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Phospholipase A_2 from *Trypanosoma brucei gambiense* and *Trypanosoma brucei brucei*: Inhibition by Organotins

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Activity and kinetics of phospholipase A_2 (PLA_2) from *Trypanosoma brucei gambiense* (Wellcome strain) and *Trypanosoma brucei brucei* (GUTat 3.1) were examined using two different fluorescent substrates. The activity in the supernatants of sonicated parasites was Ca^{2+}-independent, strongly stimulated by Triton X-100 with optimum activity at 37°C and pH 6.5–8.5. To encourage a possible interaction between the parasite enzyme and organotin compounds, fatty acid derivatives of dibutyltin dichloride were synthesized and evaluated as potential inhibitors of PLA_2. The enzyme from the two-trypanosome species differ with respect to kinetic parameters and are noncompetitively inhibited by the organotin compounds. The Michaelis constant (K_M) for PLA_2 from *T. b. brucei* is 63.87 and 30.90 μM while for *T. b. gambiense* it is 119.64 and 32.91 μM for the substrates 1,2-bis-(1-pyrenebutanoyl)-*sn*-glycero-3-phosphocholine (PBGPC) and 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine ($NBDC_{12}$-HPC), respectively.

Keywords: Trypanosome; Phospholipase A_2; Organotin; Inhibition

INTRODUCTION

African trypanosomiasis is a parasitic disease of man and animal caused by species of the genus *Trypanosoma*. The existing trypanocides have been in use for the past 50 years and side effects and resistance against these drugs have recently been recorded with growing frequency in Western and Eastern Africa.[1,2] The understanding of the biochemistry and cellular functions of these parasites has made some progress in the identification and characterization of novel drug targets for rational chemotherapy.[3] Trypanosomes were believed to be unable to synthesize fatty acids *de novo* and therefore depend on the host's lipid for their fatty acids synthesis,[4] but a recent report[5] revealed a specialized fatty acid synthesis in African trypanosomes accounting for up to 25% of their myristate requirements. Therefore, this partial dependence of trypanosomes on host

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lipid suggests that the survival of the parasite requires an appropriate amount of lipid in the host.

The ability of the trypanosome population to evade the immune response of the host is due to a dense and uniform protective coat made of about 10^7 copies/cell of a 55 kDa VSG.[6,7] The VSG of African trypanosomes has a glycosyl phosphatidyl inositol (GPI) anchor that is unusual in that its fatty acids are exclusively myristate.[8]

Organotins are compounds which possess one or more direct tin–carbon covalent bond(s) that are responsible for the specific properties of such molecules. They are widely used in agriculture and industries; and are toxic to a variety of organisms including bacteria, fungi, protozoans etc.[9] They have been reported to have some trypanocidal potentials.[10,11] In an attempt to broaden the search for more chemotherapeutic targets and molecules, some fatty acid derivatives of dibutyltin dichloride were synthesized and targeted against phospholipases of the parasites. Phospholipases and lysophosphatidyl transferases are involved in the incorporation of lipids for the synthesis of the parasite membrane and other structural lipids. Here, we report some kinetic analysis of PLA_2, extending to inhibition studies using organotin compounds.

MATERIALS AND METHODS

Materials

Dibutyltin-dichloride (DBTC), -dimyristate (DBTM), -dipalmitate (DBTP), -dioleate (DBTO), -distearate (DBTS), and diphenyltin dichloride (DPTC) (Fig. 1) were synthesized as described by Van der Kerk and Luijten.[12] DBTC and DPTC were dissolved in ethanol, glycerol and water (26:33:42), as described by William *et al.*[13] The fatty acid derivatives were dissolved in absolute ethanol (one volume) and gradually added to a mixture of equal volumes of distilled water and glycerol (one volume). 1,2-bis-(1-pyrenebutanoyl)-*sn*-glycero-3-phosphocholine (PBGPC) and 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBDC 12-HPC) were purchased from Molecular Probes, (Eugene, OR, USA). Fetal bovine serum (FBS) was from Equitech-Bio, (Ingram, USA) and the tissue culture medium Eagles MEM was from Nissui Pharmaceutical, (Tokyo, Japan). All other reagents were either of analytical or culture grades.

