

## ***In-vitro* effects of Allopurinol - PALA combination on *Leishmania donovani* promastigotes**

**SIBARAM PARIA<sup>1\*</sup>, SUBHASIS MAITY<sup>1</sup> and MUSFIQUA MOOKERJEE<sup>1</sup>**

<sup>1</sup>NSHM College of Pharmaceutical Technology, Kolkata - 700 053 (India).

(Received: June 02, 2009; Accepted: July 04, 2009)

### **ABSTRACT**

The most effective treatment for visceral leishmaniasis which prevailed since 1930 was Sodium stibogluconate. However resistance to this drug has been a major problem and in India alone 65% of the cases are resistant. Combination therapy with Indolylquinoline derivative and Sodium antimony gluconate has been effective to a greater extent. Our study was taken up with Allopurinol and N-Phosphonacetyl-L-Aspartate (PALA) with an aim to search for effective antileishmanial drugs. Though the drugs did not show very significant antiprotozoal effect when tested individually, however when used in combination it could significantly arrest the growth of *Leishmania donovani* promastigotes.

**Key words:** *Leishmania donovani*, Leishmaniasis, Allopurinol.

### **INTRODUCTION**

*Leishmania donovani*, the causative organism of Leishmaniasis, a complex disease affecting the Indian subcontinent and East Africa, is also endemic in 62 countries putting a total of 200 million people at risk (Bora,1992).The few therapeutic options registered for visceral leishmaniasis include Amphotericin B, Antimonials, Miltefosine and Paramomycin (Griensven, 2009,). All these drugs used clinically have limitations of price, safety and drug resistance. Hence in this field there is an urgent need for new and improved treatments to replace or complement the existing ones.

Although antileishmanial activities of synthetic compounds are being continually evaluated throughout the world ( Pal *et al*,2002 and Sundar, *et al*, 1998), yet the search for newer antileishmanial drugs has not been very worthwhile. In the present study we have taken up Allopurinol

which is clinically used against both the primary hyperuricemia of gout and that of secondary level arising due to hematological disorders or in antineoplastic therapy (Goodman-Gilman, 2006) and N- Phosphonacetyl-L-Aspartate ( PALA) which is undergoing clinical trial as an anticancer agent. Our aim is to investigate their possibility of possessing any antileishmanial activity either alone or in combination. Allopurinol is reported to prevent division of protozoal cells when added to a culture of *Trypanosoma -cruzi*, *Leishmania braziliensis* (Berens *et al*;1982).The overall objective of this work is to identify a safe and effective drug combination for combating Leishmaniasis ,in short term therapy.

### **MATERIAL AND METHODS**

#### **Organism used**

*Leishmania donovani* Donovan promastigote (MHOM/IN/1978/UR-6) a clinical isolate was used in the study was obtained from Indian Institute of Chemical Biology, Kolkata.

### Drugs used

All chemicals were purchased from Sigma chemical Co.USA. Until otherwise stated, N-(Phosphon acetyl)-L-Aspartate (PALA) was obtained as a gift from Prof. G. R. Stark of Stanford University. UK.

### Maintenance of strain

The strain was grown on modified Rays blood agar medium (Bera,1987), pH 7.5 at 22-23°C for 24 hrs then the promastigotes were harvested by centrifuging at 1000 x g thrice in cold Tris sucrose salt solution (250 mM sucrose, 20 mM KCl, 1 mM EDTA, 20 mM, Tris. pH7.2) and kept at 4°C until use. Viability of the harvested cells was monitored microscopically by trypan blue exclusion method.

### Liquid growth media

The composition of the media is Glucose-1%, Peptone-1%, Sodium chloride-0.5%, Potassium dihydrogen phosphate-0.02%, Sodium hydrogen carbonate-0.01%, Magnesium sulphate-1%, Choline chloride-0.3%, Folic Acid-0.01%, Haemin-0.004%, Ampicillin-0.0013%. Also known as Haemin media as haem is an essential substrate for *Leishmania donovani* promastigote in culture.

