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ANTILEISHMANIAL ACTIVITY OF HAMYCIN : A POLYENE ANTIBIOTIC

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SUMMARY : Hamycin, a polyene antibiotic, now in extensive use in the treatment of candidiasis and otomycosis, is found to be remarkably effective in killing Leishmania donovani promastigotes in a liquid medium at a concentration of 0.2 µg/ml. The glucose stimulated respiration and the uptake of 2-deoxy-D[U-^{14}C] - glucose was inhibited in cells treated with the drug at a growth inhibitory concentration. An immediate release of isotopic glucose from preloaded cells could be demonstrated after exposure to hamycin. All the above effects could be effectively prevented in the presence of ergosterol. The primary site of action of hamycin on L. donovani promastigote cells appears to be membrane sterols that result in the loss of the permeability barrier to small metabolites. The lower minimum inhibitory concentration of hamycin compared to other established drugs warrants further study in the context of increasing reports of clinical resistance to pentavalent antimonials. © 1992 Academic Press, Inc.

Leishmania donovani is the etiologic agent of human visceral leishmaniasis or kala-azar, often a fatal disease that is widely prevalent in many parts of the tropical world (**1,2**). Apart from this visceral form, cutaneous and mucocutaneous leishmaniasis are also manifested by other species of Leishmania (**1**). All species of Leishmania in general have a digenic life cycle; an extracellular, flagellated, nonpathogenic form in the insect vector and an aflagellated oval pathogenic form in humans that reside and proliferate exclusively in the host macrophages (**2**). The cultural form corresponds to the vector form. Pentavalent antimonials introduced in early days of chemotherapy are still the drugs of choice. Current clinical reports, however, show an alarming trend. A large proportion of cases (15-25%) are becoming unresponsive to antimonial treatment (**3,4**). For such cases, pentamidine, a biguanidine and amphotericin B are only available drugs as secondary line of defence. Both these drugs have severe limitations (**5,6**). Both are highly toxic and needs monitored conditions that can only be provided in an advanced

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hospital situation. An urgent need, therefore, exists for development of new lines or for improving on existing lines of drugs that may be less toxic or may have a more convenient route of administration.

Hamycin was isolated as an antifungal polyene antibiotic from the metabolic product of Streptomyces pimprina thirum by Thirumalachur in early sixties (**7,8**). Since then considerable work has been done towards its development as a drug for various fungal infections. Hamycin was found to be considerably more potent than nystatin and amphotericin B against several strains of candida and is now accepted as a major drug for treatment of oral candidiasis and otomycosis (**8**). More interestingly, the antibiotic was found to be highly effective against vaginal infections caused by protozoal spp. such as Trichomonas vaginalis and is presently widely used in India as a preferred drug against such infections. Pharmacological and toxicological data indicates the antibiotic to be less toxic than amphotericin B (**9**). Encouraged by its antiprotozoal activity and by its toxicity status, we decided to investigate the leishmanicidal potential of this drug.

In the present paper, we demonstrate that hamycin is remarkably effective against L. donovani promastigotes. The primary site of the drug is on parasite membrane and the lethal effect is brought about by disruption of the permeability barrier possibly due to the interaction with ergosterol of the membrane.

MATERIALS AND METHODS

All the biochemicals were purchased from Sigma Chemical Co.. Hamycin was obtained as a generous gift from Hindustan Antibiotics Limited, Pune, India; Amphotericin B and Pentamidine from May and Baker Research Division, U.K. For all experiments hamycin, amphotericin B and the sterols were dissolved in dimethyl sulphoxide. 2-deoxy-D[U-^{14}C]-glucose was purchased from Amersham, U.K.

A recent clinical isolate of Indian strain (UR-6) of Leishmania donovani (MHOM/IN/1978/UR-6) was used for this work (**10**). The maintenance of promastigotes and growth inhibition studies in liquid media on this form of organism were done as described recently (**11**).

Respiration of the organism was studied by conventional monometric techniques in a Warburg apparatus (**12**). Each Warburg flask contained 0.2 ml of 10% KOH in the central well. The main reservoir contained (total volume, 2.8 ml of phosphate buffered saline) approximately 1.25x 10^7 cells and requisite amount of glucose where indicated. After equilibration at 37°C for 15 minutes hamycin and ergosterol, both in dimethyl sulfoxide solution were added within 30 seconds of each other in quick succession when needed. The final volume was made uppto 3.0 ml in the reservoir with phosphate buffered saline and allowed to equilibrate for

another 5 minutes. The vessels were then closed to the atmosphere and the reading taken at this time was counted as the zero time. Air was used as gas phase; shaking speed was 120 stokes min^{-1}.

