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Could combination chemotherapy be more effective than monotherapy in the treatment of visceral leishmaniasis? A systematic review of preclinical evidence

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Abstract

From a systematic review framework, we assessed the preclinical evidence on the effectiveness of drug combinations for visceral leishmaniasis (VL) treatment. Research protocol was based on the PRISMA guideline. Research records were identified from Medline, Scopus and Web of Science. Animal models, infection and treatment protocols, parasitological and immunological outcomes were analysed. The SYRCLE's (SYstematic Review Center for Laboratory Animal Experimentation) toll was used to evaluate the risk of bias in all studies reviewed. Fourteen papers using mice, hamster and dogs were identified. *Leishmania donovani* was frequently used to induce VL, which was treated with 23 drugs in 40 different combinations. Most combinations allowed to reduce the effective dose, cost and time of treatment, in addition to improving the parasitological control of *Leishmania* spp. The benefits achieved from drug combinations were associated with an increased drug's half-life, direct parasitic toxicity and improved immune defences in infected hosts. Selection, performance and detection bias were the main limitations identified. Current evidence indicates that combination chemotherapy, especially those based on classical drugs (miltefosine, amphotericin B antimony-based compounds) and new drugs (CAL-101, PAM3Cys, tufisin and DB766), develops additive or synergistic interactions, which trigger trypanocidal and immunomodulatory effects associated with reduced parasite load, organ damage and better cure rates in VL.

Introduction

Visceral leishmaniasis (VL) or kala-azar is a neglected infectious disease caused by infection with the protozoan parasites *Leishmania infantum chagasi*, *Leishmania donovani* and *Leishmania infantum* (Freitas *et al.*, 2012). This is a potentially fatal disease in most untreated cases (over 95%), closed correlated to poverty, precarious conditions of basic sanitation and limited access to health services (WHO, 2022). In addition to VL being endemic in more than 79 countries, about 50 000–90 000 new cases of this disease occur annually worldwide and 3813 deaths were reported between 2014 and 2020 (Ruiz-Postigo *et al.*, 2021). According to the World Health Organization (WHO), these cases were associated with 10 countries, specifically Brazil, China, Ethiopia, Eritrea, India, Kenya, Somalia, South Sudan, Sudan and Yemen (WHO, 2022). While *L. infantum* is responsible for the disease in North Africa, Europe and Latin America; *L. donovani* prevails in the East Africa and Indian subcontinent (Ready, 2014).

The disease develops after the transmission of metacyclic forms of *Leishmania* spp. to vertebrate hosts by sand flies, mainly of the genders *Phlebotomus* spp. and *Lutzomyia* spp. (Nieto *et al.*, 2011; Dostálová and Volf, 2012). At the site of infection, the parasites are phagocytosed by macrophages, within which they survive and multiply by binary fission as amastigote forms (Dostálová and Volf, 2012; de Freitas *et al.*, 2016). A broad spectrum of unspecific clinical manifestations is detected during VL development, especially chronic low-grade fever, anorexia, weight loss, weakness and hepatosplenomegaly. In addition, laboratory findings such as pancytopenia, low plasma albumin, high aminotransferase levels and hypergammaglobulinemia are often associated with VL (Serafim *et al.*, 2010; Mwololo *et al.*, 2015; WHO, 2016).

The specific treatment of VL is mainly based on pentavalent antimonies (sodium stibogluconate and meglumine antimoniate), followed by paromomycin, oral miltefosine or amphotericin B as a second choice (Corral *et al.*, 2014; Joice *et al.*, 2017; Alves *et al.*, 2018). New nanostructured lipid formulations of amphotericin B (amphotericin liposomal) have shown relevant results in preclinical (Corral *et al.*, 2014) and clinical (Sundar *et al.*, 2011) studies. However, this treatment is still expensive and often unavailable in several endemic areas

(Corral *et al.*, 2014; Ponte-Sucre *et al.*, 2017). Although these drugs are recommended by the WHO as the reference chemotherapy, marked side-effects (i.e. nausea, vomiting, arthralgia, cardiac dysrhythmias, hepatitis and pancreatitis), limited efficacy, high cost, as well as complex administration (parenteral) make VL chemotherapy a challenging task (Sundar *et al.*, 2011; Hendrickx *et al.*, 2017; Ponte-Sucre *et al.*, 2017). In addition, infections caused by parasites resistant to the reference chemotherapy represent a more recent and worrying barrier to the VL treatment (Mwololo *et al.*, 2015; Ponte-Sucre *et al.*, 2017). Thus, developing more effective and less toxic treatment protocols for VL is necessary and urgent (Khadem *et al.*, 2017; Joice *et al.*, 2017).

In the last years, therapy based on drugs association has emerged as an alternative to VL treatment (Mwololo *et al.*, 2015; Joice *et al.*, 2017; Rebello *et al.*, 2019). As combination chemotherapy allows to increase drugs half-life, reduce medication dose, treatment time, systemic toxicity and side-effects (van Griensven *et al.*, 2010; Corral *et al.*, 2014; Bhattacharjee *et al.*, 2015; Rebello *et al.*, 2019); greater adherence to the treatment protocol and better therapeutic outcomes are proposed (van Griensven *et al.*, 2010). With the increase in therapeutic failures after the administration of antimonial drugs and miltefosine, combination chemotherapy is also relevant to reduce *Leishmania* spp. resistance to treatment (Sundar *et al.*, 2012; Rijal *et al.*, 2013; Ponte-Sucre *et al.*, 2017). This approach has also emerged as a chemotherapy alternative for complicated VL cases, such as patients co-infected with HIV, for which monotherapy does not achieve satisfactory results (Alvar *et al.*, 2008; van Griensven *et al.*, 2010; Rebello *et al.*, 2019).

Drug combination has been successfully used in the treatment of several other infectious diseases, such as tuberculosis, malaria and leprosy (Nosten and Brasseur, 2002; van Griensven *et al.*, 2010; Ramón-García *et al.*, 2011). However, as the current evidence is fragmented, it is difficult to establish a clear profile of drugs and protocols administered, as well as to assess their therapeutic relevance for VL. Therefore, we use a systematic review framework to retrieve and analyse the preclinical evidence on the applicability and relevance of leishmanicidal or leishmaniostatic drug combination for the chemotherapeutic management of VL. In addition to mapping the available drug combinations and their spectrum of effectiveness, all preclinical models and treatment protocols used, as well as the risk of bias related to the studies that support the current evidence were critically analysed. By characterizing the rationale underlying the co-administration of different antileishmanial drugs, this systematic review may be relevant to support translational investigations in the search of parasitological cure for this disease.

Materials and methods

Guiding questions

The main questions to be answered in this systematic review were: Are combinations of antileishmanial drugs effective in the treatment of VL? What are the main chemotherapy protocols and primary research outcomes used to determine treatment effectiveness?

Search strategy and selection of primary studies

The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement was adopted for conducting this systematic review (Hooijmans *et al.*, 2014). In our research strategy, an extensive literature search using 3 comprehensive databases was used: (i) PubMed/Medline, (ii) Scopus and (iii) Web of Science. We used an advanced research strategy based on search filters optimized according to specific algorithms and syntaxes adopted in each database. The search filters were structured in 3 complementary levels as follows: (i) treatment: combination chemotherapy, (ii) disease: VL and (iii) research type: preclinical models *in vivo*. A search filter was initially developed for PubMed according to the standardized descriptors obtained from the platform's thesaurus MeSH (Medical Subject Headings; https://www.ncbi.nlm.nih.gov/mesh). To expand the recovery of relevant indexed studies and those in the indexing process, the commands [MeSH Terms] and [TIAB] were combined. To detect all animal studies from PubMed, a standardized animal filter was applied (Pereira *et al.*, 2017). The same search filter used to disease and treatment was adapted to Scopus and Web of Science. A search limit for animal models in Scopus, and language limit (English) for Scopus and Web of Science were applied. No chronological restrictions were adopted in the search strategy. The complete search strategies applied in both databases can be consulted in the Supplementary material (Table 1). The initial selection was independently performed by 3 investigators (DSSB, ACFS and ACS), who screened the title and abstract of all recovered papers. Duplicate studies were removed by comparing the authors, title, year and journal of publication.

