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Conventional treatments and non-PEGylated liposome encapsulated doxorubicin for visceral leishmaniasis: A scoping review

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ABSTRACT

Visceral leishmaniasis (VL), also known as Kala-azar, is caused by *Leishmania (L.) donovani* complex, which includes *L. donovani* and *L. infantum* and is associated with a high death rate as compared to the cutaneous and subcutaneous form. Treatment of VL includes chemotherapeutic agents which are associated with some major hurdles like toxicities, parenteral administration, high cost, parasite resistance and stability. Hence, there is an urgent requirement to develop novel chemotherapeutic agents or repurposing of existing drugs against VL. Developing formulation of new chemical entity for the treatment of VL is laborious, time consuming and associated with huge financial burden. However, screening of existing chemotherapeutic agents is a good alternative to avail cost-effective treatment option for VL. Non-PEGylated liposome encapsulated doxorubicin (Myocet[®]) is proposed as an alternative treatment option for VL in this review article. Here, we covered the fundamental aspects of VL, loophole associated with available current treatment strategies and non-PEGylated liposome encapsulated doxorubicin as a novel alternative formulation for treating VL, as this liposomal delivery system of doxorubicin might passively target the intra-cellular regions of macrophage.

KEYWORDS: Visceral leishmaniasis; Doxorubicin; Passive targeting; Repositioning; Non-PEGylated liposome encapsulated doxorubicin; Resistance

1. Introduction

Leishmaniasis is a poverty associated and derelict tropical microbial disease. Infected macrophages associated with reticuloendothelial system are mainly responsible for causing this infectious

leishmaniasis disease. Worldwide, it has been considered as a third most prevalent vector-borne infectious disease after malaria and lymphatic filariasis in developing countries[1]. The parasite of visceral leishmaniasis (VL) was named by its co-investigators (William Leishman and Charles Donovan) as *Leishmania (L.) donovani*. VL is rife in 89 countries, wherein it is affecting a population of 350 million, posing at high menace of contagion. It is projected that 12 million people have been infected. Annually, there is an assessment explicating that 2 million leishmaniasis cases were reported. Among them, 1.5 million cases are of VL and half a million cases are of cutaneous leishmaniasis (CL)[2–5]. VL is a devastating, chronic and deadly disease with highest death rate compared to cutaneous and subcutaneous form of leishmaniasis. The mortality is as high as 100% within 2 years if left untreated as per WHO. It is regarded as kala-azar in India. Causative organism of fatal VL is *L. donovani* complex, which includes *L. donovani* and *L. infantum*. Previously, sand fly genus *Phlebotomus* is the vector and now *Leutzomine* is the vector for transmitting VL as systemic parasitic disease. The *Leishmania* species is zoonotic and anthroponotic in nature. Parasites showing bio-genetic organisms commute between a flagellated promastigote in the gut of the sand fly and an intra-cellular amastigote in the mammalian host. VL parasite drifts to central organs (liver and spleen) and replicates in macrophages

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until the host cell explodes. VL is characterized by symptoms such as persistent bouts of high fever, cough, stomach pain, weight loss, epistaxis, progressive enlargement of liver and spleen, cachexia, pancytopenia and sometimes bronchitis and diarrhea. VL is a most guileful infection usually occurring simultaneously with human immunodeficiency virus (HIV) infection as well as human migration and resettlement are altering the epidemiology and create hurdles for diagnosis and case management[6,7]. Sometimes, VL infected patients gradually suffer from illness and die, if left untreated.

2. Ontogenesis and morphology of *Leishmania* parasite

L. donovani is a digenetic parasite persisting in two hosts during its ontogenesis, i.e. man as the primary host and sand fly as the secondary host. Morphologically, it persists in both shapes as amastigote (leishmanial) and promastigote (leptomonad)[1,2,8].

Morphologically, amastigotes are non-flagellate and are being as oval shaped (2-5 μm in length; 1-2.5 μm in width) with a central nucleus (<1 μm in diameter). Perpendicular to the nucleus, a minute structure lies called *kinetoplast* with DNA and mitochondria where as a basal body with axoneme represents the root of the flagellum. The amastigote divides and multiplies inside reticuloendothelial system of human by binary fission, and it ruptures above 50-200 cause. The liberated parasite enters blood circulation and invades other healthy cells of reticuloendothelial system[8,9].

Amastigote transforms into promastigote when female sand fly sucks blood of reservoir host. These changes occur when parasite

enters into the gut of sand fly. Promastigotes are long, slender, spindle and flagellar in nature (with 15-20 μm length, 1-2 μm width). Morphologically, it is nucleated centrally with a basal body and kinetoplast, which is present near the anterior end. A single long flagellum originates beside the axoneme, which is almost equivalent to size of the body or longer than its body size. It undergoes reproduction asexually by binary fission process. By this fission procedure, promastigotes move towards pharynx, buccal cavity and proboscis of insect host. When sand fly takes plant juice, after the first infected blood meal, it multiplies and when such parasite infected sand fly bites a healthy person, the new-fangled host shows infection. Promastigotes penetrate and invade the reticuloendothelial system of new host and transforms into amastigote form to complete its cycle (Figure 1)[10,11].

Several chemotherapeutic agents have already been available in the market for treatment of VL, however, they are associated with a few limitations including frequent administration, cost, toxicities, parasite resistance and stability[2,12,13]. Penta-valent antimonials are the most favorable leishmanicidal agents, however, they are not preferred owing to their cardio-toxicity, hepatic and renal insufficiency. Pentamidine and amphotericin B are utilized as second line agents, but toxicity, availability and majorly cost limits their use[14,15]. Thus, there is requirement and development of new drugs and therapy along-with these common chemotherapeutic agents in the therapy of VL complex disease. Due to the unavailability of vaccine, many drugs are used to treat VL, as a result, leishmanial parasite develops resistance against drugs. Hence, aiming to reduce multiple drug resistance (MDR) in protozoal research, novel target