Trypanosomes

Two species of parasites were used, *Trypanosoma brucei brucei* (GUTat 3.1) and *T. b. gambiense* (Wellcome strain). The stabilates were maintained in the Department of Protozoology, Institute of Tropical Medicine, Nagasaki, Japan. The frozen stock of the parasites was grown in female ICR mice (6–8 weeks old). At high parasitaemia, estimated from the blood of the infected mouse by the rapid matching method,[14] the mouse was sacrificed and blood removed by cardiac puncture. The trypanosomes were separated from the blood cells on a DE-52 (Whatman) anion exchange colunm[15] and counted on an improved Neubauer hemocytometer.

Enzyme Extraction

About 10^8 cells/ml were lysed by sonication twice at maximum amplitude for 500 s in the presence of lysis buffer (50 mM phosphate buffer pH 7.4 containing 50 µl each of 5 µg/ml of pepstatin, leupeptin and antipain). The lysate was centrifuged at 15,000*g* at 4°C for 20 min. The supernatant was then checked for phospholipase (PLA) activity. 10 ml of the lysate was applied to a DEAE cellulose column pre-equilibrated with either 50 mM of phosphate or tris-buffer pH 7.4 and 8.0, respectively. Fractions of 5 ml each were collected (flow rate of 1 ml/min) using 0.1–0.5 M

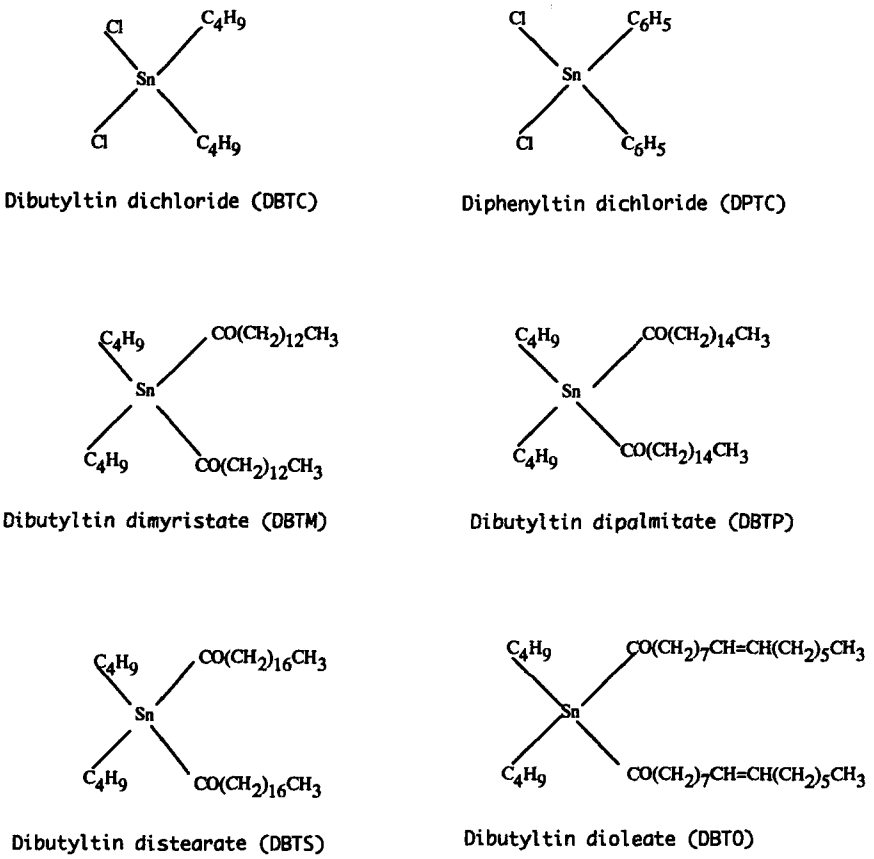

FIGURE 1 Structural formulae of the organotin compounds. DBTC and DPTC are parent compounds Derivatives were synthesized by substituting the chloride ions with myristate, palmitate, oleate and stearate.

NaCl gradient and checked for PLA_2 activity against PBGPC and $NBDC_{12}$-HPC as the substrates. The PLA_2 fractions were precipitated by NH_4SO_4 (70%) and dialyzed either against buffer or in polyethylene glycol 6000. This dialysed fraction was used as the source of the enzyme. Protein concentration was measured by Bio-Rad DC Protein Assay using a Beckman, DU Series 600 spectrophotometer at 750 nm.