### EXPERIMENTAL

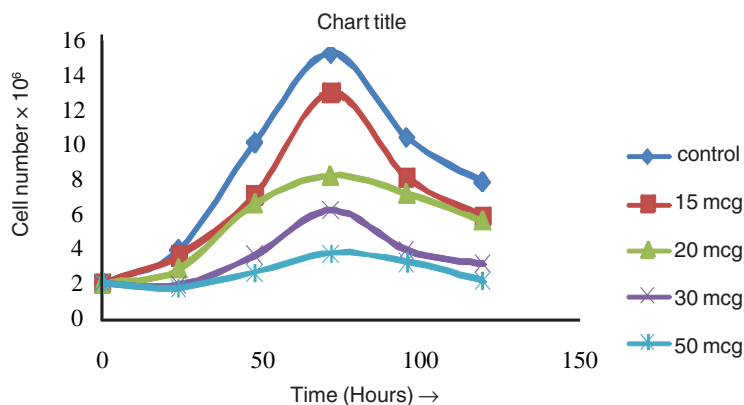
The 72 hrs growth of *Leishmania donovani* in solid blood agar was used for study. The growth was washed with normal saline and centrifuged at 3000 rpm for 3 minutes and re-suspended in

Phosphate Buffer Saline at pH 7.2 for biochemical studies. 100 ml of liquid haemin media was inoculated with the above culture and the growth was diluted till a count of  $1.04 \times 10^6$  to  $1.92 \times 10^6$  was attained which served as initial inoculums. 20ml of inoculum was distributed aseptically into 5 separate 100 ml conical flasks and from each aliquots were taken for confirming initial inoculums size which corresponded to approximately 12/field. The drug was added in four different concentration into four flasks, one flask served as control (without drug) all flasks were incubated at 22.5 °C in B.O.D incubator. At 24 hrs interval aliquots were drawn out aseptically from each flask into sterile tubes. Counts were recorded in hemocytometer using compound microscope at 150X magnification. Such cell counts were taken for 5 consecutive days and for each day an average cell count was recorded (from four different fields prepared).

### RESULTS

#### Effects of Allopurinol on the growth of *L. donovani*

The determination of cell count was carried out by using hemocytometer under compound microscope (Chatterjee,1985).The growth was exposed to Allopurinol at four different concentrations of 15µg ,20µg , 30µg & 50µg /ml for as long as 5 days. It was noted that with 50 ug / ml of Allopurinol the cell count was restricted to  $4 \times 10^6$ , at the end of 75 hrs, as compared to the control where the cell count reached  $14 \times 10^6$ . The reduction



Values are expressed as mean values obtained across a set of three independent experiment conducted

**Fig. 1: Effect of Allopurinol on *L. Donoanivani* promastigote**

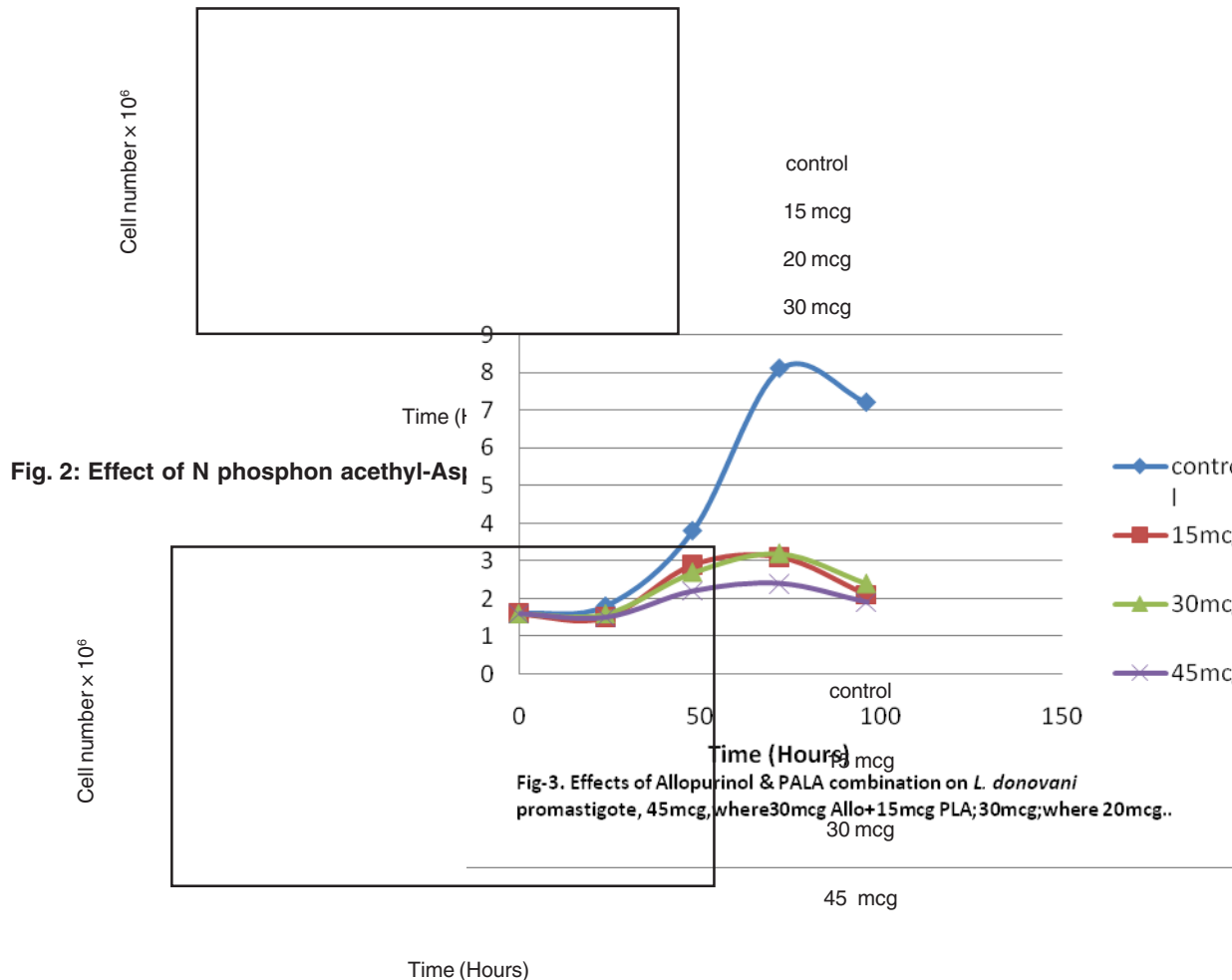
in the cell count brought about by this drug, in comparison to control (without drug) is represented in Fig-1.