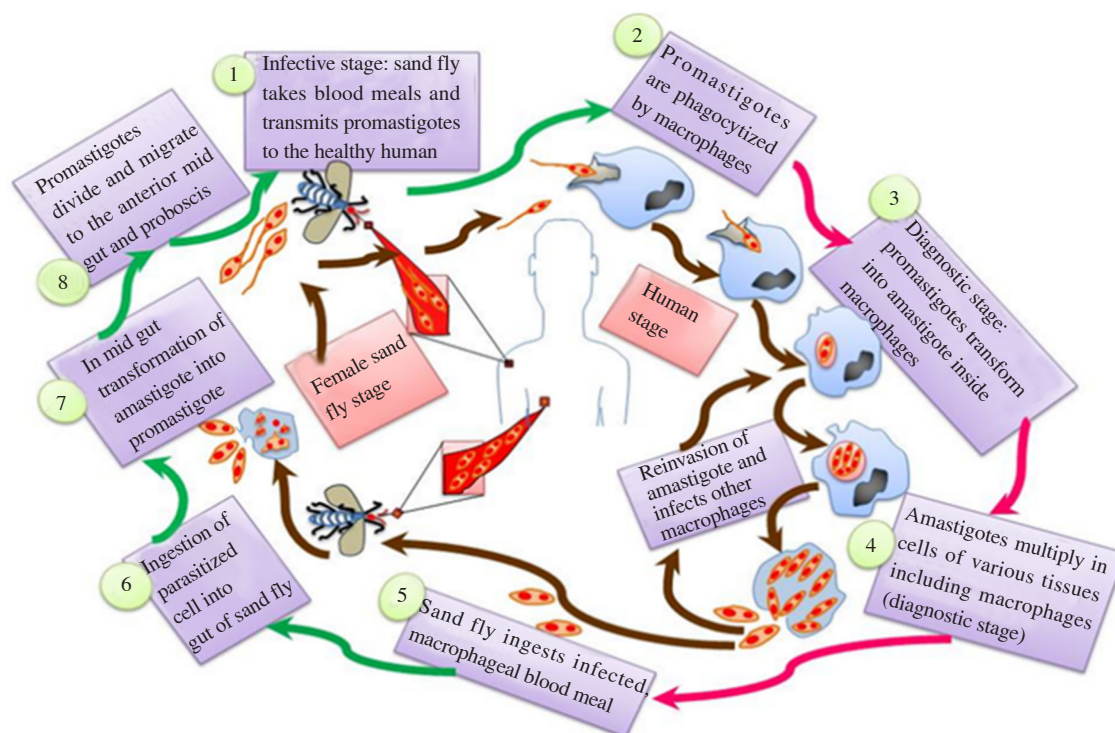


Figure 1. Life cycle of *Leishmania* parasite.

identification and development of new drugs are the two main focusing points. Development of dual drugs or multiple drugs combination therapy should be aimed to avoid the drug MDR developed by parasite. Combination therapy is used for the safety and optimization of component drugs, which are administered either concomitantly or subsequently. In combination therapy, shorter regimen is more affordable, which is easier to administer and gives better adherence than current longer regimen. It also protects the life span of individual drugs[16]. An urgent need is persisted to establish a robust, storage-stable and affordable drug delivery system for new therapeutic agents particularly for this group of disease.

In the early 1990s, the potency of doxorubicin (Dox) was discovered for VL. Several investigators studied and found the equal effectiveness of Dox (in comparison to gold standard drug; amphotericin B) as an anti-VL agent. It was reported that Dox, an anti-cancerous agent, has potent inhibitory action against VL, but its clinical use is restricted due to their several toxic responses[17–20]. In this review, we summarized the general facts and promises, loopholes associated with current treatment strategies and non-PEGylated liposome encapsulated doxorubicin (Myocet®) as a novel formulation for VL. Several research studies have considered liposomal Dox to be utilized in passively targeting the intra-cellular regions of macrophage with improved therapeutic outcomes as well as minimal toxicities as a new approach for VL treatment.

3. Conventional treatment

Development of resistance against pentamonal antimonial is an alarming issue as well as a major cause of their therapeutic discontinuation. Amphotericin B encapsulated colloidal and lipidic vesicle formulations are current choice for effective treatment of VL, however, excessive use of these formulations could lead to parasite resistance in the near future. Various chemotherapeutic agents available for the treatment of VL have been summarized in Table 1.

Since mid-1990s, pentavalent antimonial has been the foremost recommended drug for the therapy of VL and CL. Later, this situation was changed. From the last few decades, other drugs are used alternatively. Not only that many novel formulations of standard drugs came into existence and get registered for therapeutic use to treat VL, but also several drugs are in clinical trial for both diseases (VL and CL) and all these are summarized in Table 2. Currently used chemotherapeutic drugs or intended chemotherapeutic drugs to the treatment of VL are discussed below in brief.

3.1. Pentavalent antimonial

Pentavalent antimonial together with sodium stilboglucanate (SSG) and meglumine antimonite (MA) are the first line and standard

drugs used in treating VL[44]. Antimonials acts as enzyme inhibitors. They act by blocking the enzymes that are regulating the oxidation of glucose (glycolysis and citric acid cycle) and β -fatty acids. Glycolysis and citric acid cycle are majorly responsible for adenosine triphosphate (ATP) production in *Leishmania*. In glycolysis and kreb's cycle, adenosine diphosphate undergoes phosphorylation by acquiring an inorganic phosphate and forms adenosine triphosphate in the presence of nicotinamide adenine dinucleotide (NADH) enzymes. Hence antimonials block the oxidation pathways for glucose and decrease ATP level, which is an important source for the existence of *Leishmania*[45]. Antimonials interfere the *Leishmania* amastigotes bioenergetics process. Numerically, clinically and economically, unresponsiveness of antimonials against VL is a serious problem. Chemotherapy of VL through SSG started with a dose of 10 mg/kg in the initial 6-10 days and subsequently increased unresponsiveness in India led to successive upward revisions in the dose. Presently, the dose of the drug is 10 times more in comparison with the other two earlier. There are lots of clinical reports showing serious issues including emergence of resistance and over 60% treatment failures in Bihar in India render the drug useless for routine use[46]. Various studies performed by experts using antimonials are summarized in Table 3. This data also explains the enhancement of dose over a period of time. Moreover, antimonials already have several other restrictions such as requirement of 3-4 weeks of parenteral therapy along with toxic effects like arthralgia, nausea, pancreatitis and abdominal pain and long term use shows cardio-toxicity[47]. Antimonial marketed products are expensive and batch to batch variability is also reported. Relapses after inadequate treatment, resistivity is also a limitation due to wide misuse of the drugs, inappropriate use by unqualified medical practitioners and easy availability of over the counter drugs[44]. All these facts collectively make antimonials useless for the treatment of VL. It is a serious alarming situation and therapies with alternate drugs become essential.

