP. Cellular ATP was determined after extracting promastigotes (2×10^7 cells/mL) with 0.3 M perchloric acid for 15 min at 0°. The

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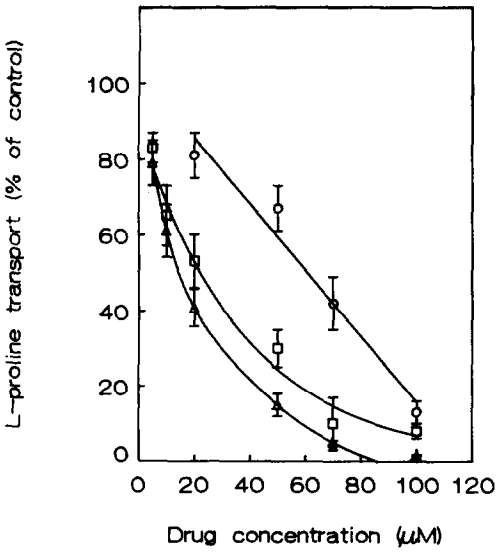

Fig. 1. Dose–response curve of the effect of antipsychotics on the transport of L-proline in *L. donovani* promastigotes. Promastigotes (4×10^7 cells/mL) in 95 μL Earle's salt solution plus 5 mM D-glucose (ESS) were preincubated for 10 min at 30°. Transport assays were initiated by the addition of 5 μL L-[^{3}H]proline (300 μM, 17 Ci/mol). Drugs were added 10 min prior to the addition of [^{3}H]proline. Chlorpromazine (□), *cis*-chlorprothixene (○) and *trans*-chlorprothixene (△) were added 10 min prior to the addition of [^{3}H]proline. Each point represents the mean ± SD of three experiment.

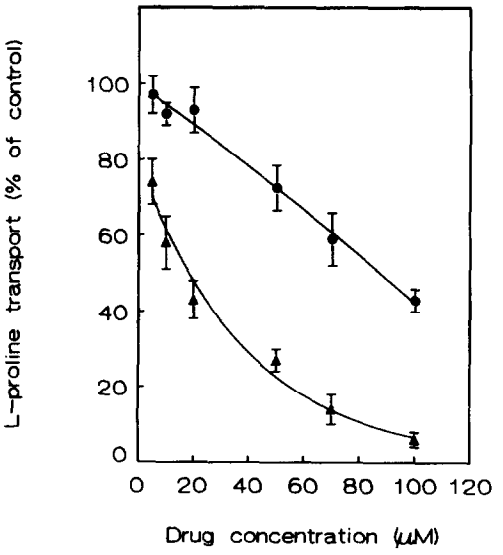

Fig. 2. Dose–response curve of the effect of antidepressants on the transport of L-proline in *L. donovani* promastigotes. Transport assays were carried as in Fig. 1. Imipramine (●), clomipramine (▲). Each point represents the mean ± SD of three experiment.

extract was neutralized with 0.3 N KOH and taken for ATP determination by the luciferase–luciferin assay.

Transport assays. Transport of L-proline was conducted essentially as described in Ref. 8.

Determination of membrane potential. Apparent membrane potential ($\Delta\psi$) was estimated from the distribution across the plasma membrane of [^{3}H]tetraphenylphosphonium (TPP^+) according to Zilberstein *et al.* [14]. Accumulation of [^{3}H]TPP^+ was determined as described for the accumulation of L-proline [8], except that [^{3}H]TPP^+ was added at 9 μM (275 Ci/mol) and the incubation lasted 15 min.

Determination of cell volume. Cell volume of promastigotes was measured as described in Ref. 15.

RESULTS

Two groups of tricyclic compounds were used in this study: antidepressants including imipramine, clomipramine and amitriptyline; and antipsychotics including chlorpromazine and chlorprothixene. Amitriptyline and chlorprothixene are tricyclic derivatives in which the nitrogen in the middle ring was substituted with a carbon atom (positions 5 and 10 in amitriptyline and chlorprothixene, respectively).

The effect of antipsychotics on steady state transport of L-proline in *L. donovani* promastigotes is summarized in Fig. 1. Chlorpromazine effectively inhibits proline transport, reaching a 50% inhibition at 22 μM and 90% at 100 μM. As shown in Fig. 1, both *cis* and *trans* isomers of chlorprothixene inhibit proline uptake, however, the *trans*-chlorprothixene is more potent than the *cis* isomer. *trans*-Chlorprothixene inhibits 50% of transport activity at 15 μM, whereas at the same concentration, *cis*-chlorprothixene inhibits only 10% of transport activity. *trans*-Chlorprothixene completely inhibits proline uptake at 85 μM. At this concentration, the *cis* isomer inhibits only 70% of transport activity. A complete inhibition of transport by this compound is achieved at 120 μM.