Inclusion and exclusion criteria

Only studies investigating the effectiveness of drug combination in the preclinical models of VL were included in the systematic review. Irrelevant studies were excluded (not related to the subject) after the initial screening, and all potentially relevant studies were recovered in full-text and evaluated for eligibility. Study exclusion was based on well-defined criteria as follows: (i) studies exclusively based on *in vitro*, *ex vivo* or *in silico* assays; (ii) studies evaluating cutaneous leishmaniasis or unrelated diseases; (iii) secondary studies (i.e. literature reviews, editorials, commentaries, short communication and letters to the editor); (iv) clinical studies; (v) absence of monotherapy as control; (vi) non-pharmacological treatments (i.e. plant extracts, cytokines, peptides) and vaccines; (vii) absence of groups treated with drug combination; and (viii) studies published in other language than English. After identifying all relevant studies in the primary search, we included a secondary screening to enhance the recovery of research records on the subject investigated. Thus, the reference lists of all papers identified in electronic databases and included in the systematic review were manually screened for additional relevant studies. In both search levels, 3 researchers (DSSB, ACS and RVG) independently analysed the eligibility criteria, and disagreements were resolved by arbitration, consulting 2 other researchers (RDN and EAMS).

Study characteristics and data extraction

Qualitative data were extracted from all included articles. For this, standardized spreadsheets (data extraction masks) were built, indicating the essential information to be collected from the reading of the individual study chain. Thus, the information summarized in data extraction masks was categorized as follows: (i) publication characteristics: authors, years and country; (ii) characteristics of the animal models: species, lineage, sex, weight and age; (iii) infection parameters: *Leishmania* species, strain, number of parasites inoculated, route of inoculation; (iv) treatment protocol: drugs co-administered, dose, frequency, route of administration; (v) complementary *in vitro* assays: parasitological/toxicity tests for drug interaction assessment; and (vi) primary research outcomes: parasitism, immunological, biochemical and survival results. For parasitism, data available in figures were digitized and the means was obtained using ImageJ software (Schneider *et al.*, 2012) after calibrating each picture to the nearest 0.01 mm.

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Table 1. Search strategies used to identify research registers in PubMed/Medline, Scopus and Web of Sciences databases

Group	Filter[a]	Date and time	No. studies
PubMed/Medline			
#1 Visceral leishmaniasis	['leishmaniasis, visceral' [mesh terms] or 'visceral leishmaniasis' [tiab] or 'leishmania infantum' [mesh terms] or 'leishmania infantum' [tiab] or 'leishmania infantum chagasi' [tiab] or 'leishmania donovani' [mesh terms] or 'leishmania donovani' [tiab]]	03/24/20	16 432
#2 Drugs	['amphotericin b' [mesh terms] or 'amphotericin b' [tiab] or 'pentavalent antimonials' [tiab] or 'pentamidine' [mesh terms] or 'pentamidine' [tiab] or 'miltefosine' [tiab] or 'paromomycin' [mesh terms] or 'paromomycin' [tiab] or 'antifungal agents' [mesh terms] or 'antifungal agents' [tiab] or 'liposomal amphotericin b' [tiab] or 'azoles' [mesh terms] or 'azoles' [tiab]]	03/24/20	704 431
#3 Animal model	Standardized filter 1 (Pereira *et al.*, 2017)	03/24/20	6 752 043
#3 Animal model	Standardized filter 2 (Pereira *et al.*, 2017)	03/24/20	98 646
#4 Combination animal filter	Standardized filter 1 OR Standardized filter 2	03/24/20	6 850 430
#5 Combination	#1 Visceral leishmaniasis AND #2 Drugs AND #4 Combination animal filter	03/24/20 06:37 pm	1084
Scopus			
#1 Visceral leishmaniasis	[title-abs-key (amphotericin b) or title-abs-key (pentavalent antimonial) or title-abs-key (pentamidine) or title-abs-key miltefosine] or title-abs-key (paromomycin) or title-abs-key (antifungal agents) or title-abs-key (liposomal amphotericin b) or title-abs-key (azoles)	03/24/20	156 236
#2 Drugs	[title-abs-key (leishmaniasis, visceral) or title-abs-key (leishmania infantum) or title-abs-key (leishmania infantum chagasi) or title-abs-key (leishmania donovani)]	03/24/20	20 583
#1 and #2	Combination #1Visceral leishmaniasis AND #2 Drugs	03/24/20	4229
#4 Combination	Search limit: Animal model	03/24/20 09:12 pm	397
Web of Science			
#1 Visceral leishmaniasis	TS = (Visceral AND leishmaniasis) OR TS = (Leishmania AND infantum) OR TS = (Leishmania AND infantum AND chagasi) OR TS = (Leishmania AND donovani)	03/23/20	18 607
#2 Drugs	TS = (Amphotericin AND B) OR TS = (Pentavalent AND Antimonies) OR TS = (Pentamidine) OR TS = (Miltefosine) OR TS = (Paromomycin) OR TS = (Antifungal AND Agents) OR TS = (Liposomal AND Amphotericin AND B) OR TS = (azoles)	03/23/20	56 560
#3 Animal model	TS = (Animal) OR TS = (Animal model) OR TS = (Murine AND model) OR TS = (Animals) OR TS = (Rodent) OR TS = (Mice) OR TS = (Rat) OR TS = (Rats) OR TS = (Guinea AND pig) OR TS = (Hamster) OR TS = (Dog) OR TS = (Dogs)	03/23/20	4 309 416
#4 Combination	Combination #1Visceral leishmaniasis AND #2 Drugs AND #3 Animal model	03/23/20 09:06 pm	758

[a]The parasite species were not written in italics and capital letters were suppressed because these variants are not considered in the search algorithms of the databases used.

Reporting quality as a risk of bias

The risk of bias in animal studies was analysed from the SYRCLE's (SYstematic Review Center for Laboratory Animal Experimentation) guidelines, which are based on the Cochrane Collaboration risk-of-bias (RoB) tool for randomized trials (Hooijmans *et al.*, 2014). This instrument is adjusted for bias aspects that play a specific role in animal intervention studies. The objective is to establish consistency and avoid discrepancies in the evaluation of methodological quality in the field of animal experimentation. In order to increase transparency and enforceability, signalling issues have been formulated to facilitate judgment based on the following levels: (1) random sequence generation, (2) baseline characteristics, (3) allocation concealment, (4) random housing, (5) blinding of participants and personnel, (6) random outcome assessment, (7) blinding of outcome assessment, (8) incomplete outcome data, (9) selective outcome reporting, and (10) other bias. The items in the RoB tool were scored with 'yes', indicating low risk of bias; 'no', indicating high risk of bias; or 'unclear', indicating that the item was not reported, and therefore, the risk of bias was unknown.

