Tropical Medicine and International Health

VOLUME 5 NO 6 PP 438–442 JUNE 2000

Inhibition of glutathione synthesis as a chemotherapeutic strategy for leishmaniasis

Preeti Kapoor, Monika Sachdev and Rentala Madhubala

School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

Summary This study focuses on the use of buthionine sulphoximine (BSO), a γ-glutamylcysteine synthetase inhibitor, on *Leishmania donovani* growth. The effect of BSO on amastigote multiplication within macrophages showed that 5 mM BSO decreased infectivity by about 50% and the mean number of amastigotes per 100 infected macrophages by 21%. The mechanism may be that BSO resulted in enhanced nitric oxide (NO) levels within macrophages, probably due to inhibition of GSH content since GSH (10 mM) given after BSO treatment led to a decrease in NO compared to macrophages treated with BSO alone which were preexposed to the *Leishmania* surface molecule lipophosphoglycan.

keywords glutathione, L-buthionine sulphoximine (BSO), *Leishmania donovani*, nitric oxide

correspondence Dr R. Madhubala, School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India. E-mail: rentala@hotmail.com

Introduction

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is a tripeptide thiol present in virtually all cells, which performs a number of vital functions such as reduction of disulphide linkages of proteins and other molecules (Meister 1983, 1988, 1995; Anderson 1997). It is also involved in the synthesis of deoxyribonucleotide precusors of DNA and in the protection of cells against the effects of free radicals and of reactive oxygen intermediates formed during metabolic processes (Anderson 1997). GSH has a role in inactivation of drugs and metabolic processing of certain endogenous compounds such as oestrogens and prostaglandins (Meister 1983). Thus inhibition of this thiol is an important mechanism for metabolic inactivation of cells.

In the case of trypanosomes and *Leishmania*, no glutathione reductase is found although GSH is present. Glutathione reductase maintains glutathione disulphide (GSSG) as glutathione (GSH) in these cells (Fairlamb & Cerami 1985). Instead these organisms reduce GSSG and other disulphides by means of nonenzymatic thiol–disulphide exchange with trypanothione, a low molecular weight dithiol unique to them (Fairlamb *et al*. 1985). Trypanothione (N^1, N^8-bis (glutathionyl) spermidine) is maintained intracellularly as a dithiol ($T[SH]_2$), due to the action of the unique enzyme, trypanothione reductase. In logarithmically growing cells, trypanothione accounts for > 68% of intracellular GSH in *Trypanosoma brucei* bloodstream and procyclic forms (Fairlamb & Henderson 1987) and in *Leishmania* species (Keithly & Fairlamb 1988). 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). Since GSH performs various protective functions within cells (Meister & Anderson 1983; Meister 1983), selective inhibition of this metabolite is an important chemotherapeutic strategy for kala-azar.

Glutathione is an integral part of most mammalian systems and its general function is oxidative defence. It is present in both 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 such as L-buthionine sulphoximine (BSO) have been successfully used *in vivo* against *Trypanosoma* in female Swiss Webster mice (Arrick *et al*. 1981). 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 & Walter 1996). This criterion may be applied in the development of drugs against parasitic infections.

This study focuses on the use of buthionine sulphoximine (BSO), a γ-glutamylcysteine synthetase inhibitor, on

Leishmania donovani amastigote multiplication within the host macrophages, and its mechanism of action.

Materials and methods

Chemicals

Tissue culture chemicals (adenine, biotin, folic acid, haemin, hepes, M-199, minimal essential medium (alpha modification), penicillin, RPMI-1640, sodium bicarbonate, streptomycin, etc.) and L-buthionine sulphoximine were procured from Sigma Chemical Co., St Louis, MO, USA. Fetal calf serum was purchased from Biological Industries, Kibbutz Beet Haemek, Israel.

Parasites

Leishmania donovani (strain AG83)

(MHOM,IN,1983/AG83) was maintained in the laboratory in medium M-199 containing 10% fetal calf serum (FCS). 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 freed of spleen cells by differential centrifugation and 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 1×10^6 promastigotes per ml of α-MEM medium and incubating them at 22 °C for 48 h. Triplicate incubations in the absence of drug were maintained in parallel as controls. The protozoal counts were taken using a Neubauer haemocytometer.

