tive class I transcripts. In particular, small RNA's generated apparently by RNA polymerase III transcription of type 2 Alu-like sequences have been detected (*20*) which may, directly or indirectly, interact with the analogous repeat sequence at the 3′ ends of the D^d and L^d genes or their mRNA's to regulate their transcription, processing, stability, or translational efficiencies.

There has been a recent suggestion that a class I transcript derived from the Qa2,3/Tla region of the MHC may also contain the type 2 Alu-like ($NC2^L$) sequence at a position analogous to that in the D^d and L^d transcripts (*21*). However, in view of the criteria that have been suggested for the identification of class I sequences (*6*), including locus-specific substitutions in both the NC1 and the transmembrane regions, we suggest that this RNA transcript may not derive from the Qa2,3/Tla region (*21*) but might more likely derive from the previously identified D^d gene (*10*). If this conclusion is correct, however, we would have expected this RNA transcript to be expressed in normal cells rather than to be induced only upon transformation (*22*).

Our study demonstrates the presence of the type 2 Alu-like sequence ($NC2^L$) within the 3′ noncoding regions of both the L^d and D^d genes, but not the K^d gene. This type 2 Alu-like sequence provides a novel polyadenylation signal which is efficiently used in transcriptional processing of these class I genes. This clearly demonstrates a biologically important function for the type 2 Alu-like sequence.

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25. We thank W. Davidson, K. Isselbacher, W. Jelinek, M. Priest, and K. Tanaka for constructive discussion. Supported in part by NIH grant AI 19148 and by an award from the Mallinckrodt Foundation to JGS.

23 July 1984; accepted 4 September 1984

Antidepressants Cause Lethal Disruption of Membrane Function in the Human Protozoan Parasite *Leishmania*

Abstract. *The antidepressant compounds clomipramine and nitroimipramine were cidal to extracellular promastigotes of both human protozoan parasites* Leishmania donovani *and* Leishmania major. *Clomipramine also killed amastigotes of both species within murine macrophages with no apparent toxicity to the host cells. Further, amastigotes were more sensitive than promastigotes to clomipramine. Clomipramine (100 micromoles per liter or 0.2 nanomole per* 1×10^6 *cells) inhibited* L*-proline transport in promastigotes. Synergistic inhibition of* L*-proline transport was observed with clomipramine after addition of either of the ionophores valinomycin or nigericin. These observations suggest that the cytotoxic effects of clomipramine result from its disruption of the proton electrochemical gradient of the parasite surface membrane.*

The parasitic trypanosomatid protozoan *Leishmania donovani* is the causative agent of human visceral leishmaniasis. This parasite has a digenetic life cycle, including an extracellular flagellated promastigote form within the alimentary tract of its insect vector and an obligate intracellular amastigote form within mammalian macrophages (*1*). The organism actively accumulates solutes such as L-proline and D-glucose via carrier-mediated transport systems (*2, 3*). Both of these transport systems are driven by a proton electrochemical gradient ($\Delta\mu_{H^+}$) across the parasite surface membrane (*3*). The presence of H^+ adenosinetriphosphatase activity in the parasite's surface membrane was suggested by both fine structure cytochemistry and the observation that transport (L-proline and D-glucose) was inhibited by *N′N′*-dicyclohexylcarbodiimide while the organisms retained high levels of cellular adenosine triphosphate (ATP) and normal respiration (*3, 4*). These functional characteristics of the *L. donovani* surface membrane are different from those of its mammalian host, and such differences might be exploited for therapeutic purposes.

The tricyclic antidepressant drugs imipramine and 3-chlorimipramine (clomipramine) are efficient inhibitors of serotonin uptake in mammalian cells (*5*). Both of these compounds also act as uncouplers of oxidative phosphorylation and at high concentrations as inhibitors of H^+ adenosinetriphosphatases in mammalian mitochondria (*6*). The latter observation prompted us to investigate the effect of these compounds on both the growth and membrane functions of *L. donovani*. For comparative purposes, the effects of these compounds were also assessed with *Leishmania major*, the agent of Old World human cutaneous leishmaniasis.

Promastigotes of a cloned strain of *L. donovani* (*7*) were maintained and grown in chemically defined RE IX medium (*8*). BALB/c mice were infected with promastigotes of a cloned strain of *L. major* (*9*). Amastigotes were isolated from the excised foot pads of these animals 4 to 5 weeks after infection. These amastigotes were allowed to transform into promastigotes in medium M199 containing 20 percent fetal calf serum and 25 m*M* Hepes at 26°C.