Results

Research records retrieved

Our primary search strategies recovered 2239 articles from PubMed, Scopus and Web of Science, of which 608 were duplicates. After title and abstract screening, 1631 studies were excluded due to inadequate research topic. Of these, 349 studies were based on only *in vitro* parasite viability assays, 595 investigated treatments without chemotherapeutic combination, and 238 studies evaluated other diseases. In addition, 386 studies corresponded to non-original papers, 5 papers were based on clinical investigations, and 23 studies were not written in English. Considering papers that investigated drug combination for VL treatment, 40 studies were selected for full-text evaluation.

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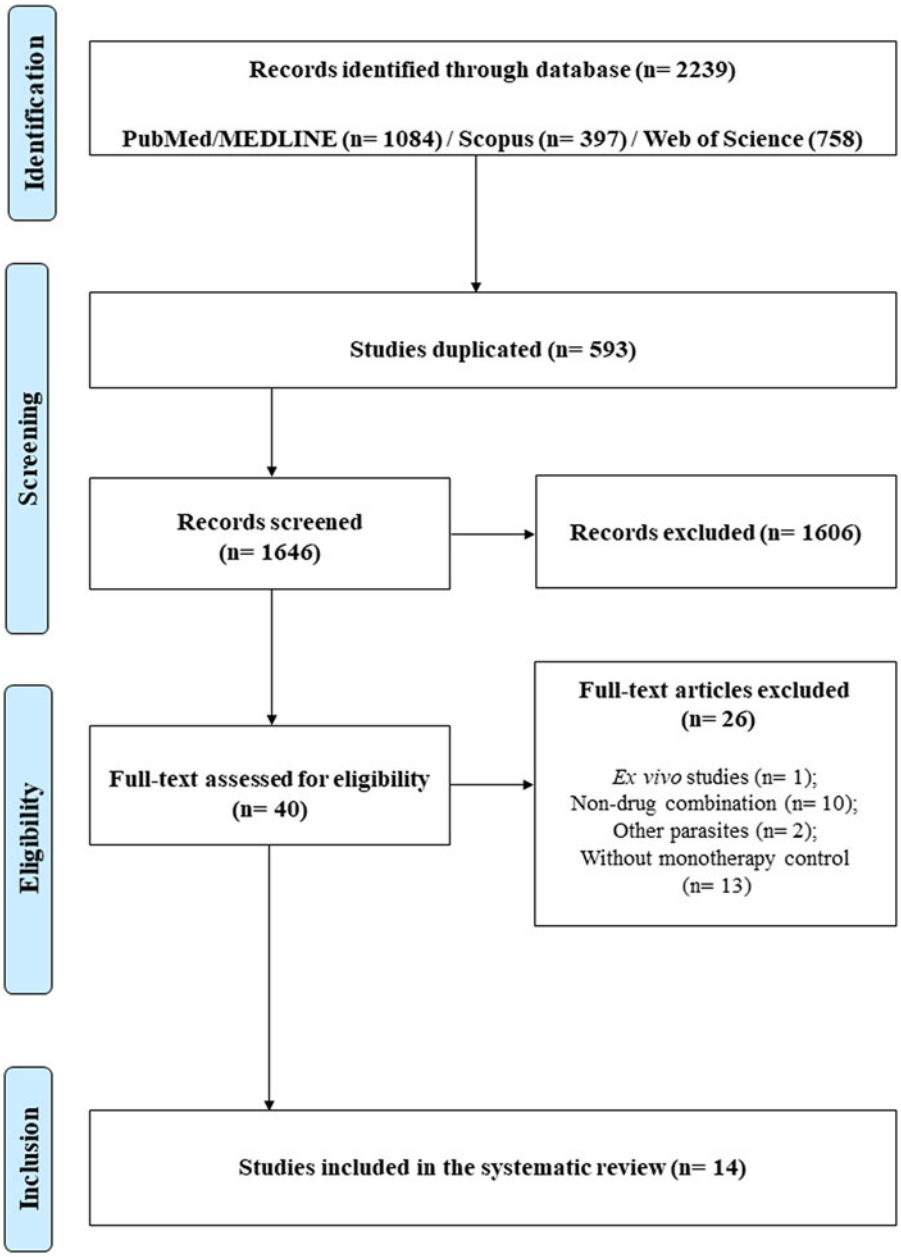

Fig. 1. Flow diagram of the systematic review literature search results. Based on PRISMA statement 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses'. www.prisma-statement.org.

Thus, 14 relevant studies were identified and included in this systematic review (Fig. 1).

Animal models of visceral leishmaniasis

General characteristics of the animal models used in all studies are showed in supplementary files (Table S1). Most studies were produced in India (35.71%, $n = 5$), followed by Kenya (14.29%, $n = 2$). The remaining studies (50%) were conducted in the USA, Italy, UK, Spain, Canada, Belgium and Brazil. BALB/c mice were used as an animal model in most studies (71.43%, $n = 10$), followed by hamster (21.43%, $n = 3$), especially the golden lineage (14.29%, $n = 2$). Hamster lineage was under-reported in 1 study. Dogs belonging to different breeds were used in only 1 study.

The report about the sex of the animals used in the studies shows that females (35.71%, $n = 5$) and works using males and females together (35.71%, $n = 5$) were the most found. Male animals were adopted in only 1 study (7.14%, $n = 1$), while this parameter was under-reported in 3 other studies (21.43%). Mice and hamster age ranged from 4 to 8 weeks, and dogs age ranged from 2 to 10 years old (7.14%, $n = 1$). This variable was neglected in 6 studies (21.43%). Animal's weight ranged from 18 to 25 g for mice, and 80 to 100 g for hamster. Most studies did not report these data (57.14, $n = 8$).

Visceral leishmaniasis characteristics

Leishmania donovani species was used in 9 studies (64.29%), followed by *L. infantum* (35.71%, $n = 5$). In studies with dogs, the animals were naturally infected and the *Leishmania* species was not identified. Four different *Leishmania* strains were used. The strains MHOM/KE/82/LRC-L445/NLB065 and MHOM/MA/67/ITMAP263 were used in 3 studies (21.43%) each, while MHOM/IN/89/GE1F8R and MHOM/ET/67/HU3 strains were found in 2 studies (14.29%) each. The remaining studies used 5 different strains. The strain used to infect the animals was not reported in 1 study (6.25%) (Table S2). Intravenous and intraperitoneal parasites inoculation was used in 5 studies (35.71%) each, followed by intracardiac route in 3 studies (21.43%) and only 1 study reported natural infection (7.14%). The inoculum size ranged from 1×10^6 to 1×10^8 parasites in studies using mice and hamsters (Table S2).

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Table 2. Control monotherapy, drug combinations, target organs, parasitism suppression and nature of pharmacological interaction