Enzyme Assay

A micro plate phospholipase assay was set up using different concentrations of PBGPC (1.25 mM) and of $NBDC_{12}$-HPC (2.5 mM) as substrates. These stock solutions were stored at −30°C until required for use. The activity of the PLA_2 towards the substrates was assayed using 50 mM phosphate or tris-buffer at pH 7.4 and 8.0, respectively, in a total reaction mixture of 60–80 µl at 37°C. This mixture consisted of 1 mM Ca^{2+}, the enzyme, the substrate, 1% Triton X-100, and made up to 60–80 µl with the corresponding buffer depending on the assay condition. After incubation for 30 min, the reaction was stopped using 60 µl of 0.0625 M NaOH. PLA_2 activity liberates the fluorescent fatty acids from the *sn*-2-position of each of the substrates. The fluorescence intensity was read using Fluoroscan II with excitation 365 nm and emission at 450 nm for PBGPC and 465 and 530 nm for $NBDC_{12}$-HPC. Results are expressed in an arbitrary unit of fluorescence intensity per min. Controls without added enzyme were run for each assay.

Similar preparations were conducted under the same conditions as described in the enzyme assay above but with different concentrations of the organotin inhibitors in a total reaction mixtures of 60–80 µl. The kinetics of the inhibitions were analysed by appropriate plots of the residual activity.

Data Analysis

All experiments were carried out in either duplicate or triplicate. Data were fitted into the appropriate equation with the use of CA-Cricket graph III Software package. In all the computed kinetic parameters a Hanes–Woolf plot was used.

In Vitro Cultivation and Lysis Studies

The trypanosome species were cultivated *in vitro* according to established procedures.[16,17] Various organotin compounds were tested against the parasites using a 24 well micro-titre plate technique. 10–200 µM of each of the test compounds was added to induce lysis and was monitored under a phase contrast microscope.

RESULTS

The activities of partially purified PLA_2 from the bloodstream form (bsf) of *T. b. brucei* and *T. b. gambiense* using PBGPC and $NBDC_{12}$-HPC as substrates were determined in phosphate (50 mM) and tris-buffers (50 mM) at pHs 7.4 and 8.0, respectively, by varying the concentration of each substrate. The PLA_2 activity from the procyclic form (pcf) of *T. b. brucei* was observed to be 3.5 times lower than that from the bsf form. The effect of temperature and pH on the activity of the enzyme was determined and showed optimum activity at 37°C and pH 6.5–8.5 (Fig. 2(a) and (b)) which is in accord with the temperature at which the parasites inhabit, infect and proliferate in the mammalian host or in culture. The observed optimum pH range and non-requirement of Ca^{2+} by the enzyme, suggest that the activity is more likely from the soluble cytosol/membrane-bound form rather than the particulate or secreted one which is assumed to lie within the acidic pH range or Ca^{2+}-dependent, respectively.

The K_M, V_{max} and K_i were computed using a Hanes–Woolf plot from the respective slopes and intercepts (Fig. 3(a) and (b)). Both enzyme activities are Ca^{2+}-independent and Triton X-100 was observed to be a requirement for the activity of the enzymes (Fig. 4). Tables I and II show the various computed kinetic parameters. DBTM, DBTP, DBTO and DBTS were observed to have mixed inhibitory effect on the PLA_2 while in the case of DBTC and DPTC the inhibition appeared to be noncompetitive using double reciprocal plots. The index of physiological efficiency (PE, V_{max}/K_M) for the enzyme from the two parasites with respect to each of the different inhibitors is shown in Fig. 5 where the PE is observed to be reduced as compared with controls without inhibitors. This reduction in PE appeared to have similar tendency for all the tin compounds in the case of *T. b. brucei* while *T. b. gambiense* appeared to be relatively more resistant to some.

Effect of the compounds on the *in vitro* cultured bloodstream forms(bsf) is almost similar and dose-dependent. However, the overall consequence of their effect (both derivatives, DBTC and DPTC) on the morphology of the parasites is lysis as observed under phase contrast microscopy (Fig. 6).