The effect of imipramine and its analogs on the growth of promastigotes was assessed. Parasite cultures were initiated at 3×10^6 cells per milliliter and when they reached 1×10^7 to 2×10^7 cells per milliliter, drugs were added at various concentrations. The number of promastigotes in each culture was then determined every 24 hours.

Table 1. Killing of *L. donovani* promastigotes by imipramine and its analogs. The number of promastigotes was assessed 24 hours after the drugs were added. N.E., no effect at up to 50 μM. Each value is the mean ± standard error of the mean (S.E.M.) of six experiments.

Drug	LD_{50} (μM)
Imipramine	N.E.
3-Chlorimipramine	24 ± 3.2
2-Nitroimipramine	9 ± 1.3
4-Nitroimipramine	5 ± 0.7
2,8-Dinitroimipramine	24 ± 4.1
4,8-Dinitroimipramine	>50
3-Cyanoimipramine	>50

Table 2. Cidal effect of imipramine and clomipramine on *L. major*. Experiments were conducted as described in Table 1 and Fig. 1. Each value is the mean ± S.E.M. of three experiments.

Drug	LD_{50} (μM)	
	Promastigotes	Amastigotes
Imipramine	>50	>10.0
Clomipramine	21 ± 1.3	1.18 ± 0.15

Growth of amastigotes of both *L. donovani* and *L. major* within peritoneal macrophages was measured as follows: macrophages were isolated from BALB/c mice, washed twice in RPMI 1640 containing 2 percent fetal calf serum by centrifugation (250*g*, 10 minutes at 4°C) and finally resuspended in this medium containing 10 percent fetal calf serum. Portions (0.4 ml) containing 5×10^5 macrophages were placed in wells of tissue culture chambers (Lab-Tek, Miles) and incubated at 37°C in room air containing 5 percent CO_2. After 12 hours the monolayers were washed to remove nonadherent cells. *Leishmania donovani* promastigotes were added at a parasite-to-macrophage ratio of 4:1 and amastigotes of *L. major* were added at a ratio of 1:1. After 5 hours of incubation (*L. donovani* at 37°C and *L. major* at 35°C), the monolayers were washed to remove residual extracellular parasites. Drugs were added to the infected macrophages 3 to 4 days after infection. The percentage of infection and the number of amastigotes per infected macrophage were then determined every 24 hours by examining at least 300 macrophages in each sample. The effects of clomipramine on macrophage phagocytosis, and oxygen burst activities were also assessed. Opsonized zymosan particles were used to measure phagocytosis, and subsequently nitro blue tetrazolium reduction was used to determine oxygen burst activity (*10*). Assessment of proline transport in *L. donovani* promastigotes was as previously described (*3*).

The parent compound imipramine at up to 50 μM had no cidal effect on *L. donovani* promastigotes, whereas its analogs, clomipramine and nitroimipramine, were both lethal to the parasites (Table 1). Clomipramine at a concentration of 24 μM killed 50 percent of the growing *L. donovani* promastigotes within 24 hours. The nitroimipramines substituted at either position 2 or position 4 of the imipramine phenylic rings, were even more toxic to the parasites than clomipramine [the median lethal dose (LD_{50}) was 9 and 5 μM, respectively]. It appears that replacing hydrogen on the phenylic rings of imipramine with either chlorine or nitro groups is requisite for cidal activity. Moreover, such substitution seems to be specific; for example, replacing the chloro group of clomipramine with a cyano group at the same position, or the addition of a nitro group to both nitroimipramines at position 8 reduced the cidal effect of both of these analogs (Table 1).

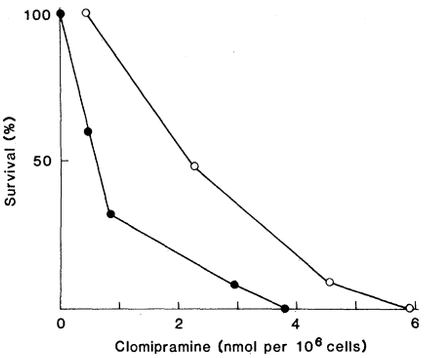

Fig. 1. Killing of *L. donovani* promastigotes and amastigotes by clomipramine. Peritoneal macrophages isolated from BALB/c mice were infected by *L. donovani* promastigotes at the ratio of four parasites per macrophage. Clomipramine was added 3 days after infection. The number of amastigotes (●) that survived after 24 hours of treatment were counted in a population of at least 300 macrophages. Promastigotes (○) were treated as in Table 1.