	Target organs	Control monotherapy (dose)	Parasitism suppression (%)	Combination chemotherapy (dose)	Parasitism suppression (%)	Drug interaction[a]
Mouse model						
(Carter *et al.*, 2003) [1]200016 strain [2]200011 strain	[1]Liver [1]Spleen [1]Bone marrow	BSO (34 mg kg^{-1}) SSG (74 mg SB^{v} kg^{-1}) SSG (282 mg SB^{v} kg^{-1})	36^{L}, 3.5^{S}, 11^{BM} 77^{L}, 5^{S} 25^{BM} 98^{L}, 40^{S}, 72^{BM}	BSO (34 mg kg^{-1}) + SSG (74 mg SB^{v} kg^{-1})	99.45^{L}, 85^{S}, 85^{BM}	–
	[2]Liver [2]Spleen [2]Bone marrow	BSO (34 mg kg^{-1}) SSG (74 mg SB^{v} kg^{-1}) SSG (282 mg SB^{v} kg^{-1})	14^{L}, 6.5^{S}, 19^{BM} 4^{L}, 4^{S}, 10^{BM} 30^{L}, 22^{S}, 6^{BM}	BSO (34 mg kg^{-1}) + SSG (74 mg SB^{v} kg^{-1})	94.5^{L}, 0^{S}, 11^{BM}	–
(Haldar *et al.*, 2009) [3]S strain [4]R strain	[3]Liver [3]Spleen	PV6 (0.5 μmol 30 g^{-1}) SAG (50 mg kg^{-1}) SAG (250 mg kg^{-1})	72.7^{L}, 75.8^{S} 47.5^{L}, 48.8^{S} 92.9^{L}, 91.0^{S}	PV6 (0.5 μmol 30 g^{-1}) + SAG (50 mg kg^{-1})	84.2^{L}, 83.4^{S}	Additive
	[4]Liver [4]Spleen	PV6 (0.5 μmol 30 g^{-1}) SAG (50 mg kg^{-1}) SAG (250 mg kg^{-1})	49.9^{L}, 53.4^{S} 17.8^{L}, 17.3^{S} 49.2^{L}, 45.8^{S}	PV6 (0.5 μmol 30 g^{-1}) + SAG (50 mg kg^{-1})	77.1^{L}, 79.2^{S}	Additive
(Mutiso *et al.*, 2011)	Spleen	DIM (12.5 mg kg^{-1}) ART (12.5 mg kg^{-1}) AMB (12.5 mg kg^{-1})	27.78^{S} 33.05^{S} 92.91^{S}	DIM (12.5 mg kg^{-1}) + ART (12.5 mg kg^{-1})	80.33^{S}	–
(Shakya *et al.*, 2012*a*)	Liver	Pam3Cys (100 μg) MTF (2.5 mg kg^{-1}) MTF (5 mg kg^{-1}) MTF (20 mg kg^{-1})	58.2^{L} 69.9^{L} 48.2^{L} 96.5^{L}	PAM3Cys (100 μg) + MTF (2.5 mg kg^{-1}) PAM3Cys (100 μg) + MTF (5 mg kg^{-1})	82.6^{L} 92.5^{L}	–

[a]Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests.
[L], Liver; [BM], bone marrow; [LN], lymph node; [1], 200016 strain; [2], 200011 strain; [3]; resistant strain; [4], susceptible strain; BSO, buthionine sulfoximine; SSG, sodium stibogluconate; MTF, miltefosine; DIM, diminazene; ART, artesunate; PV6, diperoxovanadate; Pam3Cys, N-palmitoyl-S-(2, 3-bis (palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride; AMB, amphotericin B; [1,2 and 3,4], studies using 2 different strains.

	Target organs	Control monotherapy (dose)	Parasitism suppression (%)	Combination chemotherapy (dose)	Parasitism suppression (%)	Drug interaction[a]
Mouse model						
(Shakya *et al.*, 2012*b*)	Liver	F-TUF (60 μg) L-TUF (60 μg) MTF (2.5 mg kg^{-1}) MTF (5 mg kg^{-1}) MTF (20 mg kg^{-1})	34^{L} 48^{L} 49^{L} 72^{L} 98^{L}	F-TUF (60 μg) + MTF (2.5 mg kg^{-1}) L-TUF (60 μg) + MTF (2.5 mg kg^{-1}) L-TUF (60 μg) + MTF (5 mg kg^{-1})	66^{L} 81^{L} 93^{L}	–
(Bhattacharjee *et al.*, 2015)	Liver Spleen	SAG (250 mg kg^{-1}) GA (50 mg kg^{-1})	9^{L}, 12^{S} 26^{L}, 31^{S}	GA (50 mg kg^{-1}) + SAG (250 mg kg^{-1})	92^{L}, 93^{S}	Synergistic
(Mwololo *et al.*, 2015)	Spleen	DIM (12.5 mg kg^{-1}) CHQ (12.5 mg kg^{-1}) AMB (1 mg kg^{-1})	29^{S} 8^{S} 96^{S}	DIM (12.5 mg kg^{-1}) + CHQ (12.5 mg kg^{-1})	68^{S}	Synergistic
(Khadem *et al.*, 2017)	Liver Spleen	CAL-101 (0.05 mg) AMB (0.1 mg kg^{-1})	88^{L}, 84^{S} 65^{L}, 69^{S}	CAL-101 (0.05 mg) + AMB (0.1 mg kg^{-1})	$100^{¨L}$, $100^{¨S}$	–
(Joice *et al.*, 2017)	Liver	POS (30 mg kg^{-1}) POS (15 mg kg^{-1}) POS (7.5 mg kg^{-1})	57^{L} 21^{L} 6^{L}	DB766 (75 mg kg^{-1}) + POS (30 mg kg^{-1}) DB766 (75 mg kg^{-1}) + POS (15 mg kg^{-1}) DB766 (75 mg kg^{-1}) + POS (7.5 mg kg^{-1})	86^{L} 88^{L} 75^{L}	Additive Synergistic Additive

[a]Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests.
[L], Liver; [BM], bone marrow; [LN], lymph node; MTF, miltefosine; DIM, diminazene; AMB, amphotericin B; POS, posaconazole; ART, artesunate; CHQ, chloroquine CAL-101, p110δ-specific pharmacological inhibitors; SAG, sodium antimony gluconate; GA, glycyrrhizic acid; F-TUF, free-tufisin; L-TUF, lipo-tufisin; DB766, 2,5-bis[2-(2-i-propoxy)-4-(2-pyridylimino) aminophenyl] furan hydrochloride. ¨No parasite was detected.

Protocols of combination chemotherapy

Considering all 14 studies, 23 drugs were tested in 40 different combinations (Tables 2 and S3). In mice models, 28 combinations (70%) based on 23 different drugs were used. The most used drug ($n = 3$, 13.64%) was miltefosine (MTF), followed by sodium antimony gluconate (SAG), diminazene (DIM) and DB766 compound ($n = 2$, 9.09% combinations each). Glycyrrhizic acid (GA), sodium stibogluconate (SSG), CAL-101 (CAL), amphotericin B (AMB), artesunate (ART), diperoxovanadate (PV6), N-palmitoyl-S-(2, 3-bis (palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride (Pam3Cys), buthionine sulfoximine (BSO), chloroquine (CHQ), ketoconazole (KET), lopinavir (LPV), posaconazole (POS) and tufisin (TUF) were also used in combination for VL treatment (Tables 2 and S3). For hamster models, 11 (27.5%) combinations based on 7 different drugs were investigated. The combinations were based on trans-aconitic acid (TAA), SSG, pentamidine (PET), allopurinol (ALO), allicin (ALL), AAMB, paromomycin

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	Target organs	Control monotherapy (dose)	Parasitism suppression (%)	Combination chemotherapy (dose)	Parasitism suppression (%)	Drug interaction[a]
Mouse model						
(Joice *et al.*, 2017)	Liver	DB766 (75 mg kg^{-1}) DB766 (38 mg kg^{-1}) DB766 (19 mg kg^{-1}) KET (30 mg kg^{-1}) KET (15 mg kg^{-1}) KET (7.5 mg kg^{-1}) MTF (10 mg kg^{-1})	68^{L} 40^{L} 22^{L} 76^{L} 59^{L} 41^{L} 93^{L}	DB766 (38 mg kg^{-1}) + POS (30 mg kg^{-1}) DB766 (38 mg kg^{-1}) + POS (15 mg kg^{-1}) DB766 (38 mg kg^{-1}) + POS (7.5 mg kg^{-1}) DB766 (19 mg kg^{-1}) + POS (30 mg kg^{-1}) DB766 (19 mg kg^{-1}) + POS (15 mg kg^{-1}) DB766 (19 mg kg^{-1}) + POS (7.5 mg kg^{-1}) DB766 (45 mg kg^{-1}) + KET (18 mg kg^{-1}) DB766 (30 mg kg^{-1}) + KET (12 mg kg^{-1}) DB766 (15 mg kg^{-1}) + KET (6 mg kg^{-1}) DB766 (7.5 mg kg^{-1}) + KET (3 mg kg^{-1})	83^{L} 80^{L} 69^{L} 66^{L} 67^{L} 48^{L} 92^{L} 44^{L} 36^{L} 33^{L}	Synergistic Synergistic Synergistic Synergistic Synergistic Synergistic Synergistic Antagonism Antagonism Additive

[a]Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests.
[L], Liver; [BM], bone marrow; [LN], lymph node; MTF, miltefosine; DB766, 2,5-bis[2-(2-i-propoxy)-4-(2-pyridylimino) aminophenyl] furan hydrochloride; POS, posaconazole; KET, ketoconazole.