3.2. Pentamidine isothionate

Pentamidine (isothionate or methane sulphonate), an aromatic analogue of diamidine that is employed as a second line drug in the management of VL and was tested to be beneficial in SSG resistant VL cases in India[53]. High cost, irreversible toxicities, diabetes mellitus and mortality were the limiting factors for the use of pentamidine as the therapeutic agent[54]. A rapid decrease in the response rate from more than 95% cure rate in the early 1980s to less than 70% a decade later has led to the total abandonment of this drug in India[35]. Pentamidine cellular target is still not known, however, it acts on mitochondrial level, hampering the replication and transcription of pathogen.

Table 1. Summary of characteristics of antileishmanial drugs in current use.

Class	Drug	Trade name	Regimen	Route	Duration of therapy	Resistance	Mechanism of resistance	Toxicity	Issues	Clinical efficacy (Cure at 6 months post-treatment)
Antimonial drugs	Sodium stibogluconate	Pentostam® (GlaxoSmithKline)	20 mg/kg/day	IM or IV	30 days (depending on geographical area)	60% treatment failure	Diminished biological reduction of antimony (V) to antimony (III)[17] Reduced accumulation Gene amplification[18]	Frequent fatigue[6] Nausea Muscle and joint pain Increased transaminase Changes in ECG[19] Cardiotoxicity[19] Hepatic and renal dysfunction[20] Pancreatitis[20] Shock and sudden death[21]	Length of treatment Painful injection Toxicity Resistance Availability Quality control High cost	35%-95% (depending on geographic area)[22]
	Meglumine antimonite	Glucantim (Aventis)	20 mg/kg/day	IM or IV	30 days (depending on geographical area)	39.7% treatment failure				
Polyene Antibiotic	Amphotericin B Deoxycholate	Fungizone (Bristol-Myers-Squibb, USA)	7-20 mg/kg/day	Slow IV	20 days (depending on geographical area)	Not documented			Low IV infusion Limited dose Heat stability High price Efficacy Optimum dose need to be defined Administration in hospital Current alternative second-line treatment[21,22]	0% all regions 5% in South Asia (dose of ≥ 10 mg/kg) 90% in other regions (dose of 20-30 mg/kg)[22,27]
	Liposomal Amphotericin B	AmBisome™ (Gilead Nexstar)	10-20 mg/kg/day	Slow IV	5-10 doses over 10 days	Not documented	Not documented	Nephrotoxicity[23,24] Hypokalemia[25] Icterus[26] Fever[6,21,22]		
	Amphotericin B colloidal dispersion	Amphocil / Amphotec (liposome technology, Sequesand Zeneca)	10-15 mg/kg/day	Slow IV	over 5 days	Not documented				
	Lipid complex	Abelcet (Liposome Co)	10-15 mg/kg/day	Slow IV	over 10 days	Possibility of parasitic resistance				
Alkyl phospholipid	Miltefosine	Impavido™ (Paladin)	1.5-2.5 mg/kg/day	Oral	over 28 days	Laboratory isolates	Reduced accumulation of miltefosine[28]	Nephrotoxicity[29] Hepatotoxicity[29] Teratogenicity[30] Gastrointestinal effects (in 20%-55% patient, mild effect)[31]	High price Teratogenicity[31] Patient compliance Potential for resistance	90% in South Asia not clearly established in other regions[27]

Table 1. Continued.

Class	Drug	Trade name	Regimen	Route	Duration of therapy	Resistance	Mechanism of resistance	Toxicity	Issues	Clinical efficacy (Cure at 6 months post-treatment)
Amino glycoside	Paromomycine	Paromomycin IM injection (Gland Pharma)	11 mg/kg/day	IM	21 days (mono-therapy)	Laboratory isolates	Decreased drug uptake but mechanism not clear[32]	Nephrotoxicity[33] Hepatotoxicity[34] Pain at injection site	Variable efficacy between regions	90% in mono-therapy in south Asia and in combination with SSG for 17 days in Africa[31]
Isothionate/methane sulphionate	Pentamidine	Pentam (Fresenius Kabi)	2-4 mg/kg 15-30 doses	IM	Over 3-4 weeks	Relapses in human immune deficiency virus (HIV) associated VL	Reduced uptakes and increased efflux[35] Less organelle accumulation[35]	Irreversible insulin dependent diabetes mellitus[36] Death[27]	Increasing unresponsiveness in India Toxicity Suboptimal effectiveness[37]	0% patients cured[27]

SSG-sodium stibogluconate. IM: intra muscular. IV: intra venus.

3.3. Amphotericin-B (AmB)

From the genus *Streptomyces bacterium*, AmB was produced and isolated. Chemically, it is a polyene antibiotic generally employed for treating harmful fungal infections. This antifungal agent is amphiphilic in nature and employed as an alternative drug and second line drug in the therapy of VL after antimonial. Thus, it acts as an efficient anti-leishmanial agent but still it has certain cons such as high cost, limited availability, prolonged duration of therapy (up to 30 days), toxicity like infusion related side effects (fever, chills, bone pain, thrombophlebitis), hypokalemia, renal impairments and anemia. Apart from these side effects, it cured more than 97% of the leishmaniasis[55,56].

Anti-leishmanial activities of AmB are as follows: pharmacological action of AmB is to bind with the ergosterol, a main component of leishmanial parasite and cause cell leakage of parasite thereby parasite rapidly loses many important monovalent ions. Despite the fact that in lesser degree, AmB reacts with cholesterol of host macrophages and inhibits the binding of parasites to cell. At higher concentration, it induces aqueous pore formation in cellular membrane leading to parasitic death[57-62].

Lipid-based formulations of AmB are more efficacious and well tolerated in comparison to usual preparations because their outstanding and unique physiochemical properties favor more uptake in targets of VL, *i.e.* RES (liver, spleen and bone-marrow *etc.*). United State of Food and Drug Administration (USFDA) approved the Ambisome® (unilamellar liposomal formulation), Amphocil® (colloidal dispersion) and Abelcet® (lipid complex) as some of the lipid-based formulations indicating for the therapeutic use against VL[63]. Among them, Ambisome® and Amphocil® were found more effective than Abelcet® formulation[64-66].