Effect of BSO on J774A.1 murine macrophages in vitro

J774A.1, a murine macrophage cell line, was maintained in RPMI-1640 medium with 10% FCS. Macrophages were plated in tissue culture plates at a cell density of 1×10^6 cells/ml and incubated in a CO_2 incubator (5% CO_2) at 37 °C. After 24 h, the filter-sterilized drug at different concentrations was added to the macrophages and they were again incubated at 37 °C for 48 h. At this stage, the percentage of live cells in all the groups was 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 density of 1×10^6 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 h. Thereafter the excess promastigotes were washed off and the plates incubated at 37 °C for another 12 h to permit transformation of all promastigotes 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. 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.

Nitric oxide assay

J774A.1 macrophages were seeded into 96 well tissue culture plates at a density of 5×10^5 cells/well and kept in a CO_2 incubator (5% CO_2) for 12 h at 37 °C. *Leishmania* lipophosphoglycan (LPG) (5 μg/ml) was added to the requisite wells and the plate was again incubated at 37 °C for 12 h before adding BSO (5 mM) to the wells and reincubating the plate for 12 h under similar conditions. GSH (10 mM) was added to the wells and the plate reincubated at 37 °C for 12 h, after which the samples were harvested for nitric oxide assay (Green *et al*. 1982). Results are derived from triplicate samples.

Statistical analysis

All 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 was 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 antileishmanial effect of BSO was assessed in promastigote and amastigotes. A 1.0-mM and a 5-mM concentration of BSO resulted in almost 30% and 60% inhibition of promastigote growth, respectively. No additional inhibition with higher concentration of BSO was observed (results not shown).

Table 1 Effect of BSO on intracellular amastigote multiplication of *L. donovani* within host macrophages *in vitro*

Treatment* (mM)	Mean percentage infected cells ± SD (%R)	Mean number of amastigotes per 100 infected macrophages ± SD (% R)
Control	57 ± 1	167 ± 14
BSO (5 mM)	30 ± 3 (47)[b]	132 ± 11 (21)[a]

*Cultures of macrophages were incubated with *L. donovani* at 37 °C for 3 h. After the free organisms were removed, the cultures were incubated for another 12 h 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.
*Significant decrease over control ($P < 0.01$–0.001).

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 1 shows the effect of BSO on amastigote multiplication within bone marrow macrophages. Concentrations as low as 5 mM significantly affected infectivity ($P < 0.001$). BSO (5 mM) decreased infectivity by about 50% and the mean number of amastigotes per 100 infected macrophages by 21% ($P < 0.01$).

Regarding the effect of BSO on a J774A.1 murine macrophage cell line *in vitro* at 48 h of drug treatment, a concentration of 10 mM was found to inhibit macrophage growth by 20% of control values. A concentration of 50 mM BSO decreased 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.

Table 2 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 reduced 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.

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

Table 2 Effect of BSO on nitric oxide elicitation by LPG pretreated bone marrow macrophages

Treatment*	Mean ± sd of nitric oxide (mM/10^6 cells)
Control	6.5 ± 0.1
LPG (5 μg/ml)	6.0 ± 0.1†
BSO (5 mM)	44.3 ± 6.8‡
LPG (5 μg/ml) + BSO (5 mM)	36.7 ± 0.8‡
GSH (10 mM)	0.0 ± 0.0§
BSO (5 mM) + GSH (10 mM)	4.7 ± 0.7†
LPG (5 μg/ml) + BSO (5 mM) + GSH (10 mM)	24.2 ± 1.3‡

*Nonsignificant over control values.
†Bone marrow macrophages were treated with LPG (5 μg/ml) for 12 h at 37 °C in a CO_2 incubator followed by BSO (5 mM) treatment for 12 h and treatment with GSH (10 mM) for 12 h; then nitric oxide levels were measured. These results are representative of triplicate samples.
‡Significant increase over control ($P < 0.001$).
§Significant decrease over control ($P < 0.001$).

trypanosomatid family of protozoa, which includes *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, chiefly defence against oxidative damage, GSH inhibition is a potential means for chemotherapy of these parasites.