	Target organs	Control monotherapy (dose)	Parasitism suppression (%)	Combination chemotherapy (dose)	Parasitism suppression (%)	Drug interaction[a]
Mouse model						
Rebello *et al.*, 2019	Liver Spleen	MTF (15.4 mg kg^{-1}) MTF (7.2 mg kg^{-1}) MTF (3.85 mg kg^{-1}) LPV (246.6 mg kg^{-1}) LPV (493.2 mg kg^{-1})	100^{L}, 100^{S} 100^{L}, 100^{S} 46^{L}, 67^{S} 21^{L}, 0^{S} 40^{L}, 52^{S}	LPV (493.2 mg kg^{-1}) + MTF (7.2 mg kg^{-1}) LPV (493.2 mg kg^{-1}) + MTF (3.85 mg kg^{-1}) LPV (493.2 mg kg^{-1}) + MTF (1.92 mg kg^{-1}) LPV (246.6 mg kg^{-1}) + MTF (7.2 mg kg^{-1}) LPV (246.6 mg kg^{-1}) + MTF (3.85 mg kg^{-1}) LPV (246.6 mg kg^{-1}) + MTF (1.92 mg kg^{-1})	100^{L}, 100^{S} 70^{L}, 52^{S} 44^{L}, 77^{S} $100^{¨L}$, $100^{¨S}$ 71+, 83^{S} 12^{L}, 12^{S}	Additive
Dog model						
(Oliva *et al.*, 1998)	Bone marrow Lymph node	PAR (3.5 mg kg^{-1}) MEG (30 mg Sb kg^{-1})	35^{BM}, 10^{LN} 41^{BM}, 38^{LN}	PAR (3.5 mg kg^{-1}) + MEG (20 mg Sb kg^{-1})	36^{BM}, 60^{LN}	

[a]Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests.
[L], Liver; [BM], bone marrow; [LN], lymph node; [S], spleen; MTF, miltefosine; MEG, meglumine antimoniate; PAR, paromomycin; LPV, lopinavir. ¨No parasite was detected.

(PAR) and MTF. Paromomycin and meglumine antimoniate (MEG) were combined to treat dogs with VL (Tables 2 and S3).

Intraperitoneal ($n = 12$, 48%) and oral ($n = 6$, 24%) routes were mainly used for drug administration. Intramuscular, subcutaneous and intravenous routes were reported in 24% of the studies. Only 1 study did not report these data. Most drugs were administered daily (55.56%, $n = 10$) or in a single dose (16.77%, $n = 3$). Two studies administered the drugs in alternate days (11.11%), and 2 studies twice a week (11.11%). Only 1 study chose to administer the drugs twice a day (5.56%). Most of the studies ($n = 14$, 71.42%) used doses below than those recommended for monotherapy (subdoses), to reduce the risk of toxicity (Table 3).

Main outcomes

The main outcomes were shown in Tables 2, 3 and Fig. 2. Preclinical studies demonstrated that combination chemotherapy is effective for VL treatment. In most studies, parasitism was used as a primary outcome to assess the success of antiparasitic chemotherapy. Thus, in the entire dataset with 14 studies, 100% reported a significant reduction in organ parasitism, especially in spleen ($n = 11$, 44%), liver ($n = 11$, 44%) and bone marrow ($n = 3$, 12%). Compared to monotherapy, the improved parasite clearance achieved from drug combination was often associated with the induction of a protective Th1 immunological response, which was mainly evidenced by IL-12, IL-6, TNFα, IFN-γ and IgG2 upregulation (Haldar *et al.*, 2009; Shakya *et al.*, 2012*a*, 2012*b*; Bhattacharjee *et al.*, 2015). Conversely, combined pharmacological regimens were associated with an attenuated production of Th2 and/or Treg cytokines, such as IL-10, IL-4 or TGFβ upregulation (Haldar *et al.*, 2009; Shakya *et al.*, 2012*a*, 2012*b*; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017). In addition, beneficial antiparasitic effects were associated with marked modulation of redox metabolism, especially attributed to upregulation of

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	Target organs	Control monotherapy (dose)	Parasitism suppression (%)	Combination chemotherapy (dose)	Parasitism suppression (%)	Drug interaction[a]
Hamster model						
(Kar et al., 1993) [5]8-day model [6]1-month model	Spleen	SSG (100 mg Sb kg^{-1}) SSG (50 mg Sb kg^{-1}) PET (8 mg kg^{-1}) ALO (15 mg kg^{-1}) TAA (200 mg kg^{-1}) TAA (400 mg kg^{-1})	$^{5}(-)^{S}/^{6}72^{S}$ $^{5}30^{S}/^{6}35^{S}$ $^{5}7^{S}/^{6}20^{S}$ $^{5}18^{S}/^{6}22^{S}$ $^{5}62^{S}/^{6}73^{S}$ $^{5}98^{S}/^{6}99^{S}$	SSG (50 mg Sb kg^{-1}) + TAA (200 mg) SSG (50 mg Sb kg^{-1}) + TAA (400 mg) PET (8 mg kg^{-1}) + TAA (200 mg) PET (8 mg kg^{-1}) + TAA (400 mg) ALO (15 mg kg^{-1}) + TAA (200 mg) ALO (15 mg kg^{-1}) + TAA (400 mg) SSG (50 mg Sb kg^{-1}) + ALO (15 mg kg^{-1}) SSG (100 mg Sb kg^{-1}) + ALO (15 mg kg^{-1})	$^{5}87^{S}/^{6}98^{S}$ $^{5}98^{S}/^{6}100^{¨S}$ $^{5}90^{S}/^{6}98^{S}$ $^{5}99^{S}/^{6}100^{¨S}$ $^{5}79^{S}/^{6}97^{S}$ $^{5}99^{S}/^{6}100^{¨S}$ $^{5}(-)/^{6}45^{S}$ $^{5}(-)/^{6}89^{S}$	Synergistic
(Corral *et al.*, 2014)	Liver Spleen	AMB (5 mg kg^{-1}) AMB (1 mg kg^{-1}) ALL (5 mg kg^{-1})	90.6^{L}, 94.5^{S} $\sim70^{L}$, $\sim70^{S}$ $(-)^{L}$, $(-)^{S}$	AMB (1 mg kg^{-1}) + ALL (5 mg kg^{-1})	$100^{¨}$, 96.5^{S}	Additive
(Hendrickx *et al.*, 2017)	Liver Spleen Bone marrow	MTF (10 mg kg^{-1}) MTF (20 mg kg^{-1}) MTF (40 mg kg^{-1}) PAR (180 mg kg^{-1}) PAR (350 mg kg^{-1})	18^{L}, 54^{S}, 48^{BM} 80^{L}, 94^{S}, 76^{BM} 95^{L}, 99^{S}, 86^{BM} 79^{L}, 64^{S},0^{BM} 85^{L}, 74^{S}, 84^{BM}	MTF (20 mg kg^{-1}) + PAR (350 mg kg^{-1}) MTF (10 mg kg^{-1}) + PAR (180 mg kg^{-1})	99^{L}, 99^{S}, 98^{BM} 97^{L}, 96^{S}, 88^{BM}	Indifferent

[a]Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests.
[L], Liver; [BM], bone marrow; [LN], lymph node; [S], spleen; AMB, amphotericin B; ALL, allicin; ALO, allopurinol; PAR, paromomycin; MTF, miltefosine; PET, pentamidine; MEG, meglumine antimoniate; TAA, trans-aconitic acid. ¨No parasite was detected. (–) Not reported or evaluated. [5],[6]: Study evaluated the drug combination in different models of infection.