3.4. Miltefosine

Miltefosine (hexadecyle phosphocholine) is a membrane activating alkyl phospholipid, initially developed as an anticancer drug[67]. It is a highly effective and orally active anti-leishmanial agent. In mid-1980s, its anti-leishmanial activity was discovered and Impavido™, Profounda, Inc., Orlando, United States was the first approved commercial product for the miltefosine treatment of VL. Dose dependent studies showed 95% cure rate of 100 mg/day daily dose for 4 weeks in adults and 2.5 mg/kg in children[38]. Table 4 summarized the clinical trials data using miltefosine. Miltefosine is well tolerated orally without the need of close monitoring and also suitable for ambulatory treatments due to its high efficiency, low cost and short course of treatment as well as reduced side effects. Teratogenicity and abortifacient activity are the two drawbacks associated with miltefosine, not permitting it to be used during

Table 2. Drugs under research and development pipeline.

Drug	Miltefosine	Paromomycin (Aminosidine)	Sitamaquine (WR6026)
Developing Institute	Zentaris (ex AstaMedica)	TDR (discovered by Farm Italia-Carlo Erba)	GlaxoSmithKline (discovered by Walter Reed Army Institute of Research)
Status	Registration	Phase III with new formulation. Gabbromicina (ex.Famitalia-Carlo Erba), no longer produced now	Clinical trial phase II
Expected marketing time	Registered in India	Registered in India	4 to 6 years
Issues and problems	Pricing, teratogenicity, restrictions of use in labeling	Regular dossier incomplete (lack of funding to complete studies). Potential antimony replacement. Can be used in combination with antimony or other drugs.	Walter Reed Army Institute did most of the expensive toxicology and pharmacology work. So, the drug could be quite cheap. There are serious renal adverse events
Regimen	100 mg/day for 4 weeks (adults), 2.5mg/day for 4 weeks (children)	16-20 mg/kg/day	1.5-3.0 mg/kg/day for 28 days
Cure rate	95%	93%-97%	87%-100%
Reference	[29,37,38]	[39,40]	[41–43]

Table 3. Summary of antimonials employed studies.

Study	Regimen	Duration	Clinical efficacy	Reference
In the late 1970s	10 mg/kg (small daily dose), 600 mg (maximum)	6-10 days	30% treatment failure	[48]
Expert committee	10 mg/kg (small daily dose), 600 mg (maximum)	10 days (with 10 days gap interval)	99% progression in cure rate	[49]
In 1984	10 mg/kg (small daily dose), 600 mg (maximum)	20 days of continuous treatment	86% cure rate	[50]
In 1988	20 mg/kg/day, maximum 850 mg	20 days	81% cure rate	[51]
In 1988	20 mg/kg/day, maximum 850 mg	40 days	97% cure rate	[51]
In 1991	20 mg/kg/day, maximum 850 mg	20 days	71% cure rate (decline)	[52]

Table 4. Clinical trial studies of miltefosine[68,69].

Regimen	Duration	Cure rate	Interference
50-200 mg daily	14-42 days	224 patients out of 234 (96%)	Long-term cure
50 mg twice daily (100 mg/day)	28 days	60-70 subjects (97%)	More effective
100 mg/day	21 days	Around 68-70 subjects (97%)	No decrease in efficacy
100 mg/day	14 days	89% patients	Duration affects efficacy of drug
>100 mg/day	28 days	Adverse gastro-intestinal reactions	Mild or transient adverse drug reaction

pregnancy[29]. It was reported that miltefosine showed gonadal toxicity (swollen testicles) in male dogs. Longer half-life (7 days) and low therapeutic index of drugs expand the chances of emerging resistance. Therefore, combination chemotherapy would be an alternative to avoid the development of resistance.

3.5. Paromomycin

From the microbial culture of *Streptomyces rimosus*, paromomycin (aminosidine) antibiotic was produced and isolated. Chemically, paromomycin is an aminoglycoside antibiotic which was identified as an antileishmanial compound in the 1960s and can be employed to treat both VL and CL[33]. It has poor oral absorption, therefore, it is beneficial in the development of parenteral formulation. In Bihar, India, the phase III clinical trials in Table 5 conducted revealed that paromomycin has high efficacy and safety (not inferior to AmB) because treated patients didn't show nephrotoxicity and only a small percentage of inner ear damage with an increase in hepatic transaminases. Furthermore, paromomycin is active against different pathogens including bacteria. It has short duration of administration and low cost compared to other available drugs. It acts by promoting ribosomal sub-unit association of both cytoplasmic and

mitochondrial forms, due to the dissociation caused by low Mg²⁺ concentration and it also induces respiratory dysfunctioning of leishmania promastigotes[70–72].

Table 5. Aminosidine and sodium stilboglucanate studies in Bihar[40].

Regimen	Duration	Cure rate	Inference
16-20 mg/kg/day aminosidine alone	21 days	93%-97%	Long-term cure
Conventional antimony alone	30 days	63%	Not much effective

*WHO studies in Bihar considered that aminosidine is a drug used as first-line therapy due to unresponsiveness.

3.6. Sitamaquine

Sitamaquine (8-aminoquinoline; a primaquine derivative) antimalarial drug was developed by Walter Reed Army Institute, Maryland, USA in collaboration with Glaxo Smith Kline, Philadelphia, USA for VL. For the past 8 years, it was still in the development stage and presently it is in clinical trial phase II for the therapy of VL[73]. It has unique advantage which includes oral administration and long half-life (26 h). At higher doses of drug, sitamaquine affects parasite motility, morphology and growth.

Clinical trials reported that cure rate was between the range of 87%-100% when dose was selected between 1.5 mg/kg/day and 3.0 mg/kg/day up to 28 days[74]. However, further studies are required to assess its safety issues and further development. Summary of small phase I and II clinical trial conducted using sitamaquine is listed in Table 6.

4. Need of a new chemotherapeutic agent

As mentioned in the previous section, lots of serious loopholes and drawbacks are associated with current available chemotherapeutic regimens for VL. Therefore, there is an urgent need to develop some novel and efficacious chemotherapeutic agents against VL. Development of new chemical entity is a laborious, time-consuming process and is also expensive. Screening of available chemotherapeutic moieties against VL is a good alternative chemotherapeutic option for VL.

4.1. Dox as a novel approach

Dox is water-soluble anthracycline topoisomerase inhibitor antibiotic. Dox is obtained from the immediate antecedent daunorubicin. Dox is a 14-hydroxylated derivative of daunorubicin which is a natural product, obtained from different *Streptomyces* strains, hence abundantly found in nature. Dox formulations are available as Doxil[®] (PEGylated liposomal type of nanoformulation), Myocet[®] (non-PEGylated liposomal formulation), and Caelyx. All these types of Dox are available as IV formulations[73,78–80].

