

Review Article

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Drug unresponsiveness & combination therapy for kala-azar

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Pentavalent antimonials (Sb^V) have been successfully used for treatment of kala-azar since last six decades. Since 1970s its conventional dosages have failed to achieve with 60 per cent unresponsiveness reported with WHO regimen in Bihar (India). Pentamidine initially used as a second line of drug, acquired resistance (25%) even with prolonged dosage. Newer oral drug miltefosine is a potent antileishmanial drug with longer half-life, a property likely to acquire resistance. Paromomycin has undergone extensive clinical trials in Indian kala-azar patients. Being an aminoglycoside, acquired resistance is likely to occur when used as a monotherapy. To encounter the problem of treatment failure in kala-azar and to reduce length of therapy, combination of at least two effective antileishmanial agents is a desirable option. In India sodium stibogluconate (SSG) in standard dose has been combined with other antileishmanial agents including paromomycin without encouraging result. Infection with *Leishmania donovani* depresses cell-mediated immunity. Immunological balance is tilted in favour of Th2 suppressive cytokines over Th1 producing protective cytokines. Interferon gamma ($IFN-\gamma$) has been used in combination with Sb^V in Indian kala-azar patients with unexpectedly discouraging results. Combination of two most potent leishmanicidal drugs amphotericin B and miltefosine which are not dependent on host immune system, may shorten the course of therapy besides encountering unresponsiveness. A combination therapy should be preferred when treating kala-azar associated with HIV/AIDS. Immunotherapy with exogenous Th1 stimulating cytokines or use of antileishmanial vaccine in combination with a potent chemotherapeutic agent is a future option.

Key words Antimonials - combination therapy - drug resistance - kala-azar - unresponsiveness

Visceral leishmaniasis (VL) is prevalent in more than 80 countries in Asia, Africa (*Leishmania donovani*), southern Europe (*L. infantum*) and south America (*L. chagasi*). *L. donovani* is the main causative parasite for VL. It is estimated that each year nearly 5,00,000 new cases of visceral leishmaniasis (kala-azar) occur in five countries of the world namely, India, Sudan, Bangladesh, Nepal and Brazil. Epidemics of VL have been known to

occur in India since last 100 yr, and in recent years occurred in Bihar¹. Sudan in Africa has been facing severe epidemic since last 10 yr with high morbidity and mortality. India alone contributes 50 per cent of global burden of VL yearly, of these 90 per cent cases occur in north Bihar only².

In India, pentavalent antimonials (Sb^V) meglumine antimoniate and sodium stibogluconate

Table. Changing response to pentavalent antimonials (SSG) in Bihar (1970-2001)

Study	Dose (MKD)	Duration (days)	No. of courses	No. of cases	Unresponsiveness relapse (%)
Jha (1980) ⁴	10	10	1	200	17
Jha (unpublished) 1977-80	10	10	3 with gap of 10 days	520	30 (unpublished)
Thakur (1984) ⁵	20	20 D >20 D	1 1	64 62	8 0
Jha (1986) ⁶	Child=20 Adult=10	Fresh 30 days Relapse-60 days Slow responder 42 days	1	Fresh 73 Relapse 17	1.1
Thakur (1988) ⁷	10 15 20		1 1 1		26 14 3
Jha (1992) ⁸	20	30	1	252	27.1
Jha (1995) ⁹	20	30	1	32	25
Jha (1998) ¹⁰	20	30	1	30	37
Thakur (1998) ¹¹	20	30	1	80	54
Sundar (2001) ¹²	20	30	1	184	60

Super numerals denote reference numbers
MKD, mg/kg/day

(SSG) have been the main first line drug for the treatment of all forms of leishmaniasis including VL since 1940. Meglumine antimoniate is marketed as glucantime and prostib. Sodium stibogluconate (sodium antimony gluconate, SAG) is available commercially as pentostam, solustibosan, stibanate, generic sodium antimony gluconate has been used extensively in India. Till 1970s it was used in the dosages of 10 mg/kg/day (MKD) intramuscularly (im) or intravenously (iv) for 10 days and was effective in curing >90 per cent of cases of Indian VL.