Data concerning clomipramine concentrations are given both in their molar solution values and their LD_{50} value per 1×10^6 parasites (Fig. 1). The latter are included for direct comparison of the two parasite developmental stages. Clomipramine at 0.45 nmol per 1×10^6 cells (5 μM), or less, caused no inhibition in the growth of *L. donovani* promastigotes (Fig. 1). However, clomipramine at concentrations greater than 5 μM were lethal to these cells. At 0.8 nmol of clomipramine per 1×10^6 cells (8.25 μM), 10 percent of the promastigotes were killed within 24 hours, whereas at 4.54 nmol per 1×10^6 cells (50 μM), 90 percent were killed. Promastigotes were rapidly killed at clomipramine concentrations greater than 50 μM; for example, cells exposed to 70 μM lost viability within 1 hour.

Of significance was the observation that clomipramine killed amastigotes within their host macrophages in vitro (Fig. 1). Clomipramine at 0.45 nmol per 1×10^6 intracellular amastigotes killed 40 percent of the parasites within 24 hours. The LD_{50} for intracellular amastigotes was 0.6 nmol per 1×10^6 organisms (Fig. 1). The leishmanicidal effect of clomipramine appears not to be species specific. Clomipramine was as toxic to *L. major* promastigotes and amastigotes as it was to *L. donovani* (Table 2). Clomipramine, at concentrations up to 30 μM for 24 hours, showed no apparent

Table 3. Effect of imipramine, clomipramine, and ionophores on the transport of L-proline in *L. donovani* promastigotes. Promastigotes were washed twice and resuspended in potassium phosphate buffer (0.1*M*, *p*H 7.1) containing 5 m*M* $MgSO_4$ at final concentration of 1 mg of cell protein per milliliter (3.6×10^8 cells per milliliter). The cells (100 μl) were equilibrated at 30°C for 10 minutes, and the assays were initiated by the addition of ^{3}H-labeled L-proline (50 μM, 123 mCi per mole) in the presence of 25 μM cycloheximide. Each value is the mean ± S.E.M. of four experiments.

Addition	Concentration (μM)	Transport (nmol per mg protein)	Inhibition (%)
None		14.0 ± 1.7	
Imipramine	50	13.7 ± 1.9	2.1
Clomipramine	10	10.1 ± 1.2	27.8
	50	5.6 ± 0.4	60
	100	1.3 ± 0.06	92.8
Nigericin	2	11.8 ± 1.5	15.6
Nigericin	2		
Clomipramine	10	5.2 ± 0.45	62.8
Valinomycin	0.5	10.3 ± 0.9	26.4
Valinomycin	0.5		
Clomipramine	10	4.2 ± 0.5	70
Nigericin	2		
Valinomycin	0.5	2.95 ± 0.2	79.0

deleterious effects on the phagocytosis or oxygen burst activities, the morphology, the adherence to glass, or the density of cells within monolayers of either infected or uninfected macrophages. Since clomipramine is a lipophilic compound, it would most likely be absorbed through the macrophage surface membrane, and thus the values given in Fig. 1 probably represent an underestimation of its effective intracellular cidal concentrations.

Clomipramine appears to be more toxic to *L. donovani* amastigotes within macrophages than to extracellular promastigotes ($LD_{50} = 0.6 \pm 0.08$ and 2.3 ± 0.35 μM, respectively) (Fig. 1). Since clomipramine uncouples oxidative phosphorylation in mammalian mitochondria (*6*), it is possible that this drug also disrupts the $\Delta\mu_{H^+}$ across the surface membrane of *L. donovani*. As a consequence, both promastigotes and amastigotes would cease to maintain *p*H homeostasis as well as the $\Delta\mu_{H^+}$-driven processes (for example, active transport of solutes such as L-proline and D-glucose), which would result in cell death. Further, amastigotes that reside in the extremely acidic environment of macrophage secondary lysosomes, would be rendered more sensitive to the effects of clomipramine than would promastigotes.