Uptake studies were done according to Schaefer and Mukkada (**13**) with some modifications. The uptake of 2-deoxy-D[U-^{14}C]glucose was used as a measure of 2-deoxy-D-glucose transport. Promastigotes washed twice were resuspended in the phosphate buffered saline pH 7.4 to give a final cell concentration about 0.4 mg cell protein ml^{-1}. The suspensions were equilibrated at 28°C for 10 minutes. The concentration of labelled sugar and of other additions in various experiments are indicated in the results section. Aliquotes of 1 ml samples were removed at appropriate intervals and were filtered immediately through cellulose acetic filters (0.8 µm porosity) and washed with 20 ml of phosphate buffered saline. The filters with the cells were transfered directly to scintillation vials containing 5 ml aqueous scintillation fluid and radioactivity was determined in LKB-Wallac, 1217, Rackbeta liquid scintillation counter. The results are expressed as nmole sugar (mg cell protein)$^{-1}$.

RESULTS AND DISCUSSION

Hamycin was found to have profound growth inhibitory effect on the promastigote form of Leishmania donovani. At a concentration of 0.2 µg/ml, the drug inhibited the cell division with lysis; at higher concentrations lytic effect became still more pronounced (**Fig.1**). About 80% inhibition of growth of L. donovani promastigotes was found with 0.05 µg/ml of hamycin (**Fig.1**). Vehicle control with dimethylsulfoxide at various concentrations which was used for dissolving the drug had no effect on growth (data not shown). Under identical growth conditions 2 µg/ml Pentamidine, 1 µg/ml amphotericin B and 0.1 µg/ml acivicin (**14**) were needed to arrest the growth completely.

Like other polyene antibiotics, the antifungal action of hamycin may be presumed to be by altering the permeability barrier of sensitive cells containing sterols in their plasma membrane. Lishmania spp. are known to contain ergosterol in their plasma membrane (**15**).

The glucose-stimulated respiration of Leishmania donovani promastigotes was found to be significantly inhibited at growth inhibitory concentration (**Fig.2**). Addition of about 37 times ergosterol within 30 seconds of the addition of drug almost totally restore the glucose stimulated respiration (**Fig.2**). The protection of normal respiration with the addition of ergosterol indicates that the effect on respiration may be a secondary phenomenon as a consequence of the disruption of membrane permeability barrier resulting in rapid loss of energy sources from the system.

Selective transport of metabolites and their retention is a characteristic property of biological membranes. To study the possible direct

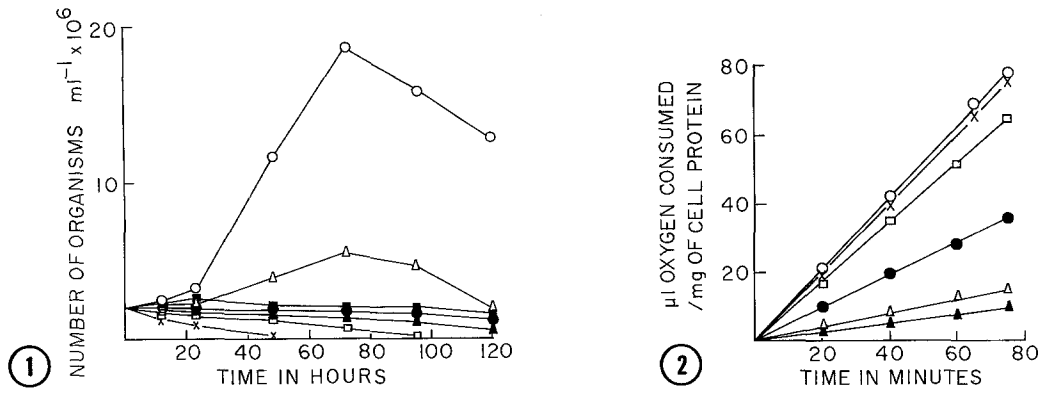

Fig. 1. Inhibition of growth of Leishmania donovani promastigotes in presence of hamycin and other antileishmanial drugs. The control growth curve in absence of any drug is represented by 'O—O'. 'Δ—Δ', '□—□' and 'X———X' indicate growth patterns in presence of 0.05 µg/ml, 0.2 µg/ml and 0.5 µg/ml of hamycin respectively. '●—●', '▲—▲' and '■—■' represent the same for 1 µg/ml of amphotericin B, 2 µg/ml of pentamidine and 0.1 µg/ml of acivicin respectively.

Fig. 2. Effect of hamycin on the respiration of Leishmania donovani promastigotes. The incubation mixture of 3 ml contained 1 ml of washed cell suspension (10 mg of protein), Phosphate buffered saline (0.15 M, pH 7.2) and other additions as indicated. '●———●' represents endogenous; 'O———O' endogenous + 2 mM glucose; 'X—X' endogenous + 2 mM glucose + 10 µl dimethylsulfoxide; 'Δ——Δ' endogenous + 2 mM glucose + 0.2 µg/ml hamycin; '▲—▲' endogenous + 2 mM glucose + 0.5 µg/ml hamycin; '□—□' endogenous + 2 mM glucose + 0.2 µg/ml hamycin + 7.5 µg/ml ergosterol.

interaction of hamycin with the membrane, the uptake of 2-deoxy-D-glucose was monitored in presence of the drug; glucose being one the prefered nutrients for the organism. Further, considerable work has been done to characterize glucose transport system in the parasite. Glucose appears to be transported by an active carrier mediated process that is driven by a proton-motive force across the membrane (**16**). **Fig.3** shows that deoxy-glucose uptake is effectively inhibited within 2 minutes of contact with the drug. The inhibition of 2-deoxy-D-glucose uptake could, however, be totally protected when ergosterol was almost simultaneously added along with the drug. The loss of permiability barrier could be shown more convincingly when parasites preloaded with radiolabelled deoxy-D-glucose were exposed to the drug. **Fig.4** shows a rapid loss of glucose from internal pool on treatment with the drug at its growth inhibitory concentration.