DISCUSSION

The chemotherapy for trypanosomiasis is unsatisfactory and attempts at vaccine development has been thwarted by the parasites remarkable property of antigenic variation[18] which enables

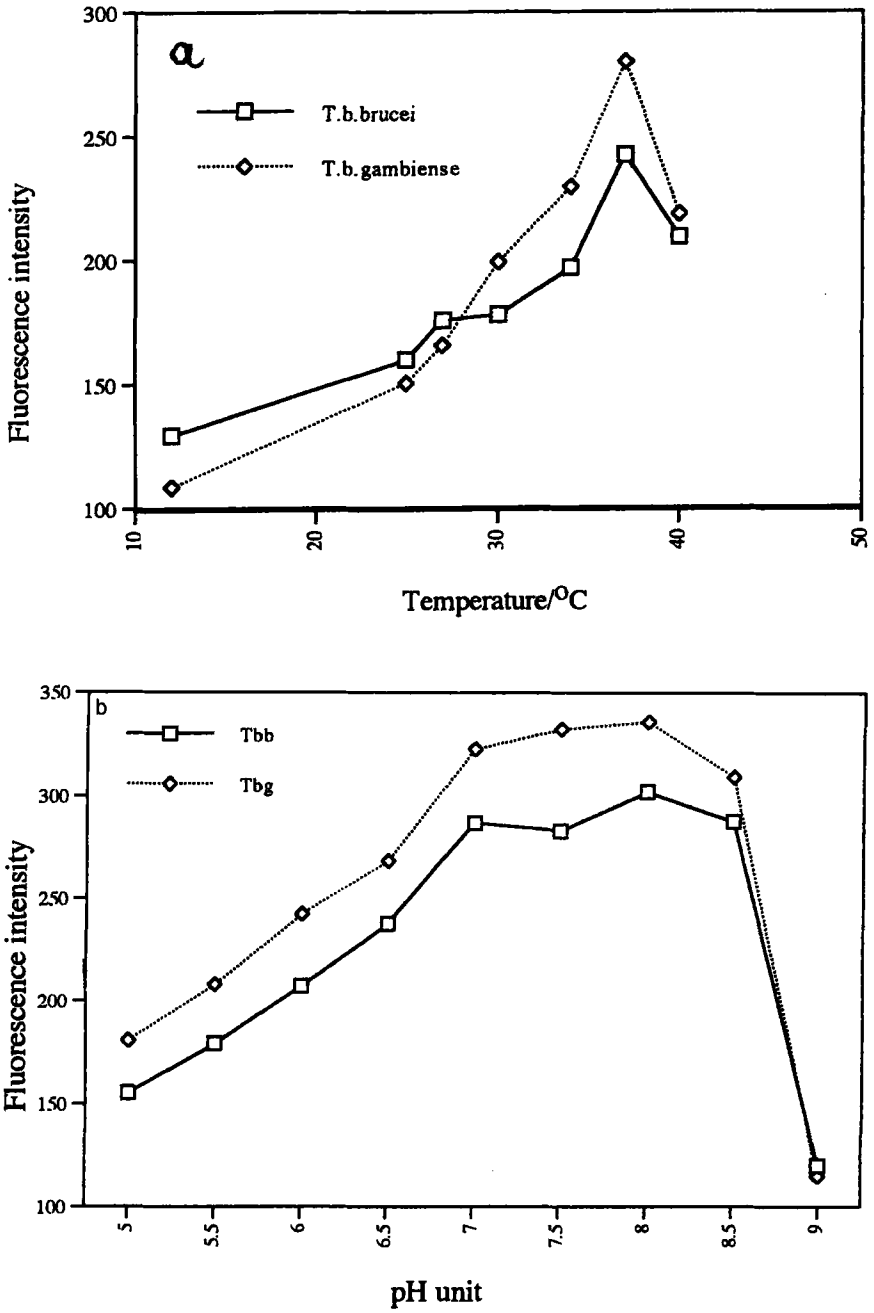

FIGURE 2 (a) The effect of temperature on the activity of PLA_2 from *T. b. brucei* and *T. b. gambiense* measured between 12–40°C, with 37°C as the optimum temperature of activity. (b) The effect of pH on the activity of the enzyme in the presence of acetate, phosphate and tris-buffers between pH 5–9, with pH optimum between 6.5–8.5. Reaction mixture was incubated for 30 min after which the fluorescence intensity was measured with Fluoroscan II at excitation 465 nm and emission at 530 nm. Results are averages of three different determinations in the presence of $NBDC_{12}$-HPC as substrate under similar conditions.