Imipramine (≤ 50 μM) had essentially no influence on proline transport of *L. donovani* promastigotes (Table 3). Clomipramine, however, at low concentrations such as 10 μM (20 pmol per 1×10^6 cells) inhibited the transport of proline by 28 percent, whereas at 100 μM (0.20 nmol per 1×10^6 cells), transport was almost completely inhibited. Clomipramine is a more potent inhibitor of serotonin transport (*11*) and oxidative phosphorylation (*6*) than is imipramine. The ionophores valinomycin and nigericin were used to assess whether clomipramine inhibited L-proline transport by uncoupling the $\Delta\mu_{H^+}$ (Table 3). Addition of either valinomycin (0.5 μM) or nigericin (2 μM) to promastigotes resulted in partial inhibition of L-proline transport, which was related to the effect of valinomycin on the membrane potential ($\Delta\psi$) and of nigericin on the *p*H gradient (ΔpH), (*3*). However, concomitant addition of nigericin (2 μM) and clomipramine (10 μM) resulted in a synergistic effect on the inhibition of proline transport. A similar significant synergistic inhibition of L-proline transport (70 percent) was observed when valinomycin (0.5 μM) and clomipramine (10 μM) were used together. These results suggest that clomipramine affects both $\Delta\psi$ and ΔpH. However, it remains to be determined whether clomipramine affects $\Delta\mu_{H^+}$ by inhibiting a surface membrane proton pump (for example, H^+ adenosinetriphosphatase), or by having an ionophore-like characteristic.

One of the objectives in the design of chemotherapeutic agents is to take advantage of physiologic differences between an infectious organism and its mammalian host. The current results with *L. donovani* have demonstrated that this approach is feasible. As we suggested (*3*), energy transduction processes similar to those of *L. donovani* may also occur in various other parasitic protozoa. Therefore, clomipramine and compounds with a similar mode of action might prove useful as antiparasitic agents.

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12. D.Z. is a recipient of the Chaim Weizmann postdoctoral fellowship award. We thank E. C. Weinbach for his suggestion to test imipramine and clomipramine, J. L. Costa for the imipramine, clomipramine, and nitroimipramine analogs, and P. A. Scott and D. Sacks for their help in initiating the macrophage and *L. major* experiments in vitro.

4 April 1984; accepted 27 July 1984

A Single Troponin T Gene Regulated by Different Programs in Cardiac and Skeletal Muscle Development

Abstract. *A cloned complementary DNA derived from a messenger RNA transiently present at low abundance levels in early chick embryonic skeletal muscle hybridizes to a messenger RNA present at high abundance levels in cardiac muscle. Genomic DNA hybridization and nucleotide sequence identity of complementary DNA's from both heart and skeletal muscle demonstrate that the messenger RNA's from both sources are encoded by the same gene. The encoded polypeptide is a troponin T sequence which is probably a cardiac isoform. This single copy troponin T isogene is governed by different regulatory programs in heart and skeletal muscle differentiation.*

The contractile proteins of muscle are members of large families of related isoproteins. In some cases the different members of an isoprotein family are so closely related that amino acid sequences differ in only small regions. For example, within the six-member actin protein family found in birds and mammals there are only 27 amino acid differences between the most widely diverged members, and the difference between muscle isoforms of actin can be due to as few as three amino acids (*1*). Despite this similarity in primary structure, each member of a contractile protein family is expressed in a highly tissue-specific, or developmental stage-specific, manner. During skeletal muscle differentiation, both the light chains (*2*) and heavy chains (*3*) of myosin as well as other muscle proteins (*4*) undergo complex, stage-specific programs of isoform switching. Thus, the expression of contractile protein isoforms appears to be precisely regulated and is often tightly coupled to specific developmental stages. Many of these switches occur within single muscle fibers (*5*), which presents an interesting problem in understanding the assembly and turnover of the myofibrillar apparatus.

Caplan *et al.* (*6*) have argued that isoprotein switching during muscle development is functionally analogous to globin isoform switching and that each successive isoprotein provides a slightly altered function which is essential to the developmental progression of muscle. While no obvious functional differences have been detected between closely related contractile protein isoforms (*7*), there may be subtle differences which have not been discerned. An alternative hypothesis would place the significance of isoformic diversity at the level of the gene rather than at the level of the protein (*8*). In this model, duplication of contractile protein genes allows them to