Dox has unquestioned massive values against a variety of neoplastic conditions such as acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilms tumor (rare kidney tumor), neuroblastoma, soft tissue and bone, breast, ovarian, transitional cell bladder, thyroid, gastric carcinoma, Hodgkin's disease, malignant lymphoma and bronchogenic carcinoma in which the small cell histologic type is the most responsive compared to other cell types. It's far frequently utilized in combination chemotherapy and as an ingredient in numerous chemotherapy regimens. Dox inhibits biosynthesis of macromolecules synthesis *via* DNA intercalation[81–83] and untimely blocks the topoisomerase II (which removes super coils in DNA

transcription process). Therefore, it is regarded as topoisomerase II inhibitor or topoisomerase II poisons.

Dox exhibits linear pharmacokinetics with wide distribution in plasma, liver, lungs, heart, and kidneys. Dox is absorbed by cells and binds to cellular components, particularly nucleic acids. Researchers reported counter intuitively activity of Dox and found that as effective as gold standard drug AmB against *Leishmania* parasite[84,85]. To overcome these limitations associated with the current therapeutic regimen choice, several drug delivery strategies have been accomplished by research teams and several opportunities would open a new door to explore the Dox against VL therapy.

4.2. Mechanism of Dox action against *Leishmania* parasite

The mammalian host redox defense framework mainly depends on glutathione oxidized and glutathione reductase and it is supplanted in *Leishmania* with the aid of an analogous, but unique framework depends on oxidized trypanothione [N1-N8-bis (glutathionyl) spermidine, T(SH)₂] and trypanothione reductase[86,87]. The di-thiol trypanothione is a chief regulatory molecule for the DNA precursors synthesis, homeostasis of ascorbate, detoxification of hydrogen peroxides, and the sequestration/export of thiol-conjugates[88]. Synthesis and reduction of trypanothione are fundamental for the upkeep of diminishing intracellular milieu, which renders the respective enzymes as attractive molecular drug targets. Trypanothione-dependent enzymes, for example, tryparedoxin peroxidase diminishes peroxides utilising electrons donated either directly from trypanothione or *via* the redox intermediate tryparedoxin. Trypanothione dependent hydrogen peroxide metabolism is especially significant in leishmaniasis for the reason that they lack peroxidase. Since this thiol is absent in humans and is essential for the continued existence of the parasites, these enzymes make molecular targets for the development of new drugs to treat these diseases[89]. Dox exhibits its anti-leishmanial activity by acting as competitive inhibitor for trypanothione reductase enzyme (trypanothione reductase is a redox-balancing enzyme of *Leishmania* spp). This enzyme is responsible for decreasing cellular oxidative stress in parasite. Dox competes for trypanothione reductase enzyme by acting as subversive substrates and inhibits enzymatic action. Anti-leishmanial potential of Dox is being exploited using numerous

Table 6. Summary of small phase I and II clinical trial for sitamaquine.

Clinical trial phase	Regimen	Duration	Cure rate	Reference
Kenya phase II	1 mg/kg/day	28 days	50%	[75]
Kenya phase II	1.7 mg/kg/day	28 days	92%	[76]
Brazilian phase II	1 mg/kg/day	28 days	Cure none of the patients	[77]
Brazilian phase II	2 mg/kg/day	28 days	67%	[77]
Brazilian phase II	2.5 mg/kg/day	28 days	Decrease efficacy due to enhanced adverse drug reactions	[77]
Multicenter phase II trial, India	1.75-2 mg/kg/day	28 days	89%	[41]
Phase II b trial, India	2 mg/kg/day with or without food	21 days	85% without food intake	[42]

drug delivery strategies. Macrophage target oriented delivery of Dox is an ideal choice of drug delivery strategies for effective management and treatment of VL. Targeted delivery to intra-cellular regions of macrophages by encapsulation of Dox into numerous carriers and biopolymers provides a more efficient way to treat VL. Several attempts of Dox delivery against VL have been performed and summarized in Table 7 and exclusively discussed in the above-mentioned section.

Table 7. Summary of various doxorubicin formulations use for visceral leishmaniasis treatment.

Formulation	Ligand	Receptor	Doxplatform	Method of preparation	<i>In-vitro</i> activity (IC ₅₀)	<i>In-vivo</i> activity (% inhibition)	Remark	Ref
Nanocapsules	Chondroitin sulphate	Mannose receptor	Chondroitin sulphate anchored Dox	Polymer electrostatic deposition method and adsorption method	Dox: 0.543 µg/mL Ligand coated Dox formulation 0.25 µg/mL	Pure Dox: 54.76 Formulation: 77.97	Improved efficacy Improved cytomaptibility Higher macrophage uptake	[90]
Nanocapsules	Sodium alginate	Fc and europilin-1 receptors	Alginate anchored Dox	Emulsification and ionic adsorption	Dox: ~0.7 µg/mL Ligand anchored formulation ~0.4 µg/mL	Pure Dox: ~43 Ligand anchored formulation: ~72	Strong Th1 responses Generation of immunity Cost effective Improved efficacy	[91]
Nanoemulsion	NA	NA	Nanoemulsion bearing Dox	Emulsion inversion point method	Dox: 0.36 µg/mL Nanoemulsion: 0.69 µg/mL	Not done	Storage stability Higher <i>in-vitro</i> activity	[92]
Nanocapsules	Phosphatidyl-serine	CD68, CD14, annexins, b2 glycoprotein I and GAS6	Phosphatidyl-serine anchored nanocapsule	Emulsion inversion point method	Dox: 691.7 ng/mL Nanocapsule formulation 354.3 ng/mL	Pure Dox: 42.85 Formulation: 85.23	Enhanced macrophage uptake Higher distribution of drug in liver and spleen Improved efficacy	[93]
Nanoparticle	NA	NA	Nanoparticle of MPEG-PLA bearing Dox	Nanoprecipitation	Not done	Not done	Higher internalization of drug to macrophages	[94]
Microparticle	Chitosan	Glucosamine carbohydrate receptors	Chitosan bearing Dox	Emulsion chemical cross-linking technique	Not done	Pure Dox: 33.3 Formulation: 78.2	Enhanced macrophage uptake Improved efficacy	[95]
Microparticle	Chitosan	*Trypanothione reductase	Dox	<i>In-silico</i> docking studies and enzyme expression, purification and activity	Trypanothione reductase inhibitory activity of Dox: Ki value 3.24 µM	Not done	<i>In-silico</i> docking studies explicate that Dox has shown positive inhibition of trypanothione reductase enzyme	[96]
Microparticle	Mannan	Mannose receptor	PLGA mannan conjugated microparticle	Solvent evaporation method	Not done	Pure Dox: 18.54 and 22.67 PLGA-PLGA: 30.45 and 38.23 Mannan-PLGA-Dox: 50.47 and 64.56	Enhanced macrophage uptake Improved efficacy	[97]
Gel-assisted layer-by-layer technology	NA	NA	Gel-assisted nanomatrix (LBL-Dox)	Layer by layer technique	Dox: 0.16 µg/mL LBL-Dox: 0.30 µg/mL	Not done	Enhanced macrophage uptake Improved efficacy	[98]
Liposome	F(ab) 2 of anti-51-kDa antibody	**51-kDa on macrophage	Antibody anchored liposome bearing Dox	Thin film hydration method	Dox: 490 ng/mL Immunoliposome of Dox: 3.1 ng/mL	Dox: 45 Immunoliposome of Dox: 86	Antibody mediated drug targeting	[85]
Liposome	Mannose	Mannose receptor	Mannosylated liposome of Dox	Thin film hydration and covalent coupling method	Dox: 480 ng/mL Mandoxosome: 3.4 ng/mL	Dox: 7.5 Mandoxosome: 90	Immunostimulation through mannose	[99]
Liposome	Mannose-human serum albumin	Mannose receptor	Dox	Coupling method	ED ₅₀ against promastigote: 0.43 µM ED ₅₀ against amastigote: 0.86 µM	Conjugated Dox: 95	Higher efficacy	[84]