Changing therapeutic response

Pentavalent antimonials: In an earlier resurgence of Indian VL which assumed epidemic proportions by 1977, an estimated 2,50,000 patient were affected in Bihar, of which 30 per cent cases were unresponsive to Sb^{v3}.

During late 1970s and early 1980's different workers used different regimens of SSG to encounter the problem of unresponsiveness (Table).

Pharmacokinetic study of SSG was conducted on Kenyan kala-azar patients. Based on this study a prolonged dosage schedule of 10 MKD parenterally up to 60 days was recommended¹³. It was also observed that children's tolerance for higher and prolonged dosage of SSG was better than adults.

In 1982, WHO¹⁴ recommended SSG in the dose of 20 MKD (maximum 850 mg) for 20-30 days in fresh cases and for double duration (40-60 days) in relapse cases; subsequently WHO (1990) recommended SSG in the dose of 20 MKD for 28 days^{1,52}.

In Bihar, 100 per cent resistance in cases of kala-azar in two villages of Darbhanga and Sitamarhi districts (182 and 59 cases, respectively) was observed who failed to respond to SSG in WHO recommended dosage. Obviously, possibility of host failure in this subset population is unlikely^{15,16}. WHO's recommendation of 20 MKD 40-60 days for relapse cases proved to be disastrous since drug induced mortality was found to be 12 per cent in this group of patients¹⁷.

In *in vitro* studies conducted on SSG resistant cases from some hyperendemic districts of Bihar¹⁸, it was observed that *L. donovani* isolates from 15 non responders required five times more dosage of Sb for killing the parasite than isolates from responders. In another *in vitro* study¹⁹, 106 (37.6%) of 282 infected macrophages and 90 (52.9%) of 170 experimental infections were cured with SAG.

Problem of unresponsiveness to diamidines: Pentamidines have been extensively used as a second line of drug for SSG unresponsive cases in Bihar since seventies till 2003. Using pentamidine isethionate (PIT) in the dose of 4 MKD iv on alternate days for 10-12 days in 82 patients a cure rate of 98.8 per cent was observed²⁰. Two of 82 died of sudden cardiac arrest. In another study PIT was used in 174 and lomidine in 59 SSG resistant cases of kala-azar in the dose 4 MKD for 20 days; cure rates of 75.2 and 100 per cent respectively were achieved²¹. Efficacy of PIT 4 MKD x20 vs amphotericin B 0.5 MKDx14 injections was compared and cure rates of 77 and 98 per cent respectively were observed²². A cure rate of 69-78 per cent was observed when PIT was used in the dose 4 MKD up to 33 days. PIT induced diabetes mellitus was observed in 10 per cent cases²³.

Amphotericin B (Amph B): At present amphotericin B is extensively used in Bihar for all SSG unresponsive cases and even as a first line drug. Used in the dose of 1 MKD x 15-20 days given on alternate days gave a cure rate of 98-100 per cent. Primary unresponsiveness and relapses are uncommon²⁴⁻²⁶.

Liposomal amphotericin B: Lipid formulation of amphotericin B deoxicholate minimize the toxicity and shortens the period of therapy targeting its delivery to parasitized RE cell. There are three different liposomal preparations, *viz.*, liposomal amphotericin B (LAMB) AmBisome (Gilead Sciences, Inc San Dimas, CA), amphotericin B lipid complex (ABLC) Abelcet, Enzon Pharma, Fairfield NJ), and amphotericin B cholesteryl dispersion (ABCD) Amphotec (Intermune corp, Brishane, CA). Of the three preparations, AmBisome used in the dosage of 3-5 MKD for 5 days or 15 MKD as a single dose iv for all refractory cases of Indian VL,

a cure rate of 90-100 per cent can be achieved²⁷. No problem of treatment failure or relapse has been encountered with this highly effective but prohibitory costly drug except when VL is associated with HIV/AIDS.

Paromomycin (PM) (aminosidine): An aminoglycoside has been used intramuscularly singly or in combination with SSG in Indian kala-azar patients. In phase II randomized controlled trial of aminosidine (paromomycin) vs sodium stibogluconate, paromomycin was used in the dose of 16 MKD or, 20 MKD x 21 days, giving cure rates of 93.3 and 96.7 per cent, respectively at six month follow up⁹. In a similar phase II study, same results were reported²⁸.

