Chapter VII

Inhibition of glutathione synthesis as a chemotherapeutic strategy for leishmaniasis
Introduction

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is a tripeptide thiol present in virtually all cells. It performs a number of vital functions within cells like reduction of disulphide linkages of proteins and other molecules. It is also involved in the synthesis of deoxyribonucleotide precursors of DNA and in the protection of cells against the effects of free radicals and of reactive oxygen intermediates that are formed during metabolic processes. GSH also has a role in inactivation of drugs and metabolic processing of certain endogenous compounds such as estrogens and prostaglandins (Meister, 1983). Thus inhibition of this thiol represents an important mechanism for metabolic inactivation of cells.

GSH biosynthesis occurs in two steps. The first step involves the combination of L-glutamate and L-cysteine in the presence of ATP to form L-γ-glutamyl-L-cysteine. The enzyme that catalyses this reaction is γ-glutamylcysteine synthetase or glutamate-cysteine ligase. L-γ-glutamyl-L-cysteine then combines with glycine in the presence of another ATP molecule to form GSH. The enzyme catalysing this reaction is GSH synthetase (Meister, 1995).

\[
\text{L-Glutamate} + \text{L-cysteine} + \text{ATP} \rightleftharpoons \text{L-γ-glutamyl-L-cysteine} + \text{ADP} + \text{Pi}
\]

\[
\text{L-γ-glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \rightleftharpoons \text{GSH} + \text{ADP} + \text{Pi}
\]

The reaction catalysed by γ-glutamylcysteine synthetase is inhibited by GSH in a feedback manner. Inhibition by GSH is not allosteric but is competitive with respect to glutamate. Inhibition is associated with the binding of the glutamyl moiety of GSH to the glutamate binding site of the enzyme. There is also evidence that the thiol moiety of GSH binds to another site on the enzyme, possibly to the cysteine binding site.

γ-Glutamylcysteine synthetase isolated from rat kidney has been characterized and it has been found have a molecular weight of 100,000 kD. It can be dissociated into subunits of 73,000 kD and 27,700 kD. The heavy subunit exhibits all of the catalytic activity of the isolated enzyme as well as feedback inhibition by GSH. However, the heavy subunit alone is not sig-
nificantly active under in vivo conditions since the heavy subunit has much lower affinity for glutamate than the native enzyme. Also it exhibits a much higher sensitivity to GSH inhibition as compared to the holoenzyme. The light subunit has a regulatory function that affects the affinity of the enzyme for glutamate. Thus feedback inhibition by GSH involves reduction of the enzyme as well as competition between GSH and glutamate for the glutamate binding site of the enzyme (Meister, 1995).

Among eukaryotes, Entamoeba histolytica is thought to be unique in the sense that it does not contain GSH or glutathione dependent enzymes (Fahey et al., 1984). Since Entamoeba histolytica does not contain mitochondria, it has been suggested that the primary function of GSH in eukaryotes is to protect against oxygen toxicity associated with these organelles. Another aerotolerant anaerobe Trypanosoma vaginalis also lacks mitochondria and GSH. Both these organisms do contain high concentrations of cysteine and other unidentified thiols (Fahey et al., 1984), which could serve to replace the functions of GSH and glutathione dependent thiols in these cells.

In the case of trypanosomes and Leishmania, though GSH is present, no glutathione reductase is found. The latter is responsible for maintaining glutathione disulphide (GSSG) as glutathione (GSH) in these cells (Fairlamb and Cerami, 1985). Instead these organisms reduce GSSG and other disulphides by means of non-enzymatic thiol-disulphide exchange with trypanothione, a low molecular weight dithiol unique to these organisms (Fairlamb et al., 1985).

Trypanothione (N¹, N⁸ - bis (glutathionyl) spermidine) is maintained intracellularly as a dithiol (T[SH]₂), due to the action of the unique enzyme, trypanothione reductase (Fig.7, Introduction chapter). In logarithmically growing cells, trypanothione accounts for greater than 68% of intracellular GSH in Trypanosoma brucei bloodstream and procyclic forms (Fairlamb and Henderson, 1987) and in Leishmania species (Keithly and Fairlamb, 1988). Trypanothione reductase is vital for the survival of the cell by maintaining the correct intracellular redox balance and by removal of peroxides. Consequently it is of great interest as a target for drug development.
Biosynthesis of trypanothione proceeds from glutathione and spermidine principally via the intermediate $N^1$-glutathionyl spermidine (GSH-SPD) (Fig. 7, Chapter I). Earlier chapters in this thesis have reported that inhibition of polyamine metabolism in Leishmania is an important means for chemotherapeutic intervention of parasite growth. Likewise a number of other drugs are thought to interfere with glutathione's role in defence against oxidative stress.