*Enzyme as a target; ** protein as a target. DOX: doxorubicin; MPEG-PLA: methoxy poly (ethylene glycol)-poly (lactide); PLGA: poly lactic co-glycolic acid; Ref: reference; NA: not available.

4.3. Chemotherapy of VL by Dox

Optimum chemotherapy and management of VL have not attended even after availability of a variety of chemotherapeutic agents due to emergence of resistance, nephrotoxicity and cardiotoxicity. Thus, alternative option is required due to continuation in dose-dependent negotiation with patient health as well as emergence of resistance and treatment failure. There is an emerging alarm for innovation of a new alternate and vital option for chemotherapy of VL. Chemotherapy of VL by Dox is an outcome of ongoing research for identification of possible alternates of current available chemotherapeutic agents against VL.

Dox shows inhibitory potential against trypanothione reductase, which is typically presented in *Leishmania* spp. compared to human. In 1992, Sett *et al.* was the first research group presented *in-vitro* and *in-vivo* therapeutic efficacy results of Dox against amastigotes and promastigotes forms of parasite. Results described the Dox efficacy through an *in-vitro* activity (IC_{50} was 0.43 μ M and 0.86 μ M against amastigotes and promastigotes, respectively) and *in-vivo* activity in infected mouse model at dose of 625 μ g/kg/day up to four consecutive days showing 95% spleen parasite burden reduction[84]. In another study, novel gel nanomatrix of Dox showed improved efficacy against *L. donovani*. In this case, IC_{50} of developed Dox formulation (0.16 μ g/mL) was 1.9 folds lesser than that of plain Dox (0.30 μ g/mL), while cell viability study demonstrated that unloaded nano-matrix was safer against macrophages represented by more than 90% cells viability[84,100].

Trypanothione reductase is a key enzyme and a principal thiol in *Leishmania* parasite and analogue of glutathione reductase, which is present in mammals. Trypanothione reductase accounts for removal of reactive oxygen species (ROS) by detoxifying the hydroperoxides, converting them into alcohol and water through trypanredoxin protein. Active sites of glutathione reductase and trypanothione reductase are different, hence they exhibit quiet specificity towards their substrates. Therefore, trypanothione reductase is considered to be a selective marker for anti-parasitic drugs development. Shukla and co-workers have explored the trypanothione reductase inhibitor ability of Dox. Authors acquired computational (*in silico* docking studies) and experimental approaches (MTT assay, hemolysis, *in-vitro* activity) to explore Dox efficacy against leishmaniasis[101]. Molecular docking studies revealed that tetracyclic (anthracyclin) moiety of Dox acts through hydrophobic interaction and is responsible for interaction with amino acids of the hydrophobic patch (Try21, Met113 and Glu467) of the active site. Dox showed binding energy comparable to potent inhibitors. Therefore, these compounds may be a potential inhibitor of trypanothione reductase. At concentration of 25 μ M, it showed approximately 60% decline in total thiol in comparison to control group. Intracellular ROS measurements using

CM-H2DCFDA probe indicated that ROS levels were sufficiently enhanced when promastigotes were incubated with 20 μ M Dox as estimated by an increase in fluorescence intensity. Increment in fluorescence intensity with respect to enhanced concentration of Dox represented the increased levels of ROS leading to oxidative stress. *In-vitro* efficacy assessment of Dox demonstrated the promising results in term of IC_{50} against promastigotes (11.76 \pm 0.11) μ M and axenic amastigotes (12.02 \pm 0.15) μ M, respectively. Kansal and co-workers developed a nanoemulsion core loaded nanocapsules entrapping Dox and efficacy was evaluated against *L. donovani*. Dox loaded nanocapsules were efficiently internalized into macrophages indicating enhanced efficacy. IC_{50} of developed Dox nanocapsules was almost 1.9 folds compared to plain drug against intracellular amastigotes[102].

4.4. Chemo-immunotherapy of VL in association with Dox

Absence of parasite specific cell-mediated immune response is a distinctive immunological aspect of active VL infection[103]. The equilibrium of specific T cell subsets, cytokines, macrophage activation, and the induction of nitric oxide (NO) sets up the impact of illness. It has been accounted that during infection, there is a raise in the number of IL-4-positive neutrophils, NK cells, and IL-10 monocytes, while the number of IFN-g-positive, IL-2, and IL-12 eosinophils is significantly diminished. But serum levels of TNF- α and IL-6 were high in patients with active VL[104,105]. Conversely, cured cases have shown a typical type 1 response with an increment in IFN- γ , IL-2 neutrophils, eosinophils, NK cells and with an increase in IL-12 monocytes. Helper and cytotoxic T cells are known to play a vital role in the immune response to this infection, connecting the innate immune response to the development of efficient adaptive cellular immunity mainly through IL-2 and IFN- γ production. These two cytokines drive the effect or functions of macrophages and elicit a Th1 immune response. These findings recommend that any intervention which can shift the immune response from Th2 type to Th1 type will have a major role in cure and prevention of VL. Lots of scientific approaches have been performed to develop an immunotherapeutic formulation of Dox for successful management of VL.