The effect of antidepressants on steady state transport of L-proline in *L. donovani* promastigotes is summarized in Fig. 2 and Table 1. Clomipramine inhibits 50% of proline uptake at 19 μM. A further increase of the concentration of clomipramine to 100 μM caused an almost complete inhibition of transport. Imipramine is found less effective than clomipramine in the inhibition of proline transport (IC_{50} = 76 μM). A complete inhibition by imipramine is achieved at 160 μM (not shown). Amitriptyline, on the other hand, is highly active causing a 50% inhibition of proline transport at a concentration as low as 5 μM (Table 1). Increasing the concentration of amitriptyline to 12 μM caused a complete inhibition of proline transport activity (not shown). The results in Figs 1 and 2 indicate that the substitution of the nitrogen by a carbon atom in the middle ring of both imipramine and promazine results in a greater inhibition of proline uptake.

The membrane potential is the main driving force of L-proline transport in *L. donovani* promastigotes at pH 7 [5, 8]. It was therefore interesting to determine the relation between the effect of the tricyclic drugs on transport and on membrane potential (Fig. 3). All drugs used in this work reduced the membrane

Table 1. IC_{50} of tricyclic compounds on various cell functions in *L. donovani* promastigotes and their relation to LD_{50}

Compound	IC_{50} (μM) ΔpH	Transport	Cellular ATP	LD_{50} (μM)
Imipramine	80 ± 7.3	76.8 ± 9.2	50 ± 6.2	>50
Clomipramine	25 ± 2.6	19 ± 2.1	24 ± 1.8	24
Amitriptyline	6.3 ± 0.9	5 ± 0.63	12 ± 1.5	5
Chlorpromazine	20 ± 2.6	22 ± 3.2	ND	28
cis-Chlorprothixene	15.9 ± 1.7	60 ± 7.1	23 ± 2.2	20
trans-Chlorprothixene	3.8 ± 0.4	15 ± 2.1	13 ± 2.1	10

Transport and ΔpH were measured as in Figs 1 and 4, respectively.
Cellular ATP was measured as described in Materials and Methods.
ND, not determined.

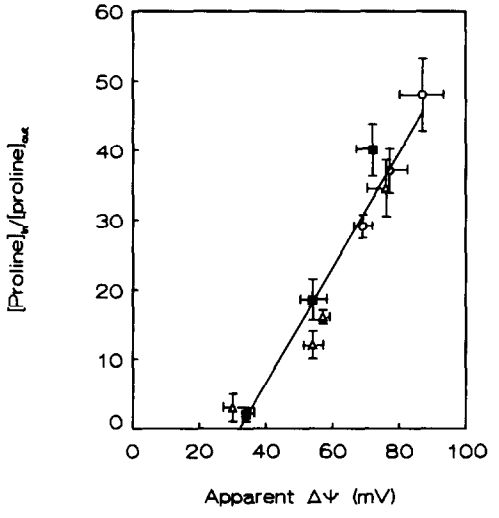

Fig. 3. Steady state of L-proline transport as function of membrane potential at various drugs concentrations. Cells (4×10^7 cells/mL) in 95 μL Earle's salt solution supplemented with 5 mM D-glucose were equilibrated at 30° for 10 min. Assays were initiated by the addition of 5 μL of [^{3}H]tetraphenylphosphonium (TPP^+) to a final concentration of 9 μM (250 Ci/mmol) and lasted 15 min. Imipramine (○), clomipramine (■) and chlorpromazine (△) were added 10 min prior to the addition of [^{3}H]TPP^+. Each point represents the mean ± SD of three experiment.

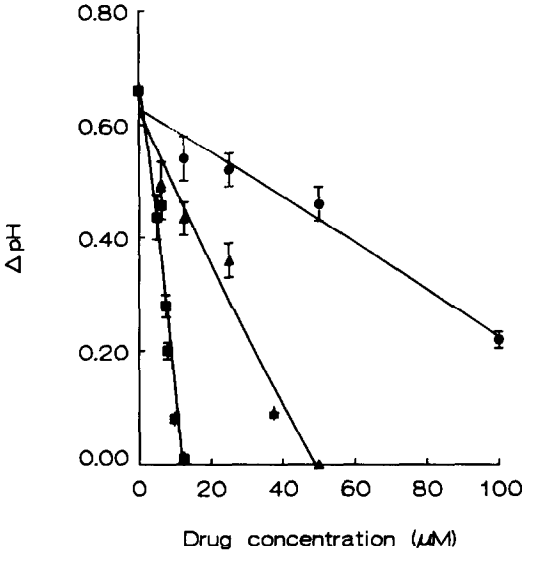

Fig. 4. The effect of antidepressants on ΔpH in *L. donovani* promastigotes. Promastigotes were added to 2.5 mL of solution which contained 136 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 10 mM Tris–succinate at pH 7, and 4 μM acridine orange to a final density of 2.4×10^7 cells/mL. Excitation and emission wavelengths were 492 and 530 nm, respectively. Imipramine (●), clomipramine (▲) and amitriptyline (■) were added when accumulation of acridine orange reached steady state. Each point represents the mean ± SE (N = 4).