Since GSH performs various protective functions within cells (Meister, 1983; Meister and Anderson, 1983), selective inhibition of this metabolite represents an important chemotherapeutic strategy for Kala-azar. Also a combination therapy of inhibitors of polyamine biosynthesis and inhibitors of glutathione will provide a rational approach for the development of new and better drugs.

However, if an enzyme or metabolite has to be used as a chemotherapeutic target in parasitic protozoans, it must be either absent in the mammalian host or it should be different in some way from the parasite enzyme. Glutathione is an integral part of most mammalian systems and its general function is oxidative defence. It is present in both the host and leishmanial systems. Thus if it is used as a target for chemotherapy there is a danger of host toxicity. However, some GSH inhibitors like Buthionine sulfoximine (BSO) (Fig. 1) etc. have been successfully used in vivo against Trypanosoma using female Swiss Webster mice (Arrick, Griffith and Cerami, 1981). It was observed that the administration of BSO to mammalian cells or whole animals was without apparent toxic effect. It was anticipated that bloodstream forms of $T. brucei brucei$ would suffer substantial oxidative and free radical damage when depleted of intracellular GSH. BSO is a drug with no known mammalian toxicity and with little intrinsic chemical reactivity. It apparently acts solely through inhibition of GSH synthesis and thus does not directly affect other cellular thiols (Griffith, 1981). Earlier reports have shown that parasites and hosts have markedly different requirements for GSH (Hussein and Walter, 1996). This criterion may have application in the development of drugs against parasitic infections.

This study focusses on the use of Buthionine sulfoximine (BSO) (Fig. 1),
a γ-Glutamylcysteine synthetase inhibitor, on *Leishmania donovani* growth *in vitro*. The effect of this inhibitor has also been studied on amastigote multiplication within host macrophages. Inhibition of host macrophages has also been explored with this compound. An attempt has been made to study the mechanism of action of this inhibitor. The effect on GSH content and on superoxide dismutase (SOD) activity in leishmanial promastigotes and on nitric oxide levels in bone marrow macrophages has been studied to address this issue.

**Materials and methods**

**Chemicals**

All the biochemicals like cytochrome C, EGTA, 2-morpholino-ethane-sulphonic acid (MES), bovine superoxide dismutase, xanthine, xanthine oxidase and trypan blue were purchased from Sigma Chemical Co., USA. The tissue culture chemicals like adenine, biotin, folic acid, hemin, hepes, M-199, minimal essential medium (alpha modification), penicillin, RPMI-1640, sodium bicarbonate, streptomycin etc. were also procured from Sigma Chemical Co., USA. Buthionine sulfoximine (BSO) was purchased from Sigma Chemical Co., USA. All the other chemicals and materials were purchased locally and were of analytical grade.

**Parasites**

*Leishmania donovani* (strain AG83) (MHOM/IN/1983/AG83) was maintained in the laboratory as described in the general materials and methods section. Golden hamsters were infected intracardially with $10^7$ *Leishmania donovani* (AG83, MHOM/IN/1983/AG83) promastigotes. After 3 months of infection, amastigotes from infected spleens were transformed into promastigotes in M-199 medium containing 30% FCS at 22°C with continuous shaking. These freshly transformed promastigotes were then made free of spleen cells by differential centrifugation. They were suspended in RPMI-1640 medium with 10% FCS before using them for the amastigote transformation study.
Drug Study

BSO was dissolved in autoclaved water and filter sterilized before addition to the medium containing parasites. The toxicity of the inhibitor was determined by adding a range of drug concentrations (0-20 mM) to a suspension of 1 X 10^6 promastigotes per ml of α-MEM medium and incubating them at 22°C for 48 hr. The minimum concentration of the drug which caused inhibition of approximately 50% of the parasites at 48 hr of drug treatment was designated as IC_{50}. Triplicate incubations in the absence of drug were maintained in parallel as controls. The protozoal counts were taken using a Neubauer hemocytometer.

