J. Pharm. Pharmacol. 1988, 40: 370–373
Communicated December 2, 1987

The therapeutic effect of sodium stibogluconate in BALB/c mice infected with *Leishmania donovani* is organ-dependent

K. C. CARTER*†, A. J. BAILLIE*, J. ALEXANDER†, T. F. DOLAN*, **Pharmacy Department, Royal College Building, and †Immunology Division, Todd Centre, Strathclyde University, Glasgow, UK*

Abstract—A study of the antileishmanial efficacy of sodium stibogluconate was carried out in BALB/c mice. The drug was administered to *Leishmania donovani*-infected animals on days 7 and 8 post-infection in one of three forms; free (40–50 mg $Sb^v\ Kg^{-1}$), liposomal, or niosomal (6·4–8·0 mg $Sb^v\ Kg^{-1}$) drug. On day 14 post-infection counts of the number of parasites present in the liver, spleen and bone marrow of treated and control animals showed that although all three drug preparations significantly reduced parasite numbers in the liver (≃99% suppression) they had little effect on those residing in the spleen or bone marrow. The carrier forms of the drug were therefore significantly more effective than free drug in reducing liver parasite burdens. Increasing the concentration and the number of doses of free drug (maximum of 500 mg $Sb^v\ Kg^{-1}$), and reducing the size of the vesicles used to deliver the drug had a minimal effect on parasite numbers in the spleen and bone marrow. It is proposed that because of the resistance of spleen and bone marrow parasites to drug therapy, the BALB/c mouse infected with *L. donovani* provides an excellent model system for the study of drug delivery to these deeper tissue sites.

Leishmania donovani is a human protozoan parasite which causes disseminated disease, characterized by fever, hepatosplenomegaly, anaemia, leukopenia and hyperglobinaemia. If untreated, the disease is usually fatal. There are marked therapeutic problems which in large measure arise from the intracellular nature of the infection and because the pathogen is eukaryotic and similar in many respects to the host cell. The drugs available at present are toxic and carry the risk of side effects, although organic pentavalent antimony compounds, such as stibogluconate, which are the drugs of choice are well-tolerated. Therapy involves a multiple dosing regimen of a minimum of 20 daily intramuscular or intravenous injections of 20 mg $Sb^v\ Kg^{-1}$ up to a maximum of 850 mg (WHO 1984; Bryceson 1987). This is associated with patient compliance problems in underdeveloped areas where the disease is endemic. In the absence of a new 'single dose' drug, one approach to the solution of these problems is to use a drug carrier system, such as liposomes or niosomes, which might allow the drug dose and the number of doses to be reduced. Preferential uptake of drug-loaded vesicles by cells of the reticuloendothelial system target the drug passively to the infected phagocytes of this tissue. Experiments in various animal models using sodium stibogluconate have shown that, compared with the free form of the drug, the dose for parasite clearance from liver can be lowered significantly by using liposomal (review, Alving 1986) and niosomal formulations (Baillie et al 1986).

In most antileishmanial drug studies in animal models of the disease, efficacy of drug therapy has been based purely on parasite liver burdens (Black et al 1977; Alving et al 1978; Adinolfi et al 1985; Baillie et al 1986), which may not accurately indicate the drug's effect on the disseminated disease and parasite survival in other tissues. In this study the efficacy of three formulations of sodium stibogluconate was assessed against parasites in liver, spleen and bone marrow in an attempt to investigate if there were any anatomical or physiological barriers which might prevent the drug being equally effective at all sites.

Correspondence to: A. J. Baillie, Dept of Pharmacy, Strathclyde University, 204 George Street, Glasgow G1 1XW, U.K.