potential in a fashion similar to their inhibition of proline transport. Figure 3 summarizes the concentration gradients of proline formed at a steady state transport as a function of apparent membrane potential ($\Delta\psi$) at various drug concentrations. The data obtained using all three drugs imipramine, clomipramine and chlorpromazine fall on a common straight line indicating that there is a linear correlation between the concentration gradient of proline formed at steady state transport and membrane potential. Zero transport was obtained at $\Delta\psi$ equals 30 mV. This suggests that the minimal membrane potential required for maintaining active transport of proline is −30 mV. These results also suggest that all three drugs effect proline transport via the same mechanism, as they all fall on the same straight line.

The effect of the tricyclic drugs on the proton gradient and on intracellular pH was determined (Figs 4 and 5). It is expressed as ΔpH ($pH_{in} - pH_{out}$) at various drug concentrations. ΔpH of untreated promastigotes suspended in the medium at pH 7 is 0.66 (acid inside). All three antidepressants tested in this work reduce ΔpH. Imipramine is the least effective drug reducing ΔpH to zero at 160 μM and 50% (ΔpH = 0.33) at 80 μM. Clomipramine reduces ΔpH to zero at a concentration of 50 μM and to 50% at 25 μM. Amitriptyline is the most effective inhibitor of the pH gradient. At a concentration as low as 12 μM, amitriptyline equilibrates pH_i with pH_o. A 50% reduction of ΔpH was reached at an amitriptyline concentration of 6.25 μM (Fig. 4).

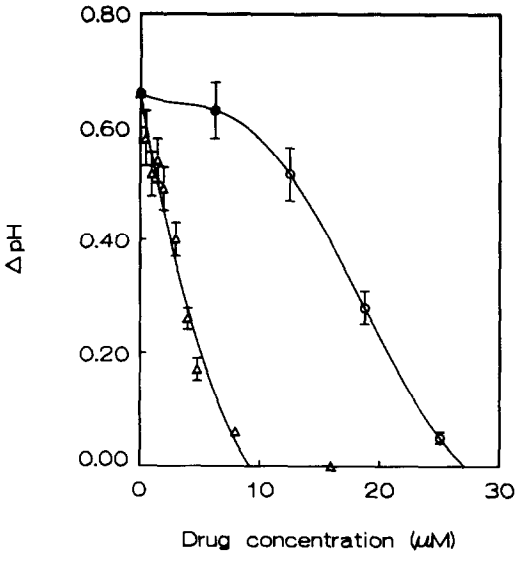

Fig. 5. The effect of antipsychotics on ΔpH in *L. donovani* promastigotes. Experiments were carried out as described in Fig. 4. *cis*-Chlorprothixene (○), *trans*-chlorprothixene (△). Each point represents the mean ± SD (N = 4).

The effect of chlorpromazine on ΔpH is similar to that of clomipramine. This compound reduces ΔpH by 50% at the concentration of 20 μM (Fig. 5). Both isomers of chlorprothixene are effective inhibitors of ΔpH. However, as observed in the transport experiments, *cis*-chlorprothixene is less effective than the *trans* isomer. While *cis*-chlorprothixene abolishes ΔpH at 28 μM, *trans*-chlorprothixene does the same at only 9 μM. Similarly, the IC_{50} of *cis*-chlorprothixene is 15.9 μM, whereas that of *trans*-chlorprothixene is 3.8 μM (Fig. 4 and Table 1).

Table 1 summarizes the IC_{50} values of all drugs used for ΔpH, proline transport and cellular ATP in *L. donovani* promastigotes and compares them with the LD_{50} values. As shown, all drugs used in this study reduce the cellular levels of ATP. Except for amitriptyline, there are good correlations between the IC_{50} for transport, ΔpH, cellular ATP and the LD_{50} values of each drug. Amitriptyline affects cellular ATP at concentrations which are twice the IC_{50} values for ΔpH and transport and LD_{50}.

DISCUSSION

The maintenance of intracellular pH and the accumulation of various nutrients in *Leishmania* cells are dependent on the existence of a proton electrochemical gradient across the plasma membrane [5, 16]. The maintenance of this gradient is therefore essential for the survival of this organism. Hence, compounds that can abolish this gradient might be toxic to *Leishmania*. Previously, we suggested that the toxicity of tricyclic compounds to *Leishmania* is due to their inhibition of $\Delta\mu_{H^+}$ formation [1, 17]. This work examined the effect of tricyclic drugs on the proton electrochemical gradient and its relation to the toxicity of these drugs to *L. donovani* promastigotes.