GSH biosynthesis is most effectively inhibited by buthionine sulphoximine (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). 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 *et al.* 1981).

The antitrypanosomal 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 could not be identified in blood samples collected 16–18 h after BSO administration was begun.

We found BSO to be an effective antileishmanial agent against *Leishmania donovani* promastigotes *in vitro* which also inhibited intracellular amastigote multiplication. Macrophage inhibitory studies showed that at the concentrations used for promastigote inhibition, the inhibitor had minimal effect on the macrophages. Weldrick *et al.* (1999) have recently reported that amastigotes were less susceptible than promastigotes to the effect of redox cyclers paraquat and menadione in the presence of 20 mM BSO which blocked the synthesis of glutathione. The results reported by this group suggest that there are factors other than, or in addition to, possession of abundant levels of intracellular thiols that may be responsible for the survival and replication of amastigotes in culture (Weldrick *et al.* 1999).

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 & Marletta 1985, 1987; Hibbs *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 *et al.* 1994). We studied the effect of pre-exposure to LPG, a surface molecule of *Leishmania* on nitric oxide synthesis and the effect of BSO on NO production in LPG pre-exposed macrophages. GSH has a protective role against the antiproliferative effects of nitric oxide in tumour cells (Petit *et al.* 1996). BSO reverses the protective effect of GSH in tumour cells (Petit *et al.* 1996). Pretreatment with BSO, which is an inhibitor of GSH synthesis, greatly increases the sensitivity of tumour cells to the antiproliferative effects of several NO-donating compounds (Petit *et al.* 1996). The results of this study clearly show that the *Leishmania* surface molecule 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 *et al.* 1995, 1996). Previous reports have shown that GSH is a scavenger of reactive oxygen (ROS) and nitrogen species (RNOS) (Luperchio *et al.* 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). Our study confirms this since GSH (10 mM) completely depleted macrophages of nitric oxide. BSO (5 mM), on the other hand, resulted in enhanced NO levels within macrophages, probably due to inhibition of GSH content. GSH (10 mM), when given after BSO treatment, led to a decrease in NO compared to macrophages treated with BSO alone which were pre-exposed to LPG. 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.

Acknowledgements

We wish to thank the University Grants Commission for providing financial support for this work. PK was supported by a grant from the University Grants Commission and MS was supported by a grant from the Department of Biotechnology, Government of India, New Delhi. *Leishmania* lipophosphoglycan was kindly provided by Dr S. Turco, USA.

References

Anderson ME (1997) Modulation of glutathione. In: *Handbook of Antioxidants* (eds L Packer & E Cadenas) Marcel Dekker, New York, pp. 321–328.

Arrick BA, Griffith OW & Cerami A (1981) Inhibition of Glutathione synthesis as a chemotherapeutic strategy for trypanosomiasis. *Journal of Experimental Medicine* **153**, 720–725.

Baumann RJ, Hanson WL, McCann PP, Sjoerdsma A & Bitonti AJ (1990) Suppression of both antimony susceptible and antimony resistant *Leishmania donovani* by a bis (benzyl) polyamine analogue. *Antimicrobial Agents and Chemotheapy* **34**, 722–727.

Evans TG, Thai L, Granger DL & Hibbs JB (1993) Effect of *in vivo* inhibition of nitric oxide production in murine leishmaniasis.

Journal of Immunology **151**, 907–915.

Fairlamb AH, Blackburn P, Ulrich P, Chait BT & Cerami A (1985) Trypanothione: a novel bis (glutathionyl) spermidine cofactor for glutathione reductase in trypanosomatids. *Science* **227**, 1485–1487.

Fairlamb AH & Cerami A (1985) Identification of a novel thiol containing cofactor essential for glutathione reductase enzyme activity in trypanosomatids. *Molecular and Biochemical Parasitology* **14**, 187–198.