**Effect of N-(phosphon acetyl)-L-Aspartate (PALA) on *L. donovani***

In a similar way when the four different drug concentrations of PALA were exposed to the promastigotes the inhibitory effect was similar at both 30µg and 20µg/ml levels. The effect of PALA on the growth of *L. donovani* as compared to control is shown in Fig. 2.

**Effects of Allopurinol and PALA combination on *L. donovani***

Allopurinol and PALA in 2:1 ratio at three different concentrations were exposed to the promastigotes, when the combination was used at 45 µg/ml i.e 30 µg/ml Allopurinol and 15 µg PALA, there was a sharp fall in the count of the promastigotes. The initial count of  $1.5 \times 10^6$  could only double, under the inhibitory effect of the combination which was much less compared to the control where the maximum count attained was



**Fig. 3: Effect of Allopurinol & PALA combination on *L. donovani* promastigote, 45 mcg, where 30 mcg, PLA 30 mcg, where 20 mcg**

Values are expressed as mean values obtained across a set of three independent experiment conducted

$8 \times 10^6$ . The combined effect on the cell count as compared to control shown in Fig. 3.

### DISCUSSION

The growth inhibitory effect of the drug (Allopurinol) is evident from Fig .2. However the vectoral form is not so suitable for ultimate drug screening. Therefore the pathogenic amastigote form still remains the major target for effective experimental research. It has been noted that the basic mechanism of action involved in case of these drugs is one which interferes with the biosynthetic cycle of purine/pyrimidine ultimately creating blockage in the nucleic acid synthesis. It has already been shown in *E.coli* that PALA is a powerful analogue of the transition state in the reaction catalyzed by aspartate trans-carbamylase. From the results shown in Fig.3 carried out with PALA, it is clear that this compound also inhibits *L.donovani*.

Our preliminary combination experiment, involving PALA and Allopurinol clearly showed that at low doses (10mcgAllo+5mcg PALA) distinct synergistic effect was produced, i.e. the inhibition produced by the combined concentration of the two drugs is greater compared to inhibition produced by either of the drugs at equivalent concentration (Fig.2, 3, & 4).

### CONCLUSION

The result of the experiments indicate that there is a distinct possibility of combination therapy in Leishmaniasis .The drug to be identified may not necessarily be PALA and Allopurinol, any other purine/pyrimidine pathway inhibitors may be experimented for this purpose. This preliminary finding opens-up a new direction for screening newer effective drug moiety to combat Leishmaniasis.

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