Effect of BSO on J774A.1 murine macrophages in vitro

J774A.1, a murine macrophage cell line was used for the inhibitor study. It was maintained in RPMI-1640 medium with 10% FCS as described in Chapter II. The macrophages were plated in tissue culture plates at a cell density of 1 X 10^6 cells/ml and they were incubated in a CO_2 incubator (5% CO_2) at 37°C. After 24 hr, the filter sterilized drug at different concentrations was added onto the macrophages and they were again incubated at 37°C for 48 hr. At this stage, the percentage of live cells in all the groups were determined. Triplicate samples were used for these studies and the data was expressed as mean ± SD of percentage of control values.

Effect of BSO on intracellular amastigote multiplication

The effect of BSO on intracellular amastigote multiplication was studied using the method of Baumann et al. (1990). Briefly, macrophages were plated onto adherent petridishes at a cell density of 1 X 10^6 cells/ml and incubated overnight at 37°C in a CO_2 incubator (5% CO_2). Parasites were added onto the macrophages at a ratio of 10 : 1 and the plates were reincubated at 37°C for 3 hr. After 3 hr, the excess promastigotes were washed off and the plates were incubated at 37°C for another 12 hr to enable all the promastigotes to get transformed into amastigotes. At this stage, the drug was added to the plates and they were kept at 37°C. The drug was replenished on the 3rd, 5th and 7th days. On the 10th day, the plates were harvested for microscopic examination as
described in the general materials and methods section. The data was expressed as the percentage of infected macrophages ± SD and the mean number of amastigotes per 100 infected macrophages ± SD. These results are representative of triplicate samples.

**GSH content along the growth curve of Leishmania donovani promastigotes**

*Leishmania donovani* promastigotes at a cell density of 3 X 10^6 cells/ml were resuspended in α-MEM medium and incubated at 22°C. 1 X 10^7 cells were withdrawn at every 24 hr of growth and assayed for GSH. Promastigotes were harvested by centrifugation at 5000 rpm for 15 min at 4°C. They were washed once with PBS (pH-7.5) and resuspended in 297.5 µl harvest buffer. The cell debris was removed by centrifugation at 12,000 rpm for 10 min at 4°C. 200 µl of this suspension was collected in a tube and used for the GSH assay as described in detail in Chapter II. The data was expressed as nM of GSH/10^7 cells (Moron, Depierre and Mannervik, 1979). These results are representative of triplicate samples.

**Effect of BSO on GSH content in Leishmania donovani promastigotes**

*Leishmania donovani* promastigotes at a cell density of 3 X 10^6 cells/ml were resuspended in α-MEM medium and incubated at 22°C for 24 hr. BSO (5 mM) was added to the cells and 1 X 10^7 cells were withdrawn 0 hr, 12 hr and 24 hr later and assayed for GSH activity as described in detail in Chapter II. The data was expressed as nM of GSH/10^7 cells. These results are representative of triplicate samples.

**SOD activity activity along the growth curve of Leishmania donovani promastigotes**

*Leishmania donovani* promastigotes were inoculated in α-MEM at a density of 3 X 10^6 cells/ml and incubated at 22°C. 2 X 10^7 cells were withdrawn at every 24 hr of growth and assayed for SOD activity. The cells were centrifuged at 5000 rpm for 15 min at 4°C, washed with PBS (pH-7.4) and finally suspended in 150 µl of cold phosphate buffer (50 mM sodium-potassium
phosphate buffer, pH-7.8). They were snap frozen in liquid nitrogen and stored at -70°C until the assay. The cells were lysed by freeze-thaw method repeated thrice before the actual assay. The cell debris was removed by centrifuging the cells at 12,000 rpm for 10 min at 4°C and the SOD activity was measured in the supernatant according to the method described in the general materials and methods section (Flohe and Otting, 1984). The SOD activity was reported as Units of SOD per 10^7 cells of triplicate samples at each time point of growth.

**Effect of BSO on superoxide dismutase activity in Leishmania donovani promastigotes**

Promastigotes were inoculated in 1×-MEM at a density of 3 X 10^6 cells/ml. BSO (5 mM) was added at 24 hr and approximately 2 X 10^7 cells from the control-or treated groups were harvested at 48 hr and assayed for SOD activity as described earlier in the general materials and methods section. The SOD activity was reported as Units of SOD per 10^7 cells. These results are representative of triplicate samples.