Active targeting of Dox is achieved by encapsulating it in mannose-fabricated liposomes and synergistic activities of INF- γ were studied in a BALB/c mice model by Kole *et al.*[106]. *L. donovani* infected macrophages were treated with free drug Dox, doxosome (liposomal Dox), and mandoxosome (mannose coated liposomal Dox). Representative IC_{50} values of manodoxosome, free Dox and doxosome were 3.4, 480 and 9.6 ng/mL, respectively. Prior INF- γ treatment showed synergistic activity represented by 55%, 1% and 44% suppression of infection in macrophages by manodoxosome

(1 ng/mL), doxosome (3 ng/mL) and free Dox (150 ng/mL), respectively. In *L. donovani* infected mice model, treatment with various forms of Dox resulting in 50% reduction of infection when combined with INF- γ . These results remarked the synergistic inhibitory effect of INF- γ with Dox for suppression of parasitic burden[103,106].

Kansal and co-workers studied the immunotherapeutic potential of sodium alginate coated nanocapsules loaded with Dox. They developed sodium alginate anchored nanocapsules bearing Dox for an immunomodulatory assisted anti-leishmanial activity. Developed formulation showed 1.5 folds higher cellular internalization followed by significantly enhanced *in-vitro* and *in-vivo* efficacy in J774A cells and infected hamster.

Sodium alginate is a potent substance having an excellent property to activate macrophages and also an inducer of releasing cytokines and cytotoxic agents[91]. Alginate coated nanocapsules of Dox uptaken by J774A.1 macrophages was improved by 1.5 folds when compared with uncoated nanocapsules. Alginate coated nanocapsules of Dox showed higher efficacy than uncoated Dox nanocapsules against intra-macrophagic amastigotes. Coated formulation treatment exhibits enhanced apoptotic efficiency as compared with nanoemulsion of Dox and free Dox as evidenced by cell cycle flow cytometric analysis, and decline in mitochondrial membrane potential, ROS and NO production. *In-vivo* results demonstrated that the increased levels of iNOS, TNF- α , IFN- γ and IL-12 when treatment was given with alginate coated nanocapsules of Dox compared with Dox nanoemulsion. Parasitic burden was diminished in *Leishmania* infected hamsters treated with alginate coated formulation in comparison to uncoated formulation.

Mukherjee *et al.* designed and fabricated an actively targeted immunotherapeutic formulation of Dox (immune-doxosome) using 51 kDa proteins (a parasite specific protein isolated from infected macrophages)[85]. Immuno-doxosomes were prepared by grafting F(ab)2 of anti-51 kDa antibody onto the liposomal surface bearing Dox *via* covalent bonding interaction mechanism. Time-dependent cellular internalization was studied with infected and normal macrophages cells, which revealed that phagocytosis of immune-doxosomes by infected macrophages reached equilibrium at 30 min, whereas uptake of immune-doxosome by normal macrophages was significantly lesser and independent of time was shorter. Higher internalization of immunodoxosomes ultimately assured higher activity with lesser non-specific tissue toxicity of Dox. *In-vitro* activity of immune-doxosomes within macrophage was compared with free Dox, doxosomes and non-specific doxosomes. IC₅₀ of immune-doxosomes (3.1 ng/mL) was significantly lesser compared with free Dox (490.0 ng/mL), doxosomes (9.8 ng/mL) and non-specific immune-doxosomes (8.0 ng/mL). Immunodoxosomes showed 100% suppression of parasite in spleen with subsequent

decline in spleen weight to normal value when treatment was given with a dose equivalent to 250 μ g/kg/day of the Dox. On the other hand, free doxorubicin showed only 45% reduction in splenic parasite burden. Doxosome and non-specific immunodoxosome caused 84% and 86% reduction in infection, respectively. These results demonstrated that immunodoxosomes were more effective than doxosome, non-specific immune-doxosomes and free Dox for suppressing parasite infection in macrophages and infected animals[85].

4.5. Macrophages targeted delivery of Dox

VL parasite invades, resides and multiplies in macrophages of mammalian host cell. Mastcell presenting cell plays a fundamental role in the etiology of VL and it is a major target for the treatment of VL. The ultimate strategy and goals of formulation development for targeting mastcell presenting cell are crucial. It could usher an unorthodox but highly effective therapeutic paradigm for treatment of VL with envisioned targeting of antigen presenting cells (macrophages) to overcome the limitation of current available marketed options. Macrophages express diverse ranges of engulfment receptors that are able to bind modified polysaccharides, lipoproteins, senescent and apoptotic cells, and a range of poly-anionic molecules. Several approaches have been already tried on the basis of receptor ligand delivery of Dox to achieve a macrophage targeted delivery limiting non-specific tissue toxicity. Sett and co-workers developed and utilized mannosylated neo-glycoprotein carrier for facilitating receptor-mediated endocytosis of Dox[84]. Here, mannose-human serum albumin was conjugated with Dox. Result of *in-vitro* activity suggested that conjugated Dox was found to be more efficiently eliminated (12.5 times) from intracellular amastigotes of *L. donovani* in peritoneal macrophages compared to free drugs. Furthermore, conjugated Dox greatly eliminated splenic intracellular parasites burden when treatment was given in four consecutive dosages (5 μ g/kg/day) up to 45 days[99].

Similarly, mannose conjugated polylactic-co-glycolic acid microparticles loaded with Dox was prepared and evaluated against experimental VL for selective macrophages targeting of liver and spleen[97]. In another study, chitosan anchored micro-particulate system with a vision for vectoring Dox to reticuloendothelial system *via* passive targeting through particle size and active targeting by receptor ligand association concept. *In-vivo* activity in syrian golden hamsters showed that ligand anchored microparticles have significant higher reduction (78.2 \pm 10.4)% in parasite load compared to free Dox (33.3 \pm 2.4)%[97].

The diblock biodegradable co-polymer bearing Dox nanoparticles were prepared using nano-precipitation method for macrophage targeting. Developed nanoparticles were evaluated for macrophage

targeting potential and obtained results depicted the higher internalization of nanoparticles in macrophages as compared to free drug. Slow release of Dox from nanoparticles resulted in availability of Dox for longer time at the parasitic site of infection, thus leading to greater efficacy[95].