Table 3. Effective drug combinations able to induce an efficient parasite clearance in different animal models of visceral leishmaniasis

	Control monotherapy (dose)	Effective combination chemotherapy (dose)	Subdose	Main outcomes[a]
Mice model				
(Carter *et al.*, 2003)	BSO (34 mg kg^{-1}) SSG (74 or 282 mg SB^{V} kg^{-1})	BSO (34 mg kg^{-1}) + SSG (74 mg SB^{V} kg^{-1})	Yes	↑ Reduced and total glutathione ↑ Drug efficacy in subdoses ↓ Efficacy in clear parasitism in liver and bone marrow ↓ Efficiently against resistant strain
(Shakya *et al.*, 2012*a*)	Pam3Cys (100 μg) MTF (2.5, 5 or 20 mg kg^{-1})	PAM3Cys (100 μg) + MTF (5 mg kg^{-1})	Yes	↑ NO, ROS, H_2O_2; ↑ drug efficacy in subdoses; ↑ phagocytic index ↑ IL-12, TNFα, IFN-γ ↓ IL-6, IL-10
(Shakya *et al.*, 2012*b*)	F-TUF (60 μg) L-TUF (60 μg) MTF (2.5, 5 or 20 mg kg^{-1})	L-TUF (60 μg) + MTF (5 mg kg^{-1})	Yes	↑ TNFα, IL-12 IFN-γ ↑ NO, ROS; ↑ drug efficacy in subdoses ↑ Phagocytic index ↓ IL-10
(Bhattacharjee *et al.*, 2015)	SAG (250 mg kg^{-1}) GA (50 mg kg^{-1})	GA (50 mg kg^{-1}) + SAG (250 mg kg^{-1})	No	↑ TNFα, IL-12, IFN-γ, ↑ NO, ↓ TGF-β, IL-10, IL-4 (*in vitro* and *in vivo*) ↓ Antimony efflux ↑ Drug efficacy
(Khadem *et al.*, 2017)	CAL-101 (0.05 mg) AMB (0.1 mg kg^{-1})	CAL-101 (0.05 mg) + AMB (0.1 mg kg^{-1})	no	↓ T regulatory cells ↓ Treatment time ¨ Parasitological cure

[a]Main outcomes compared to the group treated with monotherapy.
H_2O_2, Hydrogen peroxide; IFN-γ, interferon gamma; IL, interleukin; NO, nitric oxide; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; TNF-α, tumour necrosis factor alpha. Drugs: BSO, buthionine sulfoximine; SSG, sodium stibogluconate; PAM3cys, N-palmitoyl-S-(2, 3-bis (palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride; F-TUF, free-tufisin; L-TUF, lipo-tufisin; MTF, miltefosine; SAG, sodium antimony gluconate; GA, glycyrrhizic acid; CAL-101, p110δ-specific pharmacological inhibitors; AMB, amphotericin B. ¨No parasite was detected.

reactive oxygen (ROS) and nitrogen species (RNS) biosynthesis (Haldar *et al.*, 2009; Shakya *et al.*, 2012*a*, 2012*b*; Bhattacharjee *et al.*, 2015), and increase of hepatic and splenic and reduced total glutathione levels (Carter *et al.*, 2003). Improved cellular parameters, such as macrophage phagocytic ability and T-cell proliferation, were also associated with a better parasitological control in animals treated with combination chemotherapy than monotherapy (Haldar *et al.*, 2009; Shakya *et al.*, 2012*a*, 2012*b*; Khadem *et al.*, 2017). Improved score and clinical signs (i.e. decrease of hematochemical parameters, cutaneous alterations,

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	Control monotherapy (dose)	Effective combination chemotherapy (dose)	Subdose	Main outcomes[a]
Mice model				
(Joice *et al.*, 2017)	POS (7.5, 15, 30 mg kg^{-1}) DB766 (19, 38, 75 mg kg^{-1}) KET (7.5, 15, 30 mg kg^{-1}) MTF (10 mg kg^{-1})	DB766 (45 mg kg^{-1}) + KET (18 mg kg^{-1}) DB766 (75 mg kg^{-1}) + POS (30 mg kg^{-1})	Yes	↑ Liver concentrations and half-lives of DB766 and POS ↑ Drug efficacy
Rebello *et al.*, 2019	MTF (3.85, 7.2, 15.4 mg kg^{-1}) LPV (246.6 or 493.2 mg kg^{-1})	LPV (493.2 mg kg^{-1}) + MTF (7.2 mg kg^{-1}) LPV (246.6 mg kg^{-1}) + MTF (7.2 mg kg^{-1})	Yes Yes	↓ Organ weight ↑ Drug efficacy in subdoses ¨ Parasitological cure
Hamster model				
(Kar *et al.*, 1993)	SSG (50 or 100 mg Sb kg^{-1}) PET (8 mg kg^{-1}) ALO (15 mg kg^{-1}) TAA (200 or 400 mg kg^{-1})	ALO (15 mg kg^{-1}) + TAA (400 mg) SSG (50 mg Sb kg^{-1}) + TAA (400 mg) PET (8 mg kg^{-1}) + TAA (400 mg) PET (8 mg kg^{-1}) + TAA (200 mg)	No Yes No Yes	¨ Parasitological cure (1-month model) Inhibited *Leishmania* transformation, multiplication and infectivity ↑ Drug efficacy in subdoses
(Corral *et al.*, 2014)	AMB (1 or 5 mg kg^{-1}) ALL (5 mg kg^{-1})	AMB (1 mg kg^{-1}) + ALL (5 mg kg^{-1})	Yes	↑ Drug efficacy in subdoses ¨ Parasitological cure (50% of animals)
(Hendrickx *et al.*, 2017)	MTF (10, 20 or 40 mg kg^{-1}) PAR (180 or 350 mg kg^{-1})	MTF (20 mg kg^{-1}) + PAR (350 mg kg^{-1}) MTF (10 mg kg^{-1}) + PAR (180 mg kg^{-1})	Yes Yes	No cross-resistance *in vivo* or *in vitro* after repeatedly exposed ↑ Drug efficacy in subdoses ↓ Effectivity in lower doses and short treatment

[a]Main outcomes compared to the group treated with monotherapy.
Drugs: POS, posaconazole; DB766, 2,5-bis[2-(2-i-propoxy)-4-(2-pyridylimino) aminophenyl] furan hydrochloride; KET, ketoconazole; MTF, miltefosine; LPV, lopinavir; SSG, sodium stibogluconate; PET, pentamidine; ALO, allopurinol; TAA, *trans*-aconitic acid; All, allicin; AMB, amphotericin B; PAR, paromomycin. ¨ No parasite was detected.