**Nitric oxide assay**

J774A.1 macrophages were seeded into 96 well tissue culture plates at a density of 5 X 10^5 cells/well and kept in a CO_2 incubator (5% CO_2) for 12 hr at 37°C. LPG (5 μg/ml) was added to the requisite wells and the plate was again incubated at 37°C for 12 hr. After 12 hr, BSO (5 mM) was added to the wells and the plate was reincubated for 12 hrs under similar conditions. GSH (10 mM) was added to the wells and the plate was reincubated at 37°C for 12 hr, after which the samples were harvested for nitric oxide assay (Green et al., 1982) as described in Chapter II. These results are representative of triplicate samples.

**Statistical Analysis**

All the experiments were performed in triplicate. In the microscopic studies, a minimum of 200 cells were screened in each culture and the percentage of infected cells and the average number of amastigotes per 100 infected cells were recorded. Results were expressed as mean ± SD of triplicate samples at each time point. Student’s t test was performed to determine the level of significance and P < 0.05 was considered to be significant.
Results

The effect of BSO on the growth of Leishmania donovani promastigotes is shown in Fig. 2. The effective concentration of BSO that inhibited the growth by 50% (IC$_{50}$ value) at 48 hr of drug treatment was approximately 10 mM.

Figure 3 depicts the effect of BSO on a J774A.1 murine macrophage cell line in vitro at 48 hr of drug treatment. A concentration of 10 mM was found to inhibit macrophage growth by 20% of control values and a concentration of 50 mM BSO was found to decrease macrophage growth by only 25%. This indicates that the concentrations of BSO used to inhibit the parasite are only moderately effective against the host system.

Since the parasite survives in the intralysosomal compartment of the host macrophages, it was of interest to study the effect of BSO on intracellular amastigote multiplication within bone marrow macrophages. Table I shows the effect of BSO on amastigote multiplication within bone marrow macrophages. Concentrations as low as 5 mM were found to affect percentage infectivity significantly (P < 0.001). BSO (5 mM) decreased percentage infectivity by 47% and the mean number of amastigotes per 100 infected macrophages were decreased by 21% (P < 0.01).

Since the inhibitory effect of BSO is related to GSH depletion, the GSH levels were checked in promastigotes treated with BSO. Figure 4 depicts the GSH content along the growth curve of Leishmania donovani promastigotes. The GSH content shows a decreasing pattern along the growth curve of the promastigotes. Thus the effect of BSO on GSH levels was measured at earlier time points of growth. Figure 5 shows the effect of BSO on GSH levels in promastigotes at 12 hr and 24 hr of BSO treatment. The maximum GSH depletion was observed at 12 hr of BSO treatment. BSO (5 mM) was found to decrease BSO content in promastigotes by 90% at 12 hr of treatment and by 62% at 24 hr of treatment.

The effect of BSO on SOD activity in Leishmania promastigotes was also examined. Figure 6 shows the SOD activity along the growth curve of
**Leishmania donovani** promastigotes. The SOD activity of the promastigotes was almost constant till 48 hr of growth. It decreased at 72 hr of growth and started increasing thereafter. The SOD activity reached maximal levels at 120 hr of growth, i.e., at the stationary phase of growth. The effect of BSO (5 mM) on SOD activity at the stationary phase of growth in *Leishmania donovani* promastigotes is depicted in Table II. BSO (5 mM) was found to significantly inhibit SOD activity in *Leishmania donovani* promastigotes (P < 0.001) at 24 hr of inhibitor treatment.

Table III shows the effect of BSO on the stimulation of nitric oxide in bone marrow macrophages. Since induction of NO is a mechanism involved in defence against parasites, it was of interest to study the mechanism of action of BSO against intracellular amastigotes. BSO (5 mM) treatment led to a significant increase in the nitric oxide levels within murine bone marrow macrophages (P < 0.001). LPG (5 μg/ml), the surface molecule of the parasite, had no significant effect on NO levels. However, when BSO was administered after LPG treatment, there was a significant increase in NO over LPG alone values (P < 0.001). Treatment with GSH (10 mM) completely inhibited nitric oxide levels in macrophages. GSH (10 mM) also reversed the effect of BSO on nitric oxide stimulation in macrophages. This indicates that GSH depletion of macrophages by BSO is responsible for parasite killing by increasing NO in macrophages.