Etiology of macrophage based clearance of apoptotic cells is reconciled by phosphatidylserine that is present on the apoptotic cell external surface and gets exposed which executes binding through macrophages scavenger receptors, CD-68 receptor, CD14, annexin, β -2 glycoprotein I and GAS6. Macrophage targeting potential of phosphatidylserine was explored and evaluated. Authors developed a phosphatidylserine anchored nanocapsules dosage form using layer-by-layer method for efficient macrophage targeting of Dox. Developed cargos were evaluated during *in-vitro* and *in-vivo* conditions and subsequently compared with uncoated nanocapsules and free Dox. Cellular internalization of phosphatidylserine anchored nanocapsules in J774A.1 macrophage was 1.75-fold higher than plain nanocapsules. *In-vitro* activity of phosphatidylserine anchored nanocapsules having Dox was performed against *L. donovani* intramacrophagic amastigote and results were expressed as inhibition of parasite growth (IC_{50}) observed after 48 h of incubation. The respective IC_{50} values of phosphatidylserine anchored nanocapsules, uncoated nanocapsules and free Dox were (354.33 ± 5.13), (391.67 ± 20.40) and (691.67 ± 10.41) ng/mL, respectively. Results of *in-vivo* antileishmanial activity showed that phosphatidylserine anchored nanocapsules caused (85.23 ± 4.49)% inhibition of the splenic parasite burden, while uncoated nanocapsules and free Dox caused (72.88 ± 3.87)% and (42.85 ± 2.11)% parasitic inhibition respectively in *L. donovani* infected hamsters[94]. Chaurasia *et al.* developed chitosan anchored nanoparticles bearing AmB and Dox to specifically target macrophage with two different antileishmanial agents. Cellular uptake study in macrophage cell line revealed that chitosan anchored formulation internalized 2.02 fold higher than uncoated formulation. Developed combined drug delivery anchored with chitosan showed significantly ($P < 0.05$) lower IC_{50} against *L. donovani* infected macrophages in comparison to uncoated formulation[93].

4.6. Drug repurposing of non-PEGylated liposome encapsulated doxorubicin for VL

4.6.1. Composition of non-PEGylated liposome encapsulated doxorubicin

Non-PEGylated liposome encapsulated doxorubicin consists of single unilamellar liposomes of size approximately 160 nm which is composed of egg phosphatidyl choline: cholesterol (in the molar ratio 55:45). It is commercially available as a package of 3 vials. First vial contains doxorubicin HCl-50 mg and lactose NF-250

mg; second vial contains empty liposomes in citrate buffer, *e.g.* phosphatidyl choline-142.6 mg and cholesterol NF-57.4 mg in citrate buffer (57.6 mg/mL)-2 mL; and the third vial buffer for injection contains sodium carbonate anhydrous 54.6 mg in 3.1 mL of water for injection. After preparation as mentioned in the patient information leaflet[93–95], the doxorubicin is almost completely encapsulated into the empty liposomes through active loading process, *i.e.*, through pH gradient established across the liposomal membrane (acidic pH ~4.5 inside the liposomes) to produce 2 mg/mL liposomal doxorubicin solution. The drug to lipid ratio is approximately 0.25:1 (wt:wt). The resulting solution is red-orange and opaque in appearance and the final pH of the formulation is 6.5 to 8.5.

4.6.2. Passive targeting of reticuloendothelial systems by non-PEGylated liposome encapsulated doxorubicin

It was concluded that in comparison to Doxil[®] (PEGylated liposomal formulation of doxorubicin) and doxorubicin plain solution, the non-PEGylated liposomal version of doxorubicin (Myocet[®]) is rapidly or actively taken up by the cells of the reticuloendothelial systems like macrophages and monocytes and ends up in large quantities in liver and spleen compared to other tissues. This phenomenon can be capitalized to passively target doxorubicin to intracellular parasites (VL) of macrophages in liver and spleen. The opsonisation of non-PEGylated liposomes drives phagocytosis by macrophages and drives their passive targeting into these reticuloendothelial systems. It is also interesting to note the concentration of doxorubicin in the heart tissue for both liposomal formulations (Doxil[®] and Myocet[®]) was lower compared to free doxorubicin solution which directly reflects on the lower incidence of cardiotoxicity of the liposome encapsulated doxorubicin.

Non-PEGylated liposome encapsulated doxorubicin can passively target macrophage resident parasites in VL with reduced cardiotoxicity. Hence, authors strongly propose re-purposing of non-PEGylated liposome encapsulated doxorubicin for treating VL as an alternative to other liposomal formulations like liposomal AmB.

5. Conclusions

Pharmaceutical research on various anti-leishmanial chemotherapeutic agents and their variable responses in diverse geographical areas generated an urge to explore the novel therapeutic strategy to alleviate severe and diverse VL. However, priority should be given to remedies which are safer, more inexpensive and with minimal toxicity than existing prescription medicines. Substantial progress in search for Dox as a novel chemotherapeutic agent mainly depends upon the initiative of individual academic laboratories to consortia and public-private partnerships. Various biopolymers and carriers

also potentiate the anti-leishmanial activity of Dox *via* more efficient apoptosis and would provoke the host body's immune response. Combination of immune stimulation with anti-leishmanial drug is more effective than treatment alone with a chemotherapeutic. Non-PEGylated liposome encapsulated doxorubicin, which has potential to passively target macrophages and its intracellular parasite, can be re-purposed to treat VL. Hence, this potential therapeutic approach may encourage future validation or repurposing of the other drugs for treatment of VL infection.

6. Future outlook

Leishmaniasis is a great challenge and a global burden in terms of drug discovery and drug delivery due to capability of parasite residing in cells and subsequently widespread to other areas in the body. Higher toxicity, emergence of MDR and variable responses challenged the scientists to develop new anti-leishmanial drugs as well as new strategies to control it. The future perspectives of VL treatment depend on the development of optimum anti-leishmanial Dox formulation alone or with various biopolymers which could control the vectors, monitor the efficiency of given therapeutic regimen and diagnosis method with more sensitivity and specificity. Complete eradication of leishmaniasis also depends on combined chemotherapy of doxorubicin based drug delivery systems with other drugs, so that addition and synergism property of drugs will curb the emergent MDR effectively.

Conflict of interest statement

No conflict of interest was reported by the authors of this article. There is no conflict of interest between the authors and manufacturer.

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Authors' contributions

S.R.S. developed the theoretical formalism and final version of the manuscript. R.N.S supervised the work.

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