Materials and methods

Materials. Sodium stibogluconate powder (lot 08014) equivalent to 0·32 mg Sb mg^{-1} was obtained from The Wellcome Foundation, U.K. Synthetic (>99% pure) L-α-phostatidylcholine dipalmitoyl (DPPC) and ash free cholesterol (CHOL) were obtained from Sigma. The single chain non-ionic surfactant (Surfactant I, Baillie et al 1985) was obtained from L'Oreal, France. These materials were used as received and other reagents were of analytical grade. Liposomes and niosomes comprised 70% amphiphile (DPPC or non-ionic surfactant) and 30% CHOL, on a molar basis, and were prepared using procedures already described (Baillie et al 1986).

Animals. Eight to ten week-old male and female BALB/c mice (wt 20–25 g) which originated from the colony maintained by Bantin and Kingman Ltd. (The Field Station, Grimston, Aldborough, Hull) were used throughout experiments. Golden Syrian hamsters (*Mesocricetus auratus*) obtained from the Anatomy Department, Glasgow University were used to maintain the parasite.

Parasites. Leishmania donovani strain MHOM/ET/67/L82 (previously LV9) was obtained from Dr G. H. Coombs, Glasgow University and maintained by serial passage in hamsters. Parasites were obtained by homogenizing, in medium 199 (Gibco), the spleen of a freshly killed hamster which had been infected for approximately 8 weeks. The number of amastigotes present was estimated and the concentration adjusted as required. Hamsters were injected intraperitoneally with $1–2 \times 10^8$ amastigotes of *Leishmania donovani* in approximately 0·4 mL of medium. Mice were infected via the tail vein (without anaesthetic) by injection with 2×10^7 parasites in 0·2 mL medium.

Parasite distribution. Mice were killed by cervical dislocation and impression smears of the spleen and liver taken after weighing the organs. A smear of bone marrow was obtained by cutting off the end of the right femur, nearest the 'knee', and inserting a 25G needle into the bone. The attached bone marrow was smeared on to a glass slide. Smears were fixed in methanol, stained in 10% Giemsa (Gibco) and the number of amastigotes per 1000 host cell nuclei counted. The number of Leishman-Donovan units (LDU) was calculated per organ for liver and spleen using the formula: LDU = number of amastigotes per 1000 host cell nuclei × organ weight (g) (Bradley & Kirkley 1977).

Parasite suppression. In a typical experiment, mice were treated on days 7 and 8 post-infection. Groups of 5 mice were dosed (tail vein) with 0·2 mL of one of the following preparations; distilled water, sodium stibogluconate solution, 'empty' vesicles containing 300 mM glucose, or drug-loaded vesicles. Parasite numbers in the liver, spleen, and bone marrow were assessed 6 days later, on day 14 post-infection.

In other experiments the basic protocol was modified by either determining parasite numbers at day 50 post-treatment or by drug treating for 3, 4, or 5 consecutive days starting from day 7 post-infection.

Parasite numbers were assessed 6 days after the last drug dose.

Presentation and statistical analysis of data. Unless otherwise stated the parasite burden of the spleen and liver is expressed as $\log_{10}$ (mean LDU/organ) ± standard error, whereas the bone marrow counts are expressed as $\log_{10}$ (mean number of parasties/1000 host cells) ± standard error. The reduction in parasite burden achieved in a particular animal by stibogluconate treatment was calculated relative to the mean parasite burden (n = 5) of the control group and expressed as the percentage parasite suppression. Mean % suppression is shown. Results were analysed using an independent *t*-test or a one way analysis of variance on the $\log_{10}$ transformed data.

Results

Effect of free-drug treatment on parasite burdens. Treatment with free drug, 40–50 mg Sb^v kg^{-1} day^{-1} for 2 days caused a 98% suppression in the liver parasite burden compared with controls (Table 1a). However, there was no significant effect on the parasites residing in the spleen and bone marrow. Increasing the total dose given by using a multiple dosing regime also failed to remove parasites from the spleen and bone marrow compared with controls (Fig. 1). However, a significant reduction in the liver parasite burden was obtained ($P < 0.005$). There seemed to be a dose-dependent effect on the parasite liver burden but this was not significant. Increasing total dosage by administering two doses of higher drug concentration had no effect on spleen parasites ($P > 0.05$) and a minimal effect on those in bone marrow ($P < 0.05$) (Table 1b) compared with the marked reduction of liver parasite burdens obtained ($P < 0.001$). The suppression of bone marrow parasites was not dose-dependent (> 0.05) and not consistent with other experiments using 40–50 mg Sb^v kg^{-1} day^{-1} × 2 (e.g. Table 1a), in which no suppression was observed. In the liver, doses of both 100–125 and 200–250 mg Sb^v kg^{-1} day^{-1} × 2 were significantly more suppressive than 40–50 mg Sb^v kg^{-1} day^{-1} × 2 ($P < 0.005$). There was no significant difference between the activity of these two high dose treatments against liver parasites ($P > 0.05$).

