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A NEW ANTILEISHMANIAL COMPOUND, PHASEOLINONE

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SUMMARY : Inclusion of phaseolinone, a newly described mycotoxin, at 20 μg per ml in a solid culture medium (blood agar overlay) and at 50 μg per ml in a liquid culture (medium 199) inhibited the growth of L. donovani promastigotes. About 90% of the motile promastigotes lost motility after exposure to 50 μg per ml of phaseolinone for 6-7 h and here 3-day-old culture was more sensitive than 7-day-old culture. In an in vitro assay, DNA dependent RNA polymerase activity of 3-day-old promastigotes was considerably inhibited in the presence of this toxin. Therefore, this key enzyme was suggested to be one of the sites of action of phaseolinone.

There is no effective vaccine for leishmaniasis at present. The antimonial compounds (1,2) and diamidines (3) are the accepted drugs and are used extensively for the treatment of these diseases. Patients occasionally failing to respond to these chemotherapies have been compelled to accept spleenectomy, the surgical alternative for curing kala-azar, a visceral leishmaniasis. Undesirable severe side effects are known to occur with the administration of both antimonials and diamidines, but better drugs are not yet available; however, several compounds are under going the process of evaluation for use in chemotherapy of leishmaniasis. A recent review on this aspect presented the elaborative "advances in the chemotherapy of leishmaniasis" made during 1974-1985 (4). Among the promising substances this review described, allopurinol and its nucleosides, formycin-B, the lepidine WR-6026, and the antibiotics sinefungin, amphotericin B, rifampicin, and paramomycin are expected to be useful as future antileishmanial drugs. Many aromatic compounds were cited as antileishmanial agents in this review, but the list of these compounds lacks an aromatic compound having effective epoxy groups. Thus, it would

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be worthwhile to test the effect of such an epoxy aromatic compound on antileishmanial activity.

The diepoxy aromatic compound phaseolinone (Fig.1) has recently been purified to a pure crystalline form (5,6,7) and is not mutagenic when tested on a large number of Escherichia coli and Salmonella typhimurium strains, except for the excision-repair-deficient mutants such as E. coli WP-2 and S. typhimurium TA-100 (8). In experimental mice, only chronic phaseolinone toxicity caused anemia and spleen enlargement, but the withdrawal of toxin resulted in recovery from the physiological disorder(s) (unpublished result). The toxin phaseolinone has a LD^{50} value of approximately 1 gm per kg body weight (9), but its effect on protozoan parasites such as L. donovani has not been evaluated before.

The present report demonstrated that phaseolinone inhibited the in vitro growth of L. donovani in both solid and liquid culture media and also inhibited DNA-dependent RNA synthesis, acting at a site distinctly different from the α-amanitin-sensitive site.

MATERIALS AND METHODS

In vitro growth of Leishmania donovani : L. donovani (MHOM/IN/1978/UR-6) was used throughout the study and maintained in Ray's modified medium (10) with regular subculturing at 72 h intervals. Inclusion of phaseolinone at various doses (0 to 50 µg per ml) was made in solid Ray's modified medium (10) and in a liquid culture medium consisting of medium 199 10% heat inactivated (56°C, 30 min) fetal calf serum and 25 mM HPESE (ph 7.2 7.3) (11) to investigate the effect of phaseolinone on the growth of L. donovani at 25°C for a stipulated period of time. Highly motile promastigotes of L. donovani were exposed to the toxin in an incubation at 25°C with approximately 1.4 x 10^6 cells per ml of either phosphate buffer saline containing glucose 1% (w/v) or the liquid culture medium, both having 50 µg phaseolinone per ml. The motility of the promastigotes from this and from a toxin-free control incubation system was then determined microscopically at various points in time. In fresh liquid culture medium, 3-day-old and 7-day-old promastigotes, after having been exposed to toxin and then separated, were subjected to further growth for another 3 days at 25°C. The growth was recorded after the count of viable or motile promastigotes had been estimated microscopically.