The parasite looses its transport property (**Fig.3**) and fails to retain internal metabolites (**Fig.4**) in presence of hamycin. Further, ergosterol can significantly reverse both these effects. The primary site of action of the drug therefore appears to be with the ergosterol of the

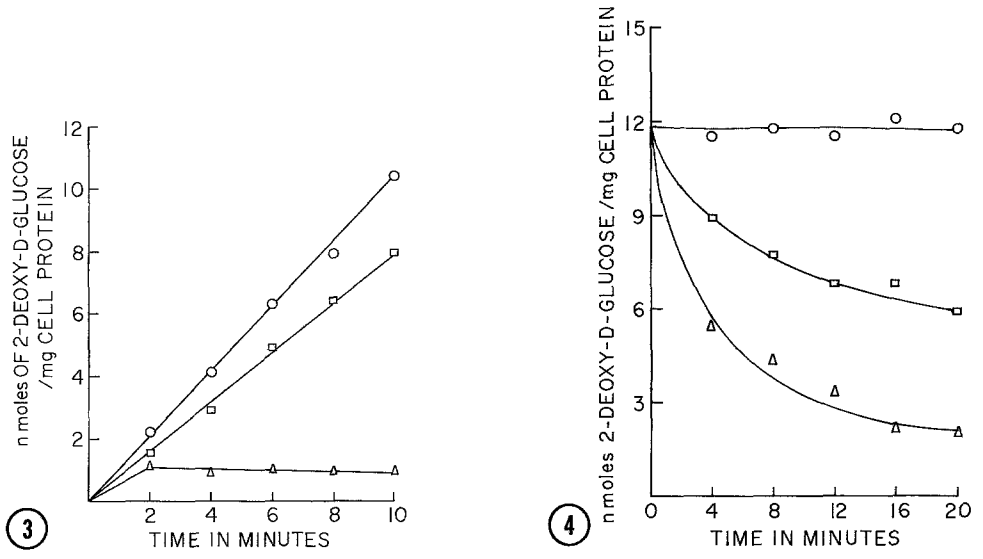

Fig. 3. Effect of hamycin on the uptake of 2-deoxy-D-glucose by Leishmania donovani promastigotes. Washed cells were resuspended in basal salt solution (5.2 g NaCl, 0.5 g KCl, 10.3 g Na_2HPO_4 in 1000 ml water, pH 7.0) to a cell density of 0.8 mg of cell protein ml^{-1}. Three flasks, one containing only cell suspension (control), another containing cell suspension and hamycin (0.2 µg/ml) and last one containing cell suspension, hamycin (0.2 µg/ml) and ergosterol (7.5 µg/ml), were incubated with gentle shaking for 10 minutes at 28°C. Individual aliquots of 0.5 ml of these incubated cell suspensions were added to the tubes of incubation mixtures of basal salt solution containing 0.1 mM 2-deoxy-D-[U-^{14}C] glucose (2.1×10^5 cpm) in a total volume of 1.0 ml. At the indicated time intervals, the contents of each tube were rapidly filtered and the radioactivity retained on the filter was counted. 'O——O' control; 'Δ——Δ' control + hamycin 0.2 µg/ml and '□——□' control + hamycin 0.2 µg/ml + ergosterol 7.5 µg/ml.

Fig. 4. Release of preloaded glucose from Leishmania donovani promastigotes on treatment with hamycin. 0.5 ml of washed cells in basal salt solution (pH 7.0) in three separate flasks were incubated with 0.1 mM 2-deoxy-D[U-^{14}C] glucose (1.2×10^6 cpm) for 30 minutes at 28°C. One of the flasks was used as control. Hamycin 0.2 µg/ml and 100 µl triton X-100 were added to two preincubated flasks respectively. Aliquots were drawn at indicated time intervals from the flasks and counts retained in the cells measured similar to **Fig. 3.** 'O——O' control; '□——□' control + 0.2 µg/ml hamycin and 'Δ——Δ' control + 100 µl Triton X-100.

plasma membrane. Leishmania parasites were earlier shown to contain ergosterol in their plasma membrane (**15**). The lysis of the parasite which is a later event may be due to some permanent disorganization of the membrane structure, the nature of which is not clear at the moment. It is, however, clear that hamycin, an established drug for some fungal and protozoal infections, has also profound antileishmanial activity.

In the context of increasing reports of clinical resistance to pentavalent antimonials search for established drugs with leishmanicidal acti-

vity should be intensified. Preliminary results with hamycin suggests that its potential as a chemotherapeutic agent against various forms of Leishmania should be further explored.

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