Fairlamb AH & Henderson GB (1987) Metabolism of trypanothione and glutathionyl spermidine in trypanosomatids. In: *Host-Parasite Cellular and Molecular Interactions in Protozoal Infections* (eds KP Chang & D Snary) Springer-Verlag, New York, pp. 29–40.

Graier WF, Simecek S, Kukovetz WR & Kostner GM (1996) High D-glucose induced changes in endothelial Ca^{2+}/EDRF signalling are due to generation of superoxide anions. *Diabetes* **45**, 1386–1395.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS & Tannenbaum SR (1982) Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Annals of Biochemistry* **126**, 131–138.

Griffith OW (1981) Depletion of glutathione by inhibition of biosynthesis. *Methods in Enzymology* **77**, 59–63.

Hibbs JB, Taintor RR, Vavnin Z & Rachlin EM (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochemical Biophysical Research Communications* **157**, 87–94.

Hussein AS & Walter RD (1996) Inhibition of glutathione synthesis of *Ascaris suum* by buthionine sulphoximine. *Parasitology Research* **82**, 372–374.

Keithly JS & Fairlamb AH (1988) Inhibition of *Leishmania* species by μ-difluoromethylornithine. In: *Leishmaniasis: the Current Status and New Strategies for Control* (ed DT Hart) Plenum Publishing Corp., New York, pp. 729–737.

Luperchio S, Tamir S & Tannenbaum SR (1996) Nitric oxide induced oxidative stress and glutathione metabolism in rodent and human cell. *Free Radial Biology Med*. **21**, 513–519.

Meister A (1983) Selective modification of glutathione metabolism. *Science* **220**, 472–477.

Meister A (1988) Glutathione metabolism and its selective modification. *Journal of Biological Chemistry* **263**, 17205–17208.

Meister A (1995) Glutathione biosynthesis and its inhibition. *Methods in Enzymology* **252**, 26–30.

Meister A & Anderson ME (1983) Glutathione. *Annual Review Biochemistry* **52**, 711–760.

Petit JF, Nicaise M, Lepoivre M, Guissani A & Lemaire G (1996) Protection by glutathione against the antiproliferative effects of nitric oxide. Dependence on kinetics of no release. *Biochemical Pharmacology* **52**, 205–212.

Proudfoot L, Nikolaev AV, Feng GJ *et al*. (1996) Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* LPG in murine macrophages. *Proceedings of the National Academy of Science* **93**, 10 984–10 989.

Proudfoot L, O'Donnell CA & Liew FY (1995) Glycosyl phosphatidylinositolphospholipids of *L. major* inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. *European Journal of Immunology* **25**, 745–750.

Severn A, Xu D, Doyle J *et al*. (1993) Pre-exposure of murine macrophages to lipopolysaccharide inhibits the induction of nitric oxide synthase and reduces leishmanicidal activity. *European Journal of Immunology* **23**, 1711–1714.

Stefani MMA, Muller I & Louis JA (1994) *Leishmania major* specific CD8+ T cells are inducers and targets of nitric oxide produced by parasitized macrophages. *European Journal of Immunology* **24**, 746–752.

Stuehr DJ & Marletta MA (1985) Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proceedings of the National Academy of Science* **82**, 7738–7742.

Stuehr DJ & Marletta MA (1987) Induction of nitrate, nitrite and N-nitrosamines: precusors and role of the respiratory burst. *Journal of Immunology* **139**, 518–525.

Suzuki K, Nakamura M, Hatanaka Y, Kayanoki Y, Tatsumi H & Taniguchi N (1997) Induction of apoptotic cell death in human endothelial cells treated with snake venom: implication of intracellular reactive oxygen species and protective effects of glutathione and superoxide dismutases. *Journal of Biochemistry* **122**, 1260–1264.

Weldrick DP, Chodacka B, Vogt R & Steenkamp DJ (1999) The effect of buthione sulphoximine on the growth of *Leishmania donovani* in culture. *FEMS Microbiology Letters* **173**, 139–146.