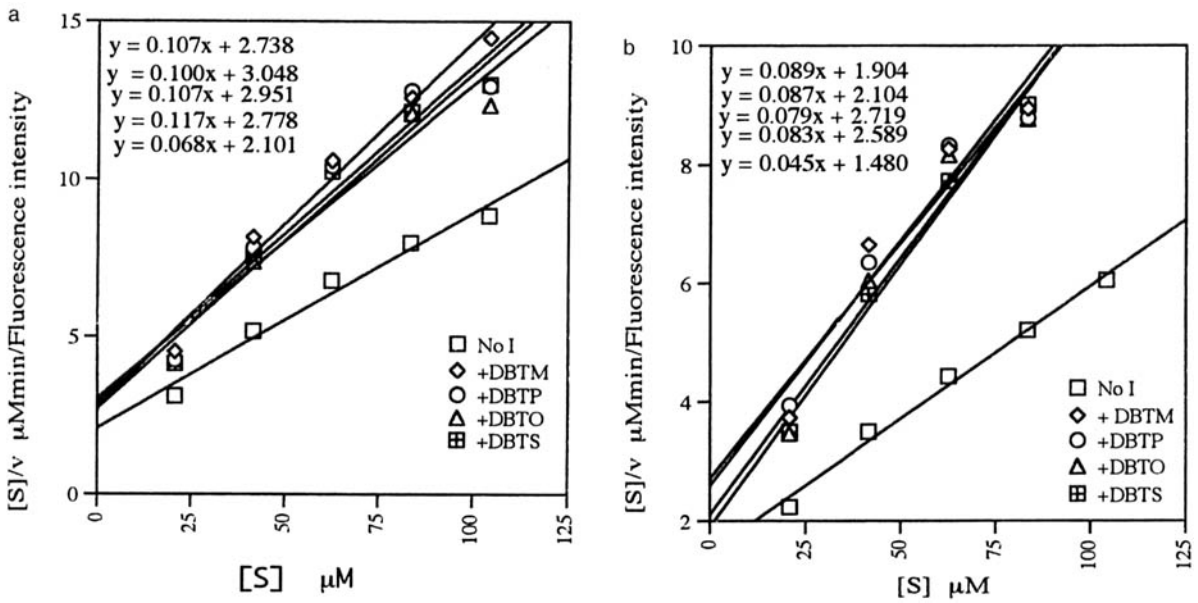

FIGURE 3 Representative Hanes–Woolf plot of substrate concentration/initial velocity versus substrate concentration. Reaction mixtures were incubated for 30 min at 37°C and activity was measured fluorometrically by varying the substrate concentration ($NBDC_{12}$-HPC) at fixed protein concentration of 20 μg/ml. Kinetic parameters and inhibition constants were calculated from the intercepts and slopes of the plots. (a) for *T. b. brucei* and (b) *T. b. gambiense*. Results are averages of three different determinations. No I = plot of activity in the absence of inhibitor. Inhibitor concentration=100 μm.

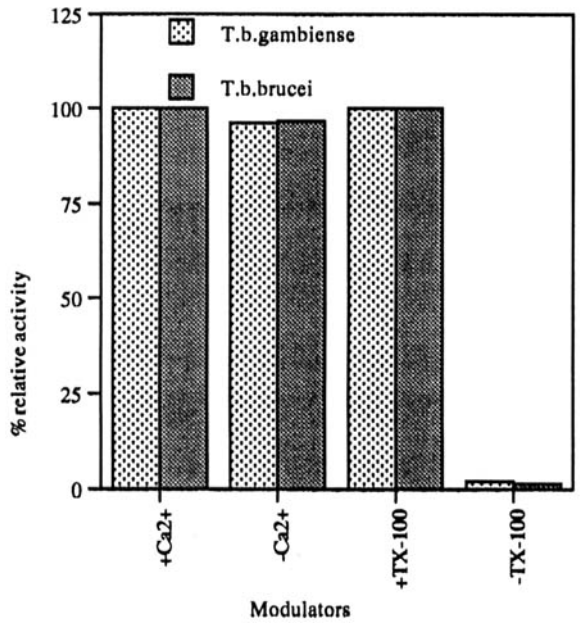

FIGURE 4 Effect of Ca^{2+} and Triton X-100 dependence on the activity of PLA_2. Reaction mixture with and without 1 mM Ca^{2+} and 1% Triton X-100 was set up, incubated at 37°C for 30 min in the presence of $NBDC_{12}$-HPC as substrate. Activity was measured fluorometrically and relative activity was calculated as a % in the presence of 1 mM Ca^{2+} and 1% Triton X-100 as maximum activity.

the parasite population to evade the host's specific humoral immune attack.[19] Because trypanosome proliferation is rapid, it has been likened to some types of cancer cells and most existing trypanocides are reported to have anti-cancer effect or *vice-versa*.[20]