Since BSO and polyamine inhibitors DFMO, CGP 40215A, CGP 48664A and MDL 27695 are all inhibitory to promastigote growth and they inhibit different metabolic pathways, a combination of the polyamine inhibitors and BSO were used against *Leishmania donovani in vitro*. Table IV shows the combined effect of BSO and the polyamine inhibitors on promastigotes. A combination of the BSO and the polyamine inhibitors were found to be more inhibitory to promastigote growth than either of the two inhibitors used alone though the effect was slightly less than additive.
Discussion

The rising incidence of visceral leishmaniasis throughout the world and the emergence of resistance to commonly used antimonials has led to an urgent need for new and more potent chemotherapeutic agents against this disease. The trypanothione biosynthetic pathway is common to the trypanosomatid family of protozoa, that include Leishmania and Trypanosoma; and is absent in the host systems. Thus this pathway constitutes an important target for chemotherapy against Kala-azar. The trypanothione pathway combines two metabolic pathways - the polyamine biosynthetic pathway and the glutathione pathway. Since glutathione (GSH) is involved in a number of vital functions within cells, chief of which is defence against oxidative damage, inhibition of GSH represents a potential means for chemotherapy of these parasites.

GSH inhibition can be brought about in a number of ways. One of these is by using substrates of glutathione transferases like α, β-unsaturated carbonyl compounds. One of the most commonly used compounds of this category is diethyl maleate (DEM). DEM has been found to reduce hepatic GSH levels of rats within 30 min of treatment. However, DEM has effects unrelated to GSH depletion and is therefore not used where only GSH depletion is required. Another method to inhibit GSH is to use thiol oxidants that convert GSH to GSSG. The diazenecarboxylic acid derivative of GSH like methylphenyldiazene carboxylate is one such compound. This inhibitor does cause GSH depletion but it also leads to undesirable side effects like functional damage and haemolysis of RBC's. In addition these inhibitors cause elevation of GSSG levels in cells leading to activation of the enzyme glutathione reductase, which regenerates GSH at the expense of NADPH. Thus these inhibitors are not generally used (Plummer et al., 1981). Yet another mechanism used to deplete GSH is to block the biosynthesis of this metabolite. GSH biosynthesis is most effectively inhibited by buthionine sulfoximine (BSO), a tightly bound inhibitor of γ-glutamylcysteine synthetase, the enzyme catalysing the first step of GSH biosynthesis. BSO has been found to have no apparent effects other than depletion of GSH when administered to mice or rats by subcutaneous or intraperitoneal injection (Griffith, 1981).
BSO has been used against certain tumor cells. GSH depletion is effective therapeutically when the normal and tumor cells have markedly different requirements for GSH. Most normal cells have a large excess of GSH but certain tumors and parasites often have levels close to those required for survival (Meister, 1988). Thus depletion of GSH in these cells would probably be more toxic to tumor cells and parasites as compared to normal cells.

The antitypanosomal effect of BSO was explored by Arrick et al. (1981). They found that when BSO was administered to Female Swiss Webster mice infected with Trypanosoma brucei brucei and trypanosomes were isolated from the blood of these mice, they were found to have depleted GSH levels. The parasite was found to be extremely fragile when isolated and examined in vitro. The parasite could not be identified in blood samples collected 16-18 hr after BSO administration was begun. In some cases, infection was also cured if plasma BSO concentrations were maintained for 27 hr. In other cases, apparently parasitemic mice were found to relapse after several days. It was postulated that some parasites persisted after the initial treatment and these multiplied once the BSO was cleared from the plasma. It was also shown that BSO does not react directly with GSH. For BSO to cause GSH depletion the parasites had to lose GSH either through some metabolic process or they had to divide to dilute the GSH pool. Metabolically quiescent trypanosomes were resistant to BSO induced lysis. There was also the possibility of recrudescence of the disease from the central nervous system (Arrick, Griffith and Cerami, 1981).

Thus the fact that BSO was effective partially in curing mice infected with Trypanosoma; even though the doses of BSO needed were quite high and recrudescence of the disease was a distinct possibility, backed its use as a probable antileishmanial agent. Another advantage in using BSO was that this inhibitor has no known mammalian toxicity and has little intrinsic chemical reactivity. Also it apparently acts solely via inhibition of GSH biosynthesis and thus does not directly affect other cellular thiols (Arrick, Griffith and Cerami, 1981).