Table 1. The effect of free stibogluconate on *L. donovani* parasite burdens of liver, spleen and bone marrow in BALB/c mice. Mice, infected with 2×10^7 *L. donovani* amastigotes, were treated on days 7 and 8 post-infection with 0·2 mL sodium stibogluconate solution, equivalent to the mg Sb^v kg^{-1} day^{-1} shown. Control mice were dosed with 0·2 mL water. Parasite burdens were determined on day 14 post-infection. Mean burdens are shown ± standard error where n = number of animals per treatment. Values in parentheses show the mean parasite suppression ± standard error.

		Amastigotes/1000 host cell nuclei		
Treatment	n	Spleen	Liver	Bone marrow
a				
Control	5	224 ± 78	3283 ± 277	34 ± 4
40–50	5	219 ± 42	56 ± 25	35 ± 13
		(2 ± 19)	(98 ± 1)	(−2 ± 37)
b				
control	6	135 ± 28	2365 ± 329	236 ± 55
40–50	5	157 ± 43	556 ± 237	77 ± 19
		(−16 ± 32)	(76 ± 10)	(67 ± 8)
100–125	6	128 ± 44	7 ± 2	103 ± 76
		(5 ± 33)	(99 ± 1)	(48 ± 35)
200–250	7	50 ± 35	8 ± 4	71 ± 12
		(49 ± 11)	(99 ± 1)	(70 ± 5)

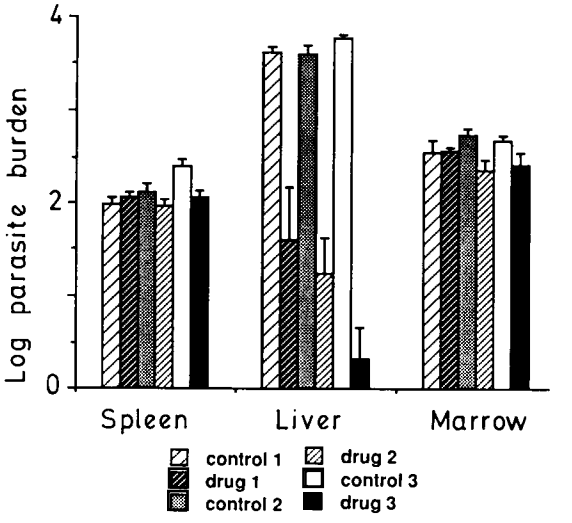

FIG. 1. The effect of multiple dosing with free stibogluconate on *L. donovani* parasite burdens of liver, spleen and bone marrow. Mice, infected with 2×10^7 *L. donovani* amastigotes, were treated with sodium stibogluconate solution, 40–50 mg Sb^v kg^{-1} day^{-1}, on days 7,8,9(1), or 7,8,9,10(2), or 7,8,9,10,11(3), post-infection. Parasite burdens were determined 6 days after the last injection. The data are shown as mean $\log_{10}$ LDU ± standard error, for liver and spleen and as mean $\log_{10}$ parasites per 1000 host cell nuclei ± standard error, for bone marrow. The minimum number of animals per treatment = 5.

The deep site parasites, or those liver parasites which survived drug treatment, apparently act as a reservoir of infection since the marked parasite suppression seen in the liver at day 6 post-drug treatment (day 14 post-infection, Table 1a) was not found when the parasite burden was determined on day 50 post-treatment (day 58 post-infection, Table 2).