RNA polymerase assay : In an in vitro DNA-dependent RNA polymerase assay using rat liver isolated nuclei, phaseolinone inhibited RNA synthesis, and it was distinct from α-amanitin (low dose) sensitivity (manuscript submitted). With this background, a study on RNA synthesis with the RNA polymerase system of L. donovani using calf thymus DNA as an exoge-

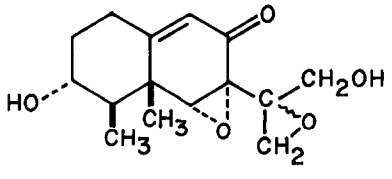

Fig.1 : The structure of phaseolinone.

neous template was undertaken to determine the effect of phaseolinone on such RNA synthesis.

Highly motile 3-day-old promastigotes were scraped from the blood agar overlay and washed twice with sterile normal saline and then once with TEGD buffer (50 mM Tris HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol (12). All steps were then carried out at 40°C. The method of enzyme preparation was similar to that used for T. cruzi RNA polymerase assay (12,13). Briefly, promastigotes, approximately 3.6×10^7 cells, were taken in 2.8 ml of TEGD buffer containing 0.3 M $(NH_4)SO_4$ and sonicated with 5 x 10 sec bursts. The sonicated sample was centrifuged at 11,000g and the clear supernatant was dialyzed against TEGD buffer to reduce the ammonium sulfate concentration to 15 mM (approximately). RNA polymerase activity was measured as described previously (12,14,15). Briefly ATP, GTP and CTP were adjusted to 600 µM and UTP to 20 µM in an assay volume of 50 µl containing 2.5 µg calf thymus DNA and 20 µl enzyme. The toxin was added when required, the assay mixture was incubated for 5 min without UTP, and the synthesis was initiated by the addition of UTP and 1.25 µCi ^{32}P-UTP (3000 Ci/mmol). The incorporation of ^{32}P-UTP (UMP) in the absence of added trinucleotides was carried out to estimate the blank value RNA synthesis was measured by terminating the reaction after 20 min and pipetting 20 µl of reaction mixture on a DAE paper as described previously (16).

RESULTS AND DISCUSSION

The growth of L. donovani in Ray's modified medium in both the absence and the presence of phaseolinone, amoxycilin, and streptomycin is shown in Table 1. No clones of L. donovani appeared in this medium

Table 1

Effect of antibiotic and phaseolinone on the growth of L. donovani on blood agar overlay

Addition	Dose	Growth on 3rd day	Growth on 7th day	Growth on 15th day
None	NA	+++	++++	
Amoxycilin	1.25 mg/ml	+++	++++	-D-
Streptomycin	50 µg/ml	+	++	+++
Amoxycilin + Streptomycin	1.25 mg + 50 µg /ml	+	++	+++
	0 µg/ml	+++	++++	-D-
Phaseolinone	5 µg/ml	++	++	-D-
	10 µg/ml	+	+	-D-
	15 µg/ml	±	-	-D-
	20 µg/ml	-	-	-
	25 µg/ ml	-	-	-
Phaseolinone +	(5 µg + 1.25 mg)/ml	++	++	-D-
	(10µg + 1.25 mg)/ml	+	+	-D-
	(15µg + 1.25 mg)/ml	±	±	-D-
	(20µg + 1.25 mg)/ml	-	-	-

(Two loopfuls of 72 hr old promastigotes were used for subculturing and the growth of the parasite in blood agar overlay to be examined in each case).
D - Drying appearance on the blood agar overlay surface.

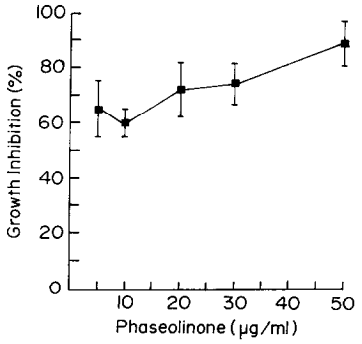

Fig.2 : The effect of phaseolinone on the growth of L. donovani promastigotes. The condition of growth has been described under the MATERIALS AND METHODS. Cells, 1.64×10^6 per ml, were used for growth. On day 50 of culture, the promastigotes with flagellar movement were counted with a hemocytometer and this gave viable counts to measure growth in each case. The growth in toxin free medium was taken as 100% to estimate growth inhibition at each dose and the data points are average ± S.D. of three sets of experiment. Triplicate was run for each dose.

containing 20 ug per ml phaseolinone. Streptomycin was effective in inhibiting the growth up to 5 days but a continuous incubation up to 15 days showed considerable development of clones of L. donovani. Thus, streptomycin inhibited in vitro growth of L. donovani when the culture period was close to or did not exceed 5 days (11). Amoxycilin, even at a dose as high as 1.25 mg per ml, had no effect on normal growth. Therefore, phaseolinone appeared to be a potent growth inhibitor of L. donovani since not a single clone developed even after 15 days of incubation.