BSO was found to be an effective antileishmanial agent when used against Leishmania donovani promastigotes in vitro. It was also found to inhibit
intracellular amastigote multiplication within host macrophages. In addition, macrophage inhibitory studies showed that at the concentrations used for promastigote inhibition, the inhibitor had minimal effect on the macrophages. Since selective action is the primary criterion for a chemotherapeutic agent, further studies were performed with this inhibitor.

Since GSH depletion is a primary feature of BSO treatment of cells, GSH levels were measured in promastigotes treated with BSO. The inhibitor was found to cause a depletion in GSH levels within 12 hr of promastigote treatment. The depletion was observed at a 24 hr time point too but it was considerably less, indicating that the effect of BSO is more prominent at earlier time points.

Earlier reports have described the effect of the parasite on nitric oxide levels in macrophages (Evans et al., 1993). Macrophages produce high levels of nitric oxide from L-arginine on stimulation by lipopolysaccharide (LPS) or cytokines such as IFN-γ (Stuehr and Marletta, 1985; Stuehr and Marletta, 1987; Hibbs Jr. et al., 1988). The killing of several intracellular pathogens has been shown to be dependent on NO production by macrophages (Evans et al., 1993). The role of nitric oxide in the killing of Leishmania major is well established (Evans et al., 1993; Severn et. al, 1993; Stefani, Muller and Louis, 1994). In the present study the effect of preexposure to LPG, a surface molecule of Leishmania on nitric oxide synthesis has been studied. The effect of BSO on NO production in LPG preexposed macrophages has also been worked out.

Previous studies have shown that GSH has a protective role against the antiproliferative effects of nitric oxide in tumor cells (Petit et al., 1996). BSO has been found to reverse the protective effect of GSH in tumor cells (Petit et al., 1996). Pretreatment with BSO, which is an inhibitor of GSH synthesis, was found to greatly increase the sensitivity of tumor cells to the antiproliferative effects of several NO-donating compounds (Petit et al., 1996). The results of this study clearly show that LPG by itself has no effect on NO synthesis, which could be the reason why the parasite successfully survives within the macrophages of the vertebrate host (Proudfoot, O'Donnell and Liew, 1995; Proudfoot et al., 1996). Previous reports have shown that GSH is a scavenger of reactive oxygen species (ROS) and reactive nitrogen species (RNOS) (Luperchio, Tamir and Tannenbam, 1996; Petit et al., 1996). Since BSO depletes
GSH content, it leads to an increase in free radicals and reactive nitrogen species in the system (Graier et al., 1996; Suzuki et al., 1997). The present study confirms the above report since GSH (10 mM) was found to completely deplete macrophages of nitric oxide. BSO (5 mM), on the other hand, resulted in enhanced NO levels within macrophages which is probably due to inhibition of GSH content. GSH (10 mM), when given after BSO treatment, led to a decrease in NO when compared to BSO alone treated macrophages which were preexposed to LPG.

BSO was also found to inhibit SOD levels in the parasite besides inhibiting the GSH content. This could be another mechanism of action of BSO whereby it results in increased ROS in the parasite leading to cell killing.

The combined effect of BSO with inhibitors of the polyamine biosynthetic pathway showed an increased inhibitory effect over either of the inhibitors used alone. This data clearly shows a combined chemotherapy using inhibitors of the polyamine biosynthetic pathway along with BSO may prove to be a more effective strategy in treating leishmaniasis.

The potent antileishmanial effect of this inhibitor at the in vitro level and its selective inhibitory activity towards the parasite make it a probable chemotherapeutic agent against Kala-azar.
Fig. 1 The structure of the glutathione inhibitor, L-Buthionine- (S,R)-Sulfoximine (BSO).
Fig. 2 Inhibition of growth of *L. donovani* promastigotes in the presence of different concentrations of BSO at 48 hr of drug treatment. Each point is mean ± SD of triplicate samples.
Fig. 3 Effect of different concentrations of BSO on the J774A.1 murine macrophage cell line at 48 hrs of drug treatment. These values are the mean ± SD of triplicate samples.
Fig. 4 GSH (Glutathione) levels along the growth curve of *L. donovani* promastigotes. Each point is the mean ± SD of triplicate samples.
Fig. 5 Effect of the GSH inhibitor BSO on GSH levels in *L. donovani* promastigotes at different intervals of parasite treatment. Results are mean ± SD of triplicate samples at each time point.
Fig. 6 SOD activity along the growth curve of *L. donovani* promastigotes. Each point represents the mean ± SD of triplicate samples.
TABLE I