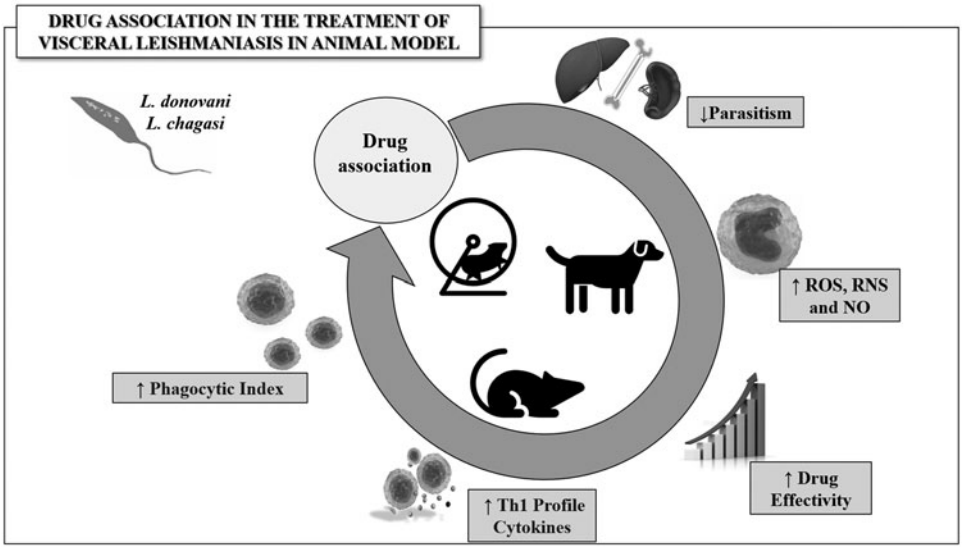

Fig. 2. Representative model of the main outcomes obtained from drug combination used in the treatment of visceral leishmaniasis. Drug combination is potentially useful in chemotherapy effectivity by upregulating the biosynthesis of reactive species and protective Th1 cytokines, stimulating proliferation and activity of immune cells, consequently reducing tissue parasitism in *Leishmania* spp.-infected animals.

anaemia, reduction in the size of spleen and/or lymph nodes and increase in weight), as well as reduction in organs hypertrophy and increase in body weight (Oliva *et al.*, 1998) and survival rates (Mutiso *et al.*, 2011) were also reported as good markers of drug combination effectiveness.

As a critical parameter in drug combination, 8 studies (57.14%) used integrated *in vitro* models to determine the nature of pharmacological interaction from specific drug combinations. From 4 studies (28.57%), 7 synergistic interactions were reported: (i) glycyrrhizic acid + sodium antimony gluconate, (ii) diminazene + chloroquine, (iii) DB766 + posaconazole (7 different combinations), (iv) DB766 + ketoconazole, (v) trans-aconitic acid + sodium stibogluconate, (vi) trans-aconitic acid + pentamidine and (vii) trans-aconitic acid + allopurinol. Additive interaction was reported in 4 studies (28.57%) with: (i) diperoxovanadate + sodium antimony gluconate, (ii) miltefosine + lopinavir, (iii) allicin + amphotericin B and (iv) DD766 + ketoconazole. Antagonism interaction was reported in 1 study using 2 different combinations with DD766 + ketoconazole. One study (7.14%) investigating paromomycin + miltefosine reported no pharmacological interaction.

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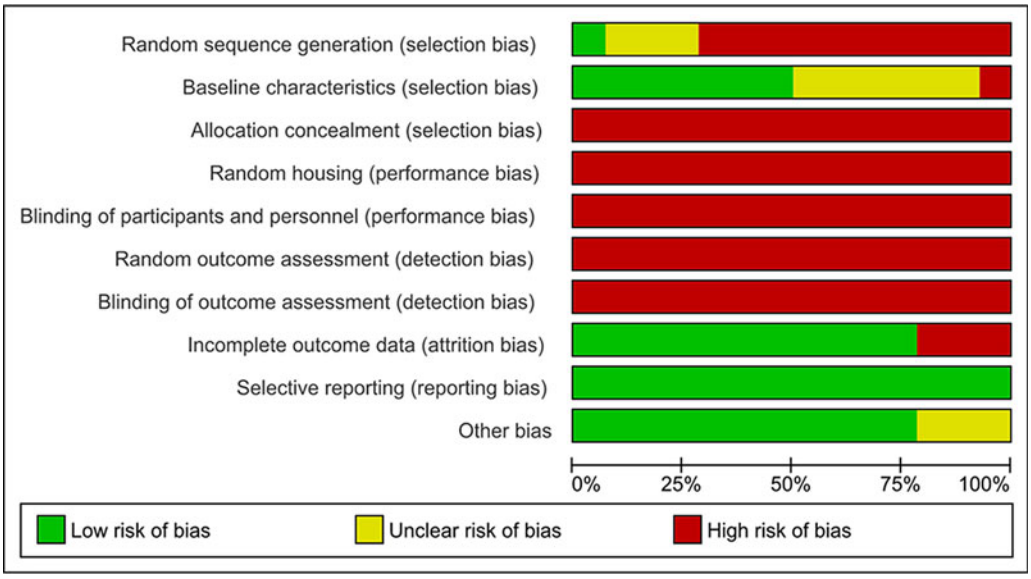

Fig. 3. Results of the risk of bias and methodological quality indicators for all studies included in this systematic review that evaluated the effect of drug combination for the treatment of visceral leishmaniasis. The items covered by the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) Risk of Bias assessment were scored with 'yes' indicating low risk of bias, 'no' indicating high risk of bias, or 'unclear' indicating that the item was not reported, resulting in an unknown risk of bias.

Risk of bias

The risk of bias analysed for all studies included in the systematic review is shown in Fig. 3. None of the studies fulfilled all methodological criteria, and a mean of 43.57 ± 6.79% items reported in SYRCLE's toll was covered from all studies reviewed. The criteria not met were predominantly under-reported, indicating an unknown or high risk of bias. The chronological analysis of all studies indicated that the risk of bias exhibited no time-dependent influence, suggesting that methodological limitations have been systematically replicated over the years of research in the area. Eight studies (57.14%) reached a below-average score (Fig. 4). Considering individually each criterion analysed, none of the studies reported information such as experimental blindness (outcome assessment, participants and personnel), allocation concealment or the criteria about randomization (housing and outcome assessment) which resulted in high risk of bias. The random sequence generator was performed in only 1 study (7.14%) (Joice *et al.*, 2017). Therefore, 3 studies (21.43%) reported randomization of animals but did not report the method used (Shakya *et al.*, 2012*a*, 2012*b*; Corral *et al.*, 2014). The baseline characteristics for animal models were reported in most studies ($n = 7$, 50%). Interestingly, 5 studies started the experiment with animals from different sexes (Carter *et al.*, 2003; Mutiso *et al.*, 2011; Shakya *et al.*, 2012*a*, 2012*b*; Mwololo *et al.*, 2015). Oliva *et al.* (1998) started the treatment using dogs from different breeds, weight, age and clinical conditions. Incomplete outcome data were adequately addressed in 11 studies (78.57%). Three studies (21.43%) unreported the parasitism data for some treatment groups (Kar *et al.*, 1993; Corral *et al.*, 2014; Khadem *et al.*, 2017). Other potential sources of bias detected were the absence of information such as strain used, route of drug administration and lineage of animals.