Table 2. The effect of free sodium stibogluconate treatment on *L. donovani* parasite burdens of liver, spleen and bone marrow.

		Number of amastigotes/1000 host cell nuclei		
Treatment	n	Spleen	Liver	Bone marrow
Controls	5	757 ± 142	3846 ± 631	596 ± 262
40–50	5	722 ± 60	1407 ± 436	1043 ± 198
		(5 ± 8)	(63 ± 11)	(−75 ± 33)

As Table 1 except that spleen, liver and bone marrow parasite burdens were determined on day 58 post-infection.

Effect of carrier-mediated therapy on parasite burdens. Treatment with 'empty' vesicles had no adverse effect on parasite survival since there was no significant difference in the parasite numbers in the spleen, liver and bone marrow of treated animals compared with controls ($P > 0.05$; Figs 2, 4).

All three drug formulations, free, liposomal and niosomal, significantly reduced *L. donovani* liver burdens ($P < 0.001$) but had no effect on spleen and bone marrow parasites ($P > 0.05$, Fig 3). Treatment with the drug-loaded carriers was more effective than free drug against liver parasites ($P < 0.01$), even though the quantity of drug administered was much lower (vesicular form, 6·4–8·0 mg Sb^v kg^{-1} day^{-1} × 2, free drug, 40–50 mg Sb^v kg^{-1} day^{-1} × 2). However, there was no significant difference between the liver parasite suppression caused by the two types of carrier.

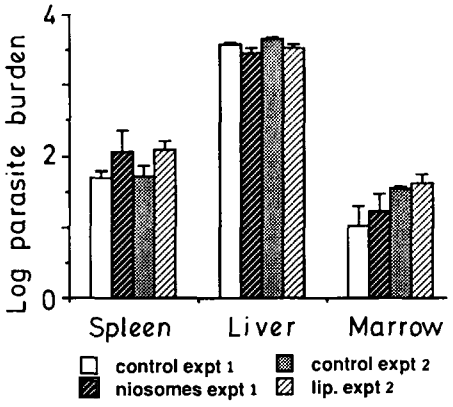

FIG. 2. The effect of treatment with empty vesicles on *L. donovani* parasite burdens. Animals were infected with 2×10^7 *L. donovani* amastigotes and treated on days 7 and 8 post-infection, with either water, or 'empty' vesicles containing 300 mM glucose. Parasite burdens were determined on day 14 post-infection. Data presented as in Fig. 1.

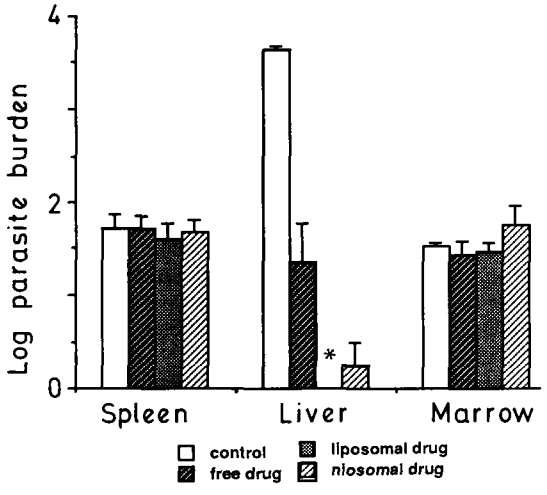

FIG. 3. The effect of treatment with three stibogluconate preparations on *L. donovani* parasite burdens. Infected animals were treated on days 7 and 8 post-infection with water (control), free drug, 40–50 mg Sb^v kg^{-1} day^{-1}, or vesicular drug, 6·4–8·0 mg Sb^v kg^{-1} day^{-1}. Parasite burdens were determined on day 14 post-infection. Data presented as in Fig. 1. (*No parasites found.)