To design and develop new active molecules for the chemotherapy of African trypanosomiasis, we synthesized fatty acid derivatives of dibutyltin dichloride (DBTC) (Fig. 1). Dibutyltin dichloride and diphenyltin dichloride (DPTC) have been reported to have some anti-cancer[21] and antitrypanosomal activity.[11] Generally organotins possess growth inhibitory properties in different biological systems by interacting with vital biomolecules,[22] and their mode of action depends on the type of biological system and the anionic groups attached to the parent organotin. The design of these derivatives appears to be an interesting approach in the sense that these compounds could be easily incorporated by the parasites in an attempt to satisfy their lipid and

TABLE I The kinetic parameters of PLA_2 from the two parasites in the presence of each of the fluorescent substrate (reaction mixtures were incubated for 30 min at 37°C after which fluorescence intensity was measured using Fluoroscan II. The values are computed from the intercepts and slopes of the Hanes–Woolf plots in the presence and absence of inhibitors. V_{max} is expressed in arbitrary unit of fluorescence intensity/min. Results are from the averages of three different determinations)

Substrate	*T. b. brucei*		*T. b. gambiense*	
	K_m (μmM)	V_{max} (unit/min)	K_m (μM)	V_{max} (unit/min)
PBGPC	63.87	43.48	119.6	90.91
$NBDC_{12}$-HPC	30.9	14.71	32.9	22.22

TABLE II Showing the inhibition constants (K_i) for the two parasites in the presence of $NBDC_{12}$-HPC and 100 μM inhibitor concentration (reaction mixtures were incubated for 30 min at 37°C after which fluorescence intensity was measured using Fluoroscan II. The values are computed from the intercepts and slopes of the Hanes–Woolf plots in the presence and absence of inhibitors. V_{max} is expressed in arbitrary unit of fluorescence intensity/min. Results are from the averages of three different determinations)

Inhibitor	*Ki* (μM)	
	T. b. brucei	*T. b. gambiense*
DBTM	138.4	136.4
DBTP	131.2	132.7
DBTO	212.3	107.1
DBTS	131.2	102.4
DBTC	486.1	100
DPTC	377.6	140.7

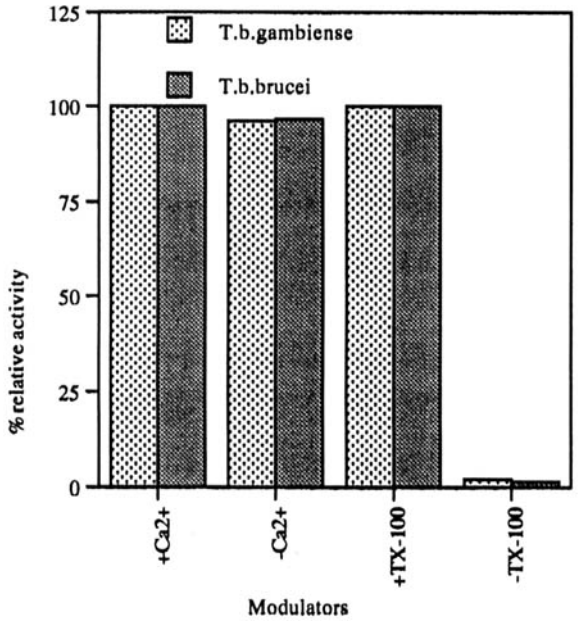

FIGURE 5 Index of physiological efficiency (PE) (V_{max}/K_M) for PLA_2 from *T. b. brucei* and *T. b. gambiense* with respect to the different inhibitors. The efficiency of the enzyme is reduced by 100 μM of each of the compounds which the parasites are sensitive to *in vitro*.

fatty acid needs. PLAs are a family of esterases that hydrolyze *sn*-ester bonds in phospholipids, releasing free fatty acid and lysophospholipid. African trypanosomes contain phospholipases, and higher activity is believed to be a function of pathogenicity.[23] Other protozoan parasites have been reported to have PLAs that are implicated in pathological and intracellular processes, and have been suggested as targets for therapeutic intervention. A Ca^{2+}-independent PLA_2 was detected in *Plasmodium falciparum*-infected erythrocytes but not in uninfected cells;[24] Saffer and Schwartzman[25] and Cassaing *et al.*[26] detected Ca^{2+}-dependent and Ca^{2+}-independent PLA_2 respectively, from supernatants of sonically disrupted *Toxoplasma gondii* and believed that these enzymes may play a role in host cell penetration. Pathogenic mechanisms in trophozoites of *Entamoeba histolytica* implicates a Ca^{2+}-stimulated PLA_2.[27] Although there appear to be variations in terms of Ca^{2+} requirement by the PLA_2 from various protozoan parasites, their ultimate role is common, to aid the parasites survive the host's hostile environment. Our observed trypanosome Ca^{2+}-independent PLA_2 may functionally, therefore not be an exception. Various types of PLA_2 with cytolytic and neurotoxic effects from venoms of different species of arthropods, insects and reptiles have been reported. The different biological func-