Discussion

In the present review, our findings are discussed considering 2 guiding questions: (1) Are combinations of antileishmanial drugs effective in the treatment of VL? and (2) What are the main chemotherapy protocols and primary research outcomes used to determine treatment effectiveness? By answering these questions, we identified that specific protocols based on drug combination can be used in realistic and rational strategies to potentiate the effectiveness of antiparasitic chemotherapy in different animal models of leishmaniasis compared to monotherapy. Interestingly, beneficial antiparasitic results were specially determined by the combination of complex pharmacological effects, especially stimulation of leucocyte replication and activity (Haldar *et al.*, 2009; Shakya *et al.*, 2012*a*, 2012*b*), upregulation of Th1 cytokines, ROS and RNS production (Haldar *et al.*, 2009; Shakya *et al.*, 2012*a*, 2012*b*; Bhattacharjee *et al.*, 2015), attenuation of the parasitic load and increase in the cure rates of infected hosts (Kar *et al.*, 1993; Carter *et al.*, 2003; Haldar *et al.*, 2009; Shakya *et al.*, 2012*a*, 2012*b*; Corral *et al.*, 2014; Hendrickx *et al.*, 2017; Khadem *et al.*, 2017; Joice *et al.*, 2017; Rebello *et al.*, 2019).

From our search strategy, we identified that studies investigating combination chemotherapy were mainly concentrated in 5 countries, such as Bangladesh, Brazil, Ethiopia, India and Sudan. Considering that VL exhibits a broad geographic distribution in 98 countries (Alvar *et al.*, 2012; WHO, 2016), this finding indicates that drug combination is not yet widely explored for VL, although studies in the field have expanded in the last 2 decades. However, the countries in which the studies were developed are consistent with endemic areas for leishmaniasis, since VL cases are especially concentrated in Latin America, Africa and the Middle East (Alvar *et al.*, 2012; Singh *et al.*, 2016; WHO, 2016). In these areas, social and environmental conditions such as humidity, temperature, rainfall, poverty, hygiene habits and limited access to health services are favourable to vector spread, making it difficult to control the disease (Oryan and Akbari, 2016). Interestingly, most of the identified studies originated from India, which is the world leader in VL cases (Alvar *et al.*, 2012; WHO, 2016). Especially in this country, the governmental strategies associated with combination chemotherapy have been successfully integrated to clinical VL management (WHO, 2015; Oryan and Akbari, 2016). Despite the high incidence of VL in South America, only 1 study was identified (Rebello *et al.*, 2019). This finding is still poorly understood, although it is potentially related to limited investments in research and development,

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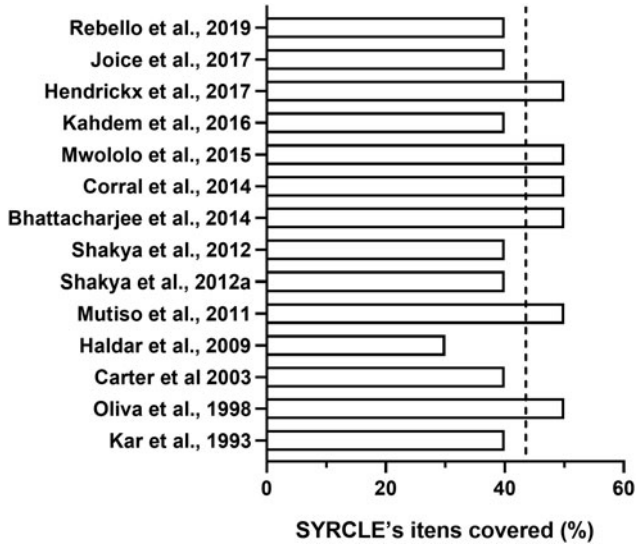

Fig. 4. Analysis of the risk of bias in each study included in the systematic review. Based on the SYRCLE's risk of bias tool for animal studies. The dotted line indicates the average score obtained for all studies reviewed.

reinforcing the neglected characteristic of this tropical disease (Hotez *et al.*, 2008; Lindoso and Lindoso, 2009). A smaller number of studies were also derived from Italy, Belgium, Spain, UK, Canada and USA. In these countries, VL cases have been frequently associated with exotic *Leishmania* species, which are introduced in these areas by humans and dogs infected and that come from endemic countries (Pérez-Ayala *et al.*, 2009).

Despite geographical divergence of the studies reviewed, the animal model choice (i.e. BALB/c mice and golden hamster) reflected a convergent research perspective. The genetic similarity from mice inbred strains (i.e. BALB/c and C57BL/6), its high reproductive efficiency and wide availability of analytical tools make these animals attractive models for preclinical research (Loria-Cervera and Andrade-Narvaez, 2014). Although extensively used (Carter *et al.*, 2003; Haldar *et al.*, 2009; Mutiso *et al.*, 2011; Shakya *et al.*, 2012*a*, 2012*b*; Mwololo *et al.*, 2015; Khadem *et al.*, 2017; Joice *et al.*, 2017), *Leishmania*-infected BALB/c mice presented limitations associated with disease development. Accordingly, these animals exhibit a natural profile of parasite resistance and, therefore, they do not represent the most adequate model to simulate human disease (Loeuillet *et al.*, 2016; McFarlane *et al.*, 2019). On the other hand, the hamster was reported as the best preclinical model for VL in 3 reviewed studies (Melby *et al.*, 2001; Loria-Cervera and Andrade-Narvaez, 2014). Characteristically, these animals exhibit immunopathological similarities of human VL, especially the limited ability in controlling parasites replication in target organs (i.e. liver, spleen and bone marrow) despite a strong Th1 response. Thus, the hamster infection culminates with the typical development of hepatosplenomegaly and hypergammaglobulinemia (Melby *et al.*, 2001; Loria-Cervera and Andrade-Narvaez, 2014). However, the applicability of hamsters is still limited due the scarcity of molecular tools available to investigate the immunological mechanisms associated with resistance or susceptibility to infection by *Leishmania* spp. (Gupta and Nishi, 2011; Loria-Cervera and Andrade-Narvaez, 2014). This limitation has been also observed in the dog model, which was reported in only study. Dogs are a realistic VL model (Quinnell and Courtenay, 2009), especially considering that these animals are natural reservoirs of *Leishmania* spp., providing a more accurate preclinical understanding of the disease immunopathology with translational potential (Loria-Cervera and Andrade-Narvaez, 2014). The limited use of dogs is not surprising, as they are generally associated with high animals cost for acquisition and maintenance, need of large facilities, and more restrictive ethical requirements compared to preclinical studies with rodent models (Loria-Cervera and Andrade-Narvaez, 2014).

In addition to the preclinical model, animals' age and sex are still highlighted as potential factors influencing the evolution of parasitic infections. There are consistent disagreements about the ideal age in animal models of *Leishmania* spp. infection. While some studies reinforce that younger animals are more susceptible to infection (Müller *et al.*, 2008; Boldizsar *et al.*, 2010; Lockard *et al.*, 2019), others pointed that extreme of ages favours the infection due to the curvilinear profile of activation and effectiveness of the innate and acquired immune responses throughout the host life cycles in animals and humans (Boldizsar *et al.*, 2010; Fuentes *et al.*, 2017). As the animals' age was often under-reported, the influence of this parameter on the immune response and on the results of antiparasitic chemotherapy cannot be accurately determined, requiring further investigation. Unlike age, the animals were consistently reported in most studies reviewed, which used female and male animals in a homogeneous proportion. There is evidence that sex hormones can interact with leucocytes such as monocytes/macrophages and lymphocytes, determining idiosyncratic immune patterns in male and female organisms (Snider *et al.*, 2009; Bhatia *et al.*, 