Reducing liposome size had no significant therapeutic effect on the spleen and bone marrow parasite burdens (Fig. 4) although the sonicated carrier form of the drug was again significantly more effective than the free drug in reducing the number of parasites in the liver ($P < 0.005$). There was no difference between the suppressive effects of sonicated and unsonicated drug-loaded vesicles in the liver ($P > 0.05$).

Discussion

The results clearly demonstrate that treatment with all three sodium stibogluconate preparations reduced the numbers of *L. donovani* parasites in the liver but had little effect on those residing in the spleen and bone marrow. This suggests that the drug was failing to reach the latter tissues, or was present in insufficient quantities or for an insufficient period to have an antiparasitic effect.

In the case of the free drug, neutron activation analyses

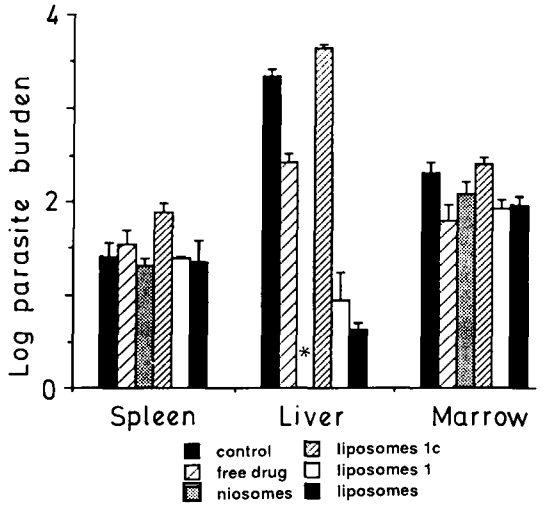

FIG. 4. The effect of vesicle size on *L. donovani* parasite burdens. Infected animals were treated on days 7 and 8 post-infection with water (control), free drug (40–50 mg Sb^v kg^{-1} day^{-1}), or vesicular drug (6·4–8·0 mg Sb^v kg^{-1} day^{-1}) in the form of niosomes with a hydrodynamic diameter $D = 333 \pm 29$ nm, liposomes, $D = 860 \pm 42$ nm, or sonicated liposomes (1), $D = 109 \pm 16$ nm. Empty, sonicated liposomes (1c) contained 300 mM glucose. Parasite burdens were determined on day 14 post-infection. Data presented as in Fig. 1. (*No parasites found.)

showed that antimony had reached the spleen and at 24 h post-dosing antimony concentrations in the liver and spleen were comparable (Hunter et al 1988). Therefore either the parasites' hepatic and splenic micro-environments are so dissimilar that parasites in each organ have different drug susceptibilities or there is a difference in the ability of the drug to reach the infected macrophages at each site. The high clearance rate of free drug from the body, 95% excreted in the urine within 6 h of an intravenous injection (D'Arcy & Harron 1983), and the low rate of blood perfusion through spleen and bone marrow compared with liver, tends to support the latter proposition.

The inability of up to five stibogluconate doses of 40–50 mg Sb^v kg^{-1} day^{-1}, to reduce parasite numbers in spleen and bone marrow would be expected for a drug that is rapidly excreted from the body. There are indications (Baillie et al 1986) that treatments with niosomal sodium stibogluconate, which is less rapidly excreted, have an additive effect on liver parasite burdens which can be explained on the basis of a liver depot effect (Alving et al 1980). It would be interesting to discover if multiple dosing with the carrier forms of the drug suppressed the parasites in the deeper organs.

The minimal effect against parasites in spleen and bone marrow of antimony doses of up to 250 mg Sb^v kg^{-1} $day^{-1} \times 2$, indicates that even higher dosage levels than this are required for a significant antiparasite effect at these sites in the BALB/c mouse. Although this dose is about 15% of the LD50 in mice, and no fatalities were seen, it is well in excess of the doses of pentavalent antimony which in man are associated with the incidence of toxic side effects (Bryceson 1987).