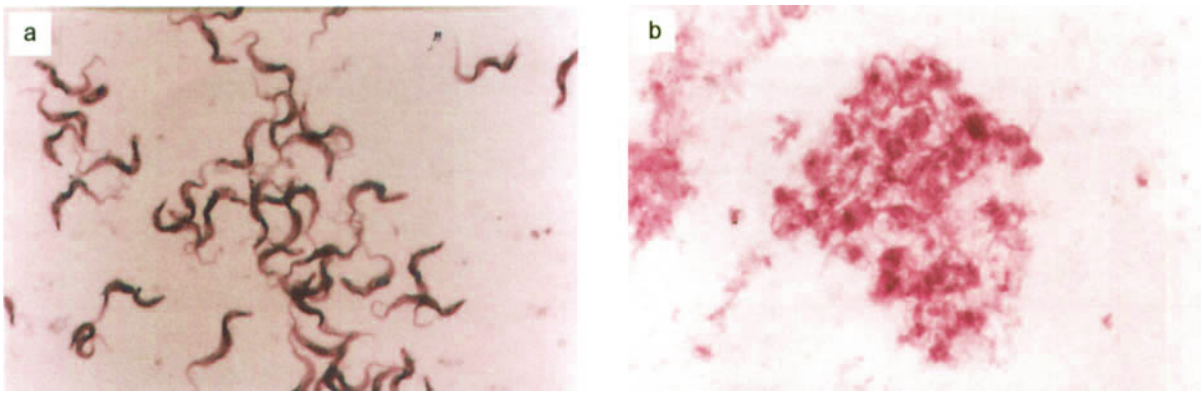

FIGURE 6 Effect of DBTC on the morphology of *in vitro* cultured *T.b. gambiense* (a) Shows the normal shape of the bloodstream cultured form of *Trypanosoma brucei gambiense* before exposure to organotins as observed under phase contrast microscopy. (b) Shows the deformed form of *Trypanosoma brucei gambiense* after exposure to lethal dose of organotin as observed under phase contrast microscopy.

tions of PLA in metazoans and protozoan parasites pre-suppose the parasites PLA to be selectively vulnerable to certain compounds. The synthesized organotins (Fig. 1) were observed to inhibit the activity of the partially purified PLA_2 from the parasites, with various inhibition constants (K_i) at a fixed inhibitor concentration (Tables I and II). The PLA_2s from the two parasites, *T. b. brucei* and *T. b. gambiense*, differ with respect to kinetic parameters and inhibitors, even though they are morphologically indistinguishable. This is not surprising as the two parasites inhabit and infect different hosts and also in consideration of the various reported molecular and biochemical differences. The synthesized organotins were also observed to induce lysis of both the *T. b. brucei* and *T. b. gambiense in vitro* in a dose-dependent fashion (data not shown). PLA_2 enhances *T. cruzi*-macrophage association in the initial step of the macrophage invasion process and quinacrine, 4-bromophenacyl bromide or phentermine (PLA_2 inhibitors) decreased host cell-parasite association.[28] Inhibition of plasmodial PLA_2 by the antimalarials chloroquine, quinine and arteether is suggested to have relevance in the chemotherapeutic action of these drugs[24]. Tributyltin-bis-oxide and extracts from *Allium sativa* are trypanocidal and were observed to inhibit trypanosomal PLA_2.[10,29] However, as organometallics interact with a range of biomolecules, it is not immediately clear whether the death of the parasites in the culture form was directly a result of the inhibition of PLA_2 but it is likely that the derivatization of the toxic organotin moiety with fatty acids, that may be required by the parasites, facilitated their uptake and subsequently lysed the parasites. Notwithstanding, as chemotherapy remains the most powerful strategy for control of trypanosomiasis, a search for effective PLA inhibitors that will starve the parasites of their extravagant requirement for fatty acid or enhance the bioavailability of toxic organoarsenics with fatty acids could probably be steps in the right direction.