In a recent phase III multicentric double blind paromomycin study on Indian visceral leishmaniasis patients with a dose of 15 MKDx21 injections, a cure rate of 94 per cent has been achieved (unpublished observation).

This is a potential new drug, which may replace SSG as a first line of drug in visceral leishmaniasis. Problem of treatment failure and relapse case be anticipated in future when used as a monotherapy.

Oral drug

The first novel oral antileishmanial compound miltefosine has been approved in India for the treatment of Indian VL in 2003. Miltefosine is an active phospholipid derivate hexadecylephosphocholine has undergone different phases of study on Indian VL in Bihar, India. Several multicentric clinical trials on adults and children have been conducted in Bihar. With 587 fresh and resistant cases a cure rate of 94 per cent was achieved at 6 months follow up. The recommended regimen for adults weighing more than 25 kg is 50 mg capsule twice daily for 28 days and in children about 12 yr weighing 25 kg 50 mg daily for 28 days, and in paediatric group between 2-11 yr a dose of 2.5 mg/kg for 28 days is recommended^{26,29,30-32}.

A multicentric phase IV study sponsored by Indian Council of Medical Research (ICMR)/WHO(TDR) /

Zentaris at 13 sites in 4 laboratories, a total of 1167 VL patients were enrolled. At 6 months follow up, 84 per cent cure rate was achieved (personal communication). However, there were 52 dropouts, 9 withdrawn, 11 had Serious Adverse Events (SAE) and 57 relapsed. Though miltefosine is the only oral effective agent against Indian VL, its long half-life of about 150 h may create a problem of resistance and relapse when used as a monotherapy.

Sitamaquine (WR 6026): It is a primaquin analogue with high antileishmanial activity. Oral sitamaquin has been tried in phase II study on 120 Indian VL patients in Bihar. Thirty patients were enrolled in each arm of 1.5, 1.75, 2.0 and 2.5 MKD for 28 days. Final cure rates of 81, 89, 100 and 80 per cent were achieved at 6 months follow-up with the four doses. Common adverse events observed were vomiting 8 per cent, dyspepsia 8 per cent, cynosis 3 per cent, nephrotoxicity like nephrotic syndrome 3 per cent and glomerulonephritis 2 per cent. However, further clinical trials need to be done to assess its safety before used in combination therapy with other antileishmanial agent³³.

Unresponsiveness

Causes: Causes of unresponsiveness include delayed diagnosis, treatment and prolonged duration of illness, failure to follow WHO regimen of treatment, low dose interrupted treatment, use of drug by unqualified practitioners, poor quality of drug, and kala-azar associated with HIV/AIDS^{12,16}.

Mechanism of unresponsiveness: It is now clear that the age old standard drug, the pentavalent antimonial, sodium stibogluconate is threatened by the development of drug resistance. This problem is more relevant for the State of Bihar in India which has nearly 40 per cent of global incidence of visceral leishmaniasis (global incidence 500,000 yearly new cases³⁴ where 40-60% cases of VL are unresponsive to Sb^v). Antileishmanial therapy can be complicated by variations in sensitivity of *Leishmania* species (*L. donovani* for Indian VL) and variation in host immune response and pharmacokinetics of the drugs used³.

Intracellular environment: After entering the reticuloendothelial (RE) system amastigotes multiply within the phagolysosome of the macrophages. The pH of this vacuole is 4.5-5.0. This environment is unsuitable for amastigote survival as it has to encounter unfavourable environment like surface molecules for e.g., the lipophosphoglycan (LPG); proteases³⁵ an H⁺ - ATPase and transporters for obtaining its nutrients and pH homeostasis³⁶.

The drug delivery system and targeting the parasites on its different sites are dependent on understanding the biology of the parasite especially *L. donovani* which resides in a tight vacuole.

Role of thiols in host cell and parasite: Thiols of host cell macrophage reduce Sb^v to more active Sbⁱⁱⁱ. Further studies have shown that parasite thiols play an important role in the process of detoxification of heavy metals. Trivalent antimony leishmania parasites have higher levels of thiols^{37,38}.

Recent studies have shown lack of sensitivity of promastigotes to Sb and inability of promastigotes to metabolize Sb to trivalent compound whereas this metabolism occurs in amastigotes. It may well be that resistant property once selected in promastigotes is present in amastigotes as well^{38,39}.

Resistance to various drugs: Mechanism of action of pentamidines is poorly understood. Pentamidine-resistant clones of *L. donovani* and *L. amazonensis* promastigotes have shown reduced uptake and increased efflux of the drug due to specific transporters⁴⁰. Besides there is an accumulation of drug into the *Leishmania* mitochondrion.

There is a small study on emergence of Amph B resistance in *L. infantum* /HIV infected cases^{41,42}. In experimental models and in *in vitro* study *L. donovani* showed intermediate sensitivity to paromomycin in comparison to the *in vitro* study where *L. major* and *L. tropica* was found to be more sensitive than *L. braziliensis* and *L. maxicana*⁴³.

In studies on selected population of promastigotes, resistance to paromomycin was due to a decrease in drug uptake in *L. donovani* but not

due to enzymatic modification or due to any mutation of the small unit ribosomal gene in *L. tropica*⁴⁴.

The mechanism of resistance in bacteria to aminoglycoside antibiotics through the metabolism of the drug to an inactive form by acetylases and phosphorylases is well studied. There is possibility of development of resistance to paromomycin when used as a single agents in VL in future similar to development of bacterial resistance to any aminoglycoside when used alone.

Miltefosine resistant lines of *L. donovani* promastigotes have been generated in the laboratory. Mechanism of resistance is due to defective uptake of miltefosine⁴⁵ through point mutations on a plasmamembrane aminophospholipid translocase.

Immunology of drug resistance in visceral leishmaniasis

Acquired drug resistance: Amastigotes and promastigotes of *Leishmania* have a surface coat, the glycocalyx which contains a number of glycoproteins, various glycopeptides, exometabolites derived from the surface coat form complexes within the parasitophorous vacuoles surrounding the parasite. The combination provides a camouflage for the parasite or neutralizes the host enzymes liberated from macrophages to kill the parasite. Infection with *L. donovani* depresses the cell-mediated immunity. The immunological balance is tilted more in favour of type 2 CD4+ helper (Th2) cells, which produce suppressive cytokines over type 1 CD4+helper (Th1) cells which secrete positively acting cytokines. In visceral leishmaniasis there is complex immune response mediated by various innate and environmental factors *e.g.*, nutrition and effector cells (natural killer cells, CD8 cells and neutrophils)⁴⁶⁻⁵¹.

Protective immune response: Mechanism of resistance⁵² includes (i) T(CD4+) cell dependent and involves T-cell co-stimulatory pathway; (ii) Secretion of regulatory activating cytokines (primarily Th1 cell associated including interleukin 12, IL12 and interferon gamma, IFN- γ); (iii) Induction of adhesion molecule and chemokine mediated recruitment of inflammatory mononuclear

cells into infected tissue and within assembled granulomas; and (iv) Activation of leishmanicidal mechanism in parasitized macrophages and influxing blood monocyte. If this response develops fully, the likely outcome is killing of most intracellular parasites, induction of quiescence in residual parasites and maintenance of low level of infection life long keeping the host in an asymptomatic state^{53,54}.

Activating cytokines: Five known activating cytokines through experimental visceral infections include IL12, IL2, IFN γ , granulocyte macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor (TNF). These cytokines regulate mechanism of acquired resistance, generation and maintenance of the Th1 cell response (IL12, IFN γ), induction of IFN γ secretion (IL12 IL2) mobilization of blood monocyte (GM-CSF), granuloma assembly (all 5 cytokines) and macrophage activation (IFN γ , TNF, GM-CSF). These are also expressed in VL patients. Except for TNF, all other cytokines are potentially available for testing with combination of antileishmanial drugs (immunochemotherapy).