Vesicular forms of the drug were also unable to reduce spleen and bone marrow parasite burdens significantly. These carrier forms of the drug were apparently no more successful than free drug at gaining access to the deeper sites even though they are preferentially taken up by phagocytes of the reticuloendothelial system. In this instance the avidity of the liver for systemically administered particles is probably responsible. Reducing vesicle (liposome) size also failed to reduce parasite burdens in spleen and bone marrow. However, different liver cell populations can

mediate uptake of liposomes from the bloodstream, and their relative contribution is dependent on liposome size and composition. Therefore, although small sonicated liposomes would have a greater chance of avoiding Kupffer cells since they could pass through the fenestrations in the sinusoidal endothelium which trap large liposomes (Scherphof et al 1983), they may, nevertheless, be taken up by liver endothelial cells (Spanjer et al 1986). This perhaps indicates the need for a qualitatively different carrier system with a greater affinity for spleen and bone marrow.

These results suggest that the main advantage propounded for the carrier forms of the drug, i.e. the capacity to reduce the drug dose required for parasite suppression, is, in the BALB/c mouse, restricted to liver parasites. The marked reduction in liver burdens achieved with stibogluconate loaded vesicles at a tenth of the free drug dosage is in agreement with the findings of Hunter et al (1988) and is typical of the increased efficacy against liver amastigotes achieved by administering antileishmanial drugs in the liposomal form (review, Alving 1986). This increased efficacy is in accord with a mechanism of passive targeting to the infected liver and measurements of tissue antimony levels (Baillie et al 1986) show that carrier-mediated delivery enhances uptake of antimony by the liver.

The organ-dependent antileishmanial effect of sodium stibogluconate observed here may in part explain the reports of relapse, in man. This occurs in 5–30% of cases (Chulay et al 1983; Sanyal & Arora 1979), perhaps as a result of recolonization of the patient by parasites from deep foci of infection. Indeed in a recent study, *Leishmania donovani* amastigotes were recovered from bone marrow aspirates of 2 out of 3 individuals, who had received daily injections of sodium stibogluconate for two weeks (Wickramasinghe et al 1987). This suggests that the removal of parasites from deeper sites is also difficult in man, and although newer regimens which involve both increased drug dosage and duration of treatment (WHO 1984), can largely overcome this problem, a small proportion, 2–8%, still relapse.

Other workers (Berman et al 1986) found that meglumine antimonate in both the free and liposomal forms was capable of significantly reducing *L. donovani* numbers in the livers and spleens of hamsters and squirrel monkeys. Unfortunately they did not present data for bone marrow. Early work by Goodwin (1944, 1945) showed that high dose stibogluconate treatment (500 mg $Sb^v\ kg^{-1}$) could achieve almost complete suppression of spleen parasites in the hamster. These reports, which are at variance with those presented here, suggest that clearance of parasites from the spleen may be species-dependent: a possibility which is now under investigation in this laboratory. However, in view of the resistance of bone marrow parasites to stibogluconate both in man (Wickramasinghe et al 1987) and in the BALB/c mouse, we suggest that the BALB/c system is a good model to study drug therapy and targeting to the deeper tissues.

The results presented here raise some doubts on the conclusions and claims for carrier mediated therapy based on animal experiments in which only liver parasite burdens were assessed. The ideal carrier mediated therapy for visceral leishmaniasis would be one able to eradicate parasites from all sites of infection after a single dose treatment. It would seem that as far as stibogluconate is concerned, the present generation of carrier systems is unequal to this task.

This study was supported by the Medical Research Council. J. Alexander holds a Wellcome Trust Lectureship and T. F. Dolan is supported by SERC. The donations of sodium stibogluconate and non-ionic surfactant are gratefully acknowledged.