References

[1] Bacchi, C.J. (1993), *Parasitol. Today* **9**, 190.
[2] Boudichon, A.J. (1998), *J. Protozool. Res.* **8**, 258–262.
[3] Croft, S.L. (1999), *Mem. Inst. Oswald Cruz* **94**(2), 215–220.
[4] Mellors, A. and Samad, A. (1989), *Parasitol. Today* **5**(9), 239–244.
[5] Morita, Y.S., Paul, K.S. and Englund, P.T. (2000), *Science* **288**(5463), 140–143.
[6] Cross, G.A. (1975), *Parasitol.* **71**, 393–417.
[7] Vickerman, K. and Luckins, A.G. (1969), *Nature* **224**, 1125–1126.

[8] Buxbaum, L.U., Raper, J., Opperdoes, F.R. and Englund, P.T. (1994), *J. Biol. Chem.* **269**(48), 30212–30220.
[9] Pain, G. and Cooney, J.J. (1998), *Arch. Environ. Contam. Toxicol.* **35**(3), 412–416.
[10] Nok, A.J., King, A.N.E., Adaudi, A., Achoba, I.I., Gimba, C.E., Musa, O.S. and Kagbu, J.A. (1992), *J. Clin. Biochem. Nutr.* **13**, 81–85.
[11] Shuaibu, M.N., Ameh, D.A., Bonire, J.J., Adaudi, A.O., Ibrahim, S. and Nok, A.J. (2000), *Parasite* **7**, 43–45.
[12] Van der Kerk, G.M.J. and Luijten, J.G.A. (1956), *J. Appl. Chem.* **6**, 49–55.
[13] William, S., Joseph, G.V., Van Spanje, I.N.E., Snoek, M., Brands, R. and Hooykaas, H. (1977), *Toxicol. Appl. Pharmacol.* **42**, 197–212.
[14] Herbert, W.J. and Lumsden, W.H.R. (1976), *Expt. Parasitol.* **40**, 427–431.
[15] Lanham, S.M. and Godfrey, D.G. (1970), *Expt. Parasitol.* **28**, 521–534.
[16] Hirumi, H. and Hirumi, K. (1989), *J. Parasitol.* **75**, 985–989.
[17] Hirumi, H. and Hirumi, K. (1994), *Parasitol. Today* **10**(2), 80–84.
[18] Englund, P.T., Hereld, D., Krakow, J.L., Doering, T.L., Masterson, W.J. and Hart, G.W. (1988) *MBL Lectures in Biology Volume 9. The Biology of Parasitism* (England, P. T. and Sher, A., eds.) (Alan R. Liss, Inc., New York), pp 401–412.
[19] Cross, G.A.M. (1996), *Bioassay* **18**, 283–291.
[20] Barrett, S.V. and Barrett, M.P. (2000), *Parasitol. Today* **16**(1), 7–9.
[21] Crowe, A.J., Smith, P.J. and Atassi, G. (1984), *Inorg. Chem. Acta* **93**, 179–184.
[22] Nok, A.J., Shuaibu, M.N., Bonire, J.J., Dabo, A., Wushishi, Z. and Ado, S. (2000), *J. Enz. Inhib.* **15**, 411–420.
[23] Hambrey, P.N., Mellors, A. and Tizzard, I.R. (1981), *Mol. Biol. Parasitol.* **2**, 177–186.
[24] Zidovetzki, R., Sherman, I.W. and O'Brien, L. (1993), *J. Parasitol.* **79**(4), 565–570.
[25] Saffer, L.D. and Schwartzman, J.D. (1991), *J. Protozool.* **38**(5), 454–460.
[26] Cassaing, S., Fauvel, J., Bessieres, M.H., Guy, S., Seguela, J.P. and Chap, H. (2000), *Int. J. Parasitol.* **30**, 1137–1142.
[27] Vargas-Villareal, J., Olvera-Rodriguez, A., Mata-Cardenas, B.D., Martinez-Rodriguez, H.G., Said-Fernadndez, S. and Alagon-Cano, A. (1998), *Parasitol. Res.* **84**, 310–314.
[28] Connelly, M.C. and Kierszenbaum, F. (1984), *Biochem. Biophys. Res. Commun.* **121**(3), 931–939.
[29] Nok, A.J., William, S. and Onyenekwe, P.C. (1996), *Parasitol. Res.* **82**, 634–637.