Both antidepressants and antipsychotics reduce ΔpH and inhibit the proton motive force-driven transport of L-proline in the following order of efficiency: amitriptyline, *trans*-chlorprothixene > *cis*-chlorprothixene > clomipramine, chlorpromazine > imipramine. They all also deenergize the cells in the same order of efficiency. Furthermore, antidepressants and antipsychotics which are structurally related, have similar effects on these activities. For example, clomipramine and chlorpromazine, which both have a chlorine atom on the side ring, demonstrate similar IC_{50} values for transport, ΔpH and cellular ATP. Furthermore, substituting with a carbon atom of the nitrogen in the middle ring of both imipramine and promazine results in a significant increase of the potency of these drugs. Amitriptyline is 15-fold more active than imipramine and 5-fold more active than clomipramine. Similarly, *trans*-chlorprothixene is much more active than chlorpromazine and imipramine. This increase in activity might be due to the hydrophobicity of these molecules. The addition of chlorine atom at the side ring of chlorpromazine or clomipramine neutralizes the positive charge of the nitrogen in both compounds. Moreover, substituting the positively charged nitrogen in the middle ring with a carbon will also result in a more hydrophobic compound. An increase in hydrophobicity allows a better penetration of these molecules through the lipid bilayer of the plasma membrane.

Tricyclic compounds like local anesthetics, at high concentrations uncouple oxidative phosphorylation in mitochondria of various systems [18, 19]. Moreno *et al.* [20] have demonstrated that at high concentrations (≈40 μM), crystal violet uncouples oxidative phosphorylation. A similar effect on the synthesis of ATP in mitochondria was found with local anesthetics when added at high concentrations of 1 mM and above [21]. Moreover, Weinbach *et al.* [22] showed that, when used at high concentrations (>25 μM), various antidepressants also uncouple oxidative phosphorylation in rat liver mitochondria. However, unlike classical uncouplers, the uncoupling by antidepressants was due to specific interactions of these compounds with the F_1-ATPase. All the compounds used in this work deenergize promastigotes, reducing cellular ATP at concentrations similar to those which inhibit transport and $\Delta\mu_{H^+}$. It is suggested that the deenergization is due to the uncoupling of the mitochondria.

With most of the drugs, the IC_{50} values for ΔpH, transport, and cellular ATP are similar to the values obtained for LD_{50}. This suggests a non-specific effect of these compounds. Such an effect might result from uncoupling of the mitochondria, as well as non-specific interactions of the tricyclic compounds with the lipids of the plasma membrane. It was previously shown that local anesthetics and tricyclic compounds, when added at high concentrations, interact with membrane phospholipids [23–25]. Such interactions may increase the permeability of the plasma membrane and of intracellular organelles. Such leakiness of the cells results in collapse of $\Delta\mu_{H^+}$ and consequently in the inhibition of all activities which depend on the existence of this gradient.

Tricyclic drugs rather specifically inhibit the transport of biogenic amines in chromaffin granules and in various other systems [26]. In these systems, the IC_{50} is in the nanomolar range. Moreover, it was shown that there is a direct correlation between binding of the drugs and inhibition of transport, suggesting that the inhibition of serotonin transport by imipramine or clomipramine is due to the specific binding of the drugs on the transporter [27]. Tricyclic antidepressants and antipsychotics inhibit the plasma membrane H^+-ATPase in *L. donovani* promastigotes (Zilberstein *et al.*, unpublished results). In analogy to the their effect on the nervous system, a more specific inhibition may be achieved using compounds that possess IC_{50} values which are in the nanomolar range. Evidence in support of this hypothesis is shown in the experiment with amitriptyline (Table 1). IC_{50} values of amitriptyline are more than 15-fold lower than those of imipramine and four-fold lower than those of clomipramine. Amitriptyline IC_{50} values for transport and ΔpH are lower than the concentration that decreases cellular ATP. Moreover, unlike all other drugs used in this work, amitriptyline possesses a high therapeutic index of 100 [4].

The search for drugs that specifically affect the size of the chemiosmotic energy on the plasma membrane of *Leishmania* is rational because that mechanism is essential for the survival of this organism. The results of this work demonstrate that tricyclic drugs are active against *Leishmania*. However, to obtain more specific drugs one must search for or develop compounds with much lower IC_{50} values. This will hopefully lead to anti-leishmanial drugs with low LD_{50} that possess a high therapeutic index. Attempts to find and develop such compounds are now in progress.

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