References

Adinolfi L. E., Bonventre P. F., Vander Pas M., Eppstein D. A. (1985) Synergistic effect of glucantime and a liposome-encapsulated muramyl dipeptide analog in therapy of experimental visceral leishmaniasis. Infection and Immunity 48: 409–416

Alving C. R. (1986) Liposomes as drug carriers in leishmaniasis and malaria. Parasitology Today 2: 101–107

Alving C. R., Steck E. A., Chapman W. L., Waits V. B., Hendricks L. S., Swartz G. H., Hanson W. L. (1978) Therapy of Leishmaniasis: superior efficacies of liposome encapsulated drugs. Proc. Nat. Acad. Sci. (USA) 75: 2959–2963

Alving C. R., Steck E. A., Chaman W. L., Waits V. B., Hendricks L. S., Swartz G. H., Hanson W. L. (1980) Liposomes in leishmaniasis. Therapeutic effects of antimonial drugs, 8-aminoquinolines, and tetracycline. Life Sci. 26: 2231–2238

Baillie A. J., Florence A. T., Hume L. R., Muirhead G. T., Rogerson A. (1985) The preparation and properties of niosomes–non-ionic surfactant vesicles. J. Pharm. Pharmacol. 37: 863–868

Baillie A. J., Coombs G. H., Dolan T. F., Laurie J. (1986) Non-ionic surfactant vesicles, niosomes, as a delivery system for the antileishmanial drug, sodium stibogluconate. Ibid. 38: 502–505

Berman J. D., Hanson W. L., Chapman W. L., Alving C. R., Lopez-Berestein G. (1986) Anti-leishmanial activity of liposome encapsulated amphotericin B in hamsters and monkeys. Antimicrob. Agents Chemother. 30: 847–851

Black C. D. V., Watson G J., Ward R. J. (1977) The use of Pentostam liposomes in the chemotherapy of experimental leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 71: 550–552

Bradley D. J., Kirkley J. (1977) Regulation of *Leishmania* populations within the host. 1. The variable course of *Leishmania donovani* infections in mice. Clin. Exp. Immunol. 30: 119–129

Bryceson A. D. M. (1987) in: Peters, W., Killick-Kendrick, R. (eds) The leishmaniasis in biology and medicine. Volume II. Academic Press, London, pp 847–907

Chulay J. D., Bhatt S. M., Muigai R., Ho M., Gochihi G., Were J. B., Chunge C., Bryceson A. D. (1983) A comparison of three dosage regimens of sodium stibogluconate in the treatment of visceral leishmaniasis in Kenya. J. Inf. Dis. 148: 148–155

D'Arcy P. F., Harron D. W. G. (1983) Leishmaniasis. Pharm. Int. 4: 238–243

Hunter C. A., Dolan T. F., Coombs G. H., Baillie A. J. (1988) Vesicular systems (niosomes and liposomes) for delivery of sodium stibogluconate in experimental murine visceral leishmaniasis. J. Pharm. Pharmacol. 40: 161–165

Goodwin L. G. (1944) The chemotherapy of experimental leishmaniasis. 1. The spleen as an index of infection in the Syrian hamster. Trans. R. Soc. Trop. Med. Hyg. 38: 151–160

Goodwin L. G. (1945) The chemotherapy of experimental leishmaniasis. 2. A dose response curve for the activity of sodium stibogluconate. Ibid. 39: 133–145

Sanyal R. K. Arora R. R. (1979) Assessment of drug therapy of Kala-azar in current epidemic in Bihar. J. Commun. Dis. 11: 198–202

Scherphof G. L., Roerdink F., Dijkstra J., Ellens H., De Zanger R., Wisse E. (1983) Uptake of liposomes by rat and mouse hepatocytes and Kuppfer cells. Biological Cell 47: 47–58

Spanjer H. H., Van Galen M., Roerdink F. H., Regts J., Scherphof G. L. (1986) Intrahepatic distribution of small unilamellar liposomes as a function of liposomal lipid composition. Biochem. Biophys. Acta 863: 224–230

WHO (1984) The leishmaniases. Technical report series 701. World Health Organization, Geneva

Wickramasinghe S. N., Abdalla S. H., Kaisili E. G. (1987) Ultrastructure of bone marrow in patients with visceral leishmaniasis. J. Clin. Pathol. 40: 267–275