Figure 2 illustrates the dose response curve of phaseolinone for the inhibition of growth of L. donovani in liquid culture medium. Here, growth inhibition increased more or less linearly with dose, 10 to 50 µg per ml, the highest dose used. When the viability was estimated in terms of motile count, the growth medium containing 50 µg per ml toxin had a motile count approximately 10% of that of the complete growth in toxin free control incubation. But the inoculum used here was approximately 12% of the total motile count as observed for the growth in the control. Therefore, the growth was apparently abolished in the presence of phaseolinone (50 µg per ml). Hence, phaseolinone could be treated as an effective growth inhibitor of L. donovani even in a liquid culture medium. The LD_{50} value (the concentration necessary for 50% inhibition of growth) for phaseolinone was approximately 10 µg per ml in this medium. Thus the inhibitory potency of toxin in this liquid culture appeared to be low compared to solid blood agar overlay growth.

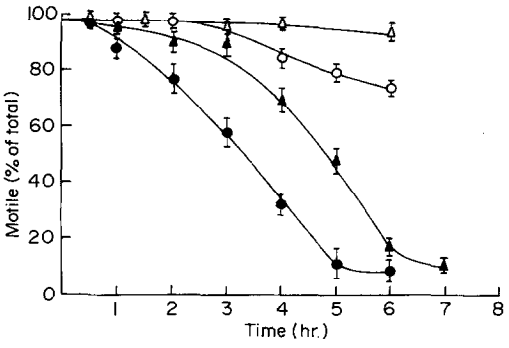

Fig.3 : The effect of phaseolinone on the motility loss of viable promastigotes of L. donovani. The viable count at each time point was determined as in Fig.1 and it was then compared with the viable count at the beginning, taken as 100%. Data points present average ± S.D. of three experiments using triplicate runs at each point.

In addition to the growth inhibition, the effect of toxin exposure on the viable promastigotes was assessed by incubating the motile cells in two different incubation systems and following the kinetics of motility loss. The results shown in Fig.3 demonstrate a sharp decrease in motility in presence of phaseolinone 50 µg per ml after 3 and 2 h of incubation in liquid culture medium and glucose supplemented phosphate buffer saline respectively. But the parasite population in both these incubations had 10-12% motile count after 6-7 h of toxin exposure. The latter incubation system without added toxin, resulted a decrease in motile count by 20% or so. While the former liquid culture medium alone, did not show such reduced motility, indicating its protective role on the motility loss even when this was due to the toxicity of pheseolinone. But these results were also indicative for killing of the promastigotes in terms of motility loss in the presence of phaseolinone. Beccause trypan blue exclusion test showed that non-motile cells were mostly dead. Further investigations were carried out to examine the growth response of the promastigotes after their separation from these incubation systems.

Promastigotes from both the 3-day-old (growing) and 7-day-old culture (stationary), exposed to phaseolinone having approximately 10% motile population showed 4 folds increase in motile count in a subsequent growth for 3 days (Table 2). But promastigotes from parallel controls (incubation in absence of toxin) resulted 8 and 4 folds increase in motile count in an identical growth experiment for 3 and 7-day-old cells respectively. Therefore, 3-day-old toxin exposed cells had half the proliferation compared to corresponding control indicating that either some promastigotes in the toxin treated motile population were capable only to resume