Effect of BSO on intracellular amastigote multiplication of *L. donovani* within host macrophages *in vitro*

<table>
<thead>
<tr>
<th>Treatment (mM)</th>
<th>Mean % infected cells ± SD (%R)</th>
<th>Mean number of amastigotes per 100 infected macrophages ± SD (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57 ± 1</td>
<td>167 ± 14</td>
</tr>
<tr>
<td>BSO (5 mM)</td>
<td>30 ± 3 (47)⁴</td>
<td>132 ± 11 (21)⁴</td>
</tr>
</tbody>
</table>

⁴ - Significant decrease over control (P < 0.01-0.001).

b - Cultures of macrophages were incubated with *L. donovani* at 37°C for 3 hr. After the free organisms were removed, the cultures were incubated for another 12 hr to enable all the promastigotes to get transformed into amastigotes. At this time, macrophages received RPMI either alone or containing BSO. The inhibitor was replenished on days 3, 5 and 7 of the experiment. Sets of triplicate samples each were terminated on day 10. The slides were processed for microscopic examination. These results are representative of triplicate samples.
### TABLE II

**Effect of BSO on superoxide dismutase levels in *L. donovani* promastigotes**

<table>
<thead>
<tr>
<th>Treatment (^b)</th>
<th>Mean ± SD of SOD (Units/10^7 cells)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>5.50 ± 0.32</td>
</tr>
<tr>
<td>BSO (5 mM)</td>
<td>3.46 ± 0.16(^a)</td>
</tr>
</tbody>
</table>

\(^a\) - Significant decrease over control (P < 0.001).

\(^b\) - *L. donovani* promastigotes (strain AG83) were treated with BSO (5 mM) for 24 hr at 22°C, after which they were harvested for the SOD assay.
TABLE III

Effect of BSO on nitric oxide elicitation by LPG pretreated bone marrow macrophages

<table>
<thead>
<tr>
<th>Treatment d (nM)</th>
<th>Mean ± SD of nitric oxide (mM/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>LPG (5 μg/ml)</td>
<td>6.0 ± 0.1 a</td>
</tr>
<tr>
<td>BSO (5 mM)</td>
<td>44.3 ± 6.8 b</td>
</tr>
<tr>
<td>LPG (5 μg/ml) + BSO (5 mM)</td>
<td>36.7 ± 0.8 b</td>
</tr>
<tr>
<td>GSH (10 mM)</td>
<td>0.0 ± 0.0 c</td>
</tr>
<tr>
<td>BSO (5 mM) + GSH (10 mM)</td>
<td>4.7 ± 0.7 a</td>
</tr>
<tr>
<td>LPG (5 μg/ml) + BSO (5 mM) + GSH (10 mM)</td>
<td>24.2 ± 1.3 b</td>
</tr>
</tbody>
</table>

a – Not significant over control values.
b – Significant increase over control (P < 0.001).
c – Significant decrease over control (P < 0.001).
d – Bone marrow macrophages were treated with LPG (5 μg/ml) for 12 hr at 37°C in a CO₂ incubator followed by BSO (5 mM) treatment for 12 hr. The macrophages were subsequently treated with GSH (10 mM) for 12 hr; after which the nitric oxide levels were measured. These results are representative of triplicate samples.
TABLE IV
Effect of BSO alone and its combination with polyamine biosynthetic pathway inhibitors DFMO, CGP 40215A, CGP 48664A and MDL 27695 on *L. donovani* growth *in vitro*.

<table>
<thead>
<tr>
<th>Group</th>
<th>DFMO (μM)</th>
<th>CGP 40215A (μM)</th>
<th>CGP 48664A (μM)</th>
<th>MDL 27695 (μM)</th>
<th>BSO (mM)</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Observed&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>31 ± 8</td>
</tr>
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<td>2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>39 ± 1</td>
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<td>62 ± 8</td>
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<td>4</td>
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<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>35 ± 5</td>
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<sup>a</sup> Values are mean ± SD of triplicate samples