Suppressor cytokines: In visceral leishmaniasis the cell mediated immune system is tilted in favour of Th2 cell mediated cytokines deactivating Th1 cell response favouring visceral infection. Th2 cell associated cytokines are IL-4, IL10, IL13 and transforming growth factor B (TGF-B). IL4 and IL-10 are implicated in progressive intracellular infection with increase in parasite burden and deregulated Th1 cell response responsible for regulating and optimizing the efficacy of chemotherapy, leading to relapses of VL after apparent cure following effective chemotherapy.

Therapeutic effect on chemotherapy -Sb

Treatment directed to neutralize IL-10 or blocking its receptor is more desirable than treating it with Th1 cytokines⁵⁵. Sb activity is dependant on host Th1 response (IL-12, IFN γ , TNF). Simultaneous induction of macrophage activation and administration of Sb may achieve optimal parasite killing. *L. donovani* infected mice deficient in activated macrophage's primary leishmanicidal

pathway, governed by inducible nitric oxidase syntheses (iNoS) and phagocyte oxidase, respond normally to Sb⁵⁶.

Combination therapy in Indian visceral leishmaniasis

To encounter the problem of resistance, relapse and to reduce the length of treatment with antileishmanial as a monotherapy, combination of at least two antileishmanial drugs is a desirable option. Such a combination therapy is being practiced for the treatment of tuberculosis and leprosy. Drugs used in combination therapy should have synergistic or additive effect without having drug interaction.

Combination of SSG with allopurinol / SSG + ketokonazole/SSG+ levamisole: In 128 untreated cases of kala-azar, patients were allocated to four groups of 32 each. Group A received SSG 20 MKD x 30 days; group B SSG 20 MKD x 30 days plus allopurinol in the dose of 20 MKD in three divided dosage for 30 days; group C received SSG in the same dose plus ketokonazole 600 mg daily in three divided dosage, while in group D SSG was combined with levamisole in single oral dose of 13 MKDx30 days. Levamisole acts by stimulating T lymphocytes therapy and helps in restoring cell mediated immunity. Results of this study revealed full cure rates of 75, 87.5, 70 and 78.1 per cent at 6 months follow up in groups A, B, C and D respectively. SSG plus allopurinol was found superior to SSG when used as monotherapy⁹.

SSG+aminosidine (paromomycin): In one study, a combination of SSG 20 MKD x 20 days + aminosidine 12 MKDx20 days was used⁵⁷. Of the 22 patients, 18 were cured at day 180 of follow up. Aminosidine has shown marked potentiation *in vitro* when used with SSG. No cross resistance was observed. An additive effect was also observed in animal model^{58,59}.

In a prospective randomized comparative open label trial on efficacy of paromomycin (PM) plus SSG versus sodium stibogluconate alone in the treatment of VL⁴⁵. Aminosidine was used in the dose of 12 or 18

MKD x21 days while SSG was used in the dose of 20 MKD x 30 days. A total of 150 patients were enrolled in this study. Final cure rates were 92.3, 93.8, 53 per cent, respectively in the three groups. Thus PM plus SSG for 21 days at 12 or 18 MKD was significantly more effective than SSG alone for 30 days⁶⁰.

SSG + interferon gamma: IFN γ , a macrophage activating T cell lymphocytes therapy can enhance the effect of conventional therapy against intracellular *L. donovani*⁶¹. In an *in vitro* study it was found that with combination therapy, the dosage of pentostam required to achieve 50 per cent inhibition or killing of visceral *L. donovani* was reduced by 10-fold and four-fold respectively. Results suggest that IFN γ therapy may be a useful adjunct in visceral leishmaniasis and illustrate one potential role for IFN- γ in the treatment of systemic intracellular infectious.

In one study, 156 untreated kala-azar patients were recruited, group (A) received SSG in the dose of 20 MKD x 30 days, (B) SSG 20 MKD x 30 days + IFN γ 100 $\mu\text{g}/\text{m}^2/\text{d}$ x30 days, (C) SSG + IFN γ in the same dose for 15 days. At six months follow up 36 per cent patients of group A, 49 per cent group B and 42 per cent of group C had complete cure. There was no statistically significance difference among the groups⁶². Beneficial effect of this combination therapy in Indian VL seems to be limited where infection shows high level of resistance to SSG.

Combination therapy in visceral leishmaniasis associated with HIV AIDS

Patients of visceral leishmaniasis associated with HIV/AIDS are more resistant to antimonial therapy. Primary failure and relapses are common and maintenance therapy is also indicated. Experiences from southern European studies showed that pentavalent antimony (Sb^v) used in the dosage of 20 MKDx28 days was not well tolerated and led to cure rate of 60 per cent in co-infected patients⁶³.

Amphotericin B given iv in the dose of 1 MKD x 28 days gives an initial cure rate of 62 per cent. Drug-induced toxicity is also common besides relapse. Amphotericin B lipid complex, in the dose

of 4 MKD on day 1-5, 10, 17, 24, 31 and 38 was well tolerated⁶⁴.

Option for combination therapies in VL with HIV/AIDS include the following: (i) SSG 20 MKD x 28 days + paromomycin 15 MKD x 28 days; (ii) SSG 20 MKD x 28 days + amphotericin B 1 MKD x 28 days; (iii) SSG 20 MKD x 28 days + allopurinol 20 MKD x 28 days; (iv) SSG 20 MKD x 28 days + interferon gamma (IFN γ) 100 μ g/m²/days x 28 days when used has synergistic effect but results are inconclusive⁶⁵ (v) Pentamidine 4 MKD 28 days + paromomycin 15 MKD x 28 days; (vi) Miltefosine 2.5 MKD (patients weighing > 25 kg - 100 mg/day x 28 days + SSG 20 MKD x 28 days or Amph B 1 MKD x 28 days; and (vii) A combination of miltefosine 100 mg/day for 28 days and Amph B 1 MKD x 28 days.

In one study, 31 cases were diagnosed to have kala-azar associated with HIV/AIDS. Three cases were treated with a combination of miltefosine orally 100 MKD x 28 days plus Amph B 1 MKD iv x 28 days. No relapse has been reported till four months follow up. Besides combined therapy patients also received highly active antiretroviral therapy (HAART)⁶⁶.

Future options of combination therapies

Immunotherapy

(i) Use of Th1 cytokines like IL12, IFN γ and TNF+Sb; (ii) Granuloma remodeling exogenous cytokines IL-2 or GM-CSF (granuloma-macrophage colony stimulating factor) + potent antileishmanial drug like paromomycin; (iii) IL-10 - receptor blockers + chemotherapy + Sb/paromomycin; and (iv) Amphotericin B + miltefosine - are directly microbicidal towards intracellular *L. donovani* amastigotes *in vitro*⁵². Both act independently of the immune response.

Conclusions

In the last three decades, there has been changing response to pentavalent antimonials (Sb^v), and unresponsiveness to this in certain part of Bihar is as high as 60 per cent. In this scenario any combination therapy with this drug is unlikely to achieve desired effect.

Amphotericin B given iv in the dose of 1 MKD x 15 days with a cure rate of nearly 100 per cent is being used extensively in Sb^v resistant cases.

Newer drug like oral miltefosine has a direct leishmanicidal property and is not dependent on host immune system. It is still debatable whether this should replace Sb^v as a first line of drug for Indian VL due to its teratogenicity in experimental *in vivo* studies. Contraceptive device will be essential when used in females of child bearing age. It has a long half-life of 150 h and when used indiscriminately is likely to produce unresponsiveness and relapse. Paromomycin (aminosidine) which has undergone phase II and III studies on Indian VL patients in Bihar, has a cure rate of 94 per cent when used as a monotherapy. This agent is also likely to have unresponsiveness as for other aminoglycosides used in different bacterial diseases. Combination of miltefosine 2.5 MKD + aminosidine 15 MKD for 14 days seems to be a better future option to encounter the problem of relapse and unresponsiveness.

Immediate future strategies should be to save two new potent antileishmanial drugs, miltefosine and paromomycin acquiring resistance in Indian kala-azar. A shorter course of combination therapy should be studied. Second generation antileishmanial vaccine with or without adjuvant together with one potent antileishmanial agent could be a future option.

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