Table 2

Sensitivity of L. donovani promastigotes to phaseolinone

Age of the promastigote used	Phaseolinone (Toxin) exposure µg/ml	No of cells per ml added for growth (cell no.)x10^{-6} Viable (motile) S.D.±S.E	Nonviable (average)	No of cells per ml grown after 72 hrs (cell no.)x10^{-6} Viable (motile) S.D.±S.E.	Nonviable (average)	Count* on growth increase in terms of viable (cell no.)x10^{-6} (motile) S.D. ± S.E.	loss in terms of nonviable (cell no.)x10^{-6} (average)
3 day old	0	2.38±0.067	0.27	20.25±0.0288	-	17.87 ± 0.048	-
	0+Amocycilin (1.25 mg/ml)	2.45±0.065	0.29	20.35±0.025	-	17.90 ± 0.046	-
	50	0.260±0.005	2.38	1.22±0.035	0.98	0.96 ± 0.0299	1.40
	50 + Amox (1.25 mg/ml)	0.25±0.0066	2.41	1.15±0.0413	0.90	0.90 ± 0.0438	1.51
7 day old	0	2.35±0.065	0.29	9.61±0.106	-	7.26 ± 0.107	-
	0 + Amox (1.25 mg/ml)	2.65±0.005	0.31	9.85±0.040	-	7.20 ± 0.04	-
	50	0.23±0.0063	2.61	1.15±0.005	1.58	0.92 ± 0.0057	1.03
	(1.25 mg/ml)	0.25±0.0066	2.38	1.01±0.0088	1.42	0.76 ± 0.0088	0.96

3 day or 7 day old promastigotes 2.65-2.85x10^{-6} cells per ml was incubated in 1 ml of liquid culture medium with 50 ug phaseolinone per ml or none for 7 hrs at 25°C. When promastigotes were approximately 10% motile the cells were separated from incubation medium and washed twice with fresh culture medium and then allowed to grow in 5 ml of liquid culture medium at 25°C for another 3 days. The results represent average ± S.E. of 5 experiments taking duplicate for each one in a set.

* The count shown here was obtained by calculation from the values in previous 2 columns.

the growth or all of them had growth rate much less than that of the promastigote in the control. This was not observed with 7-day-old cells for which toxin exposed motile population resulted a growth which is identical to that of its corresponding control. Hence, 3-day-old cells appeared to be more sensitive to phaseolinone than 7-day-old promastigotes. Some nonmotile cells might revert to motile one being responsible for the increase in motile count, one may argue in this line. In that case, 3-4 folds increase in the motile count for both the two age group cells would result an approximately equal loss in nonmotile count. But the observed loss in non-motile count, instead of being equal was significantly different (Table 2 last column). And the lethal action of phaseolinone on L. donovani was strengthened further when its inhibitory effect was identified for DNA-dependent RNA synthesis.

Cells from 3-day-old culture, appeared to be more sensitive to the toxin were used for RNA polymerase preparation. The results of RNA polymerase activity in presence and absence of phaseolinone and ∝-amanitin are shown in Table 3. Both phaseolinone and ∝-amanitin inhibited RNA synthesis but resulted an additive effect when used together. This clearly indicated that phaseolinone acted on the RNA polymerase to which ∝-amanitin, at a dose 0.2 µg per ml sufficient for complete inhibition of eukaryotic RNA polymerase II (15) was rather insensitive. The extent of ∝-amanitin inhibition indicated that the enzyme in this parasite is probably

Table 3

Effect of phaseolinone on DNA-dependent* RNA polymerase activity

Addendum	pM/10^{10} cells/hr[a]	Activity %	Inhibition %
None	643	100	0
α-amanitin (0.2 µg/ml)	435.1	68	32
Phaseolinone (50 µg/ml)	413.59	64	36
α-amanitin + Phaseolinone	224.81	35	65

* In absence of ATP, GTP, CTP, the ^{32}P-UTP incorporation was estimated and this was used as a blank for subtracting from experimental value to estimate the enzyme activity.
[a]The results are expressed here as the average of six experiments.

not effectively inhibited at a low dose. An observation similar to this was reported for T. cruzi enzyme system (12).

Phaseolinone, a newly described mycotoxin being a diepoxy aromatic compound effectively inhibited the growth of L. donovani possibly causing inhibition of an ∝-amanitin insensitive component of RNA polymerase of the parasite as one of the modes of action. Though, the inhibitory effect on RNA synthesis in an isolated rat liver in vitro assay was also demonstrated (17), its excretion and very high LD_{50} may be advantageous to use it as a chemotherapeutic agent. Extensive study in animal models would be highly useful to arrive a definite conclusion. A recent report on the mode of action of antimonials, the extensively used drug for treatment of leishmaniasis, described that DNA topoisomerase present in L. donovani is the target enzyme for these compounds (18). And this enzyme like RNA polymerase is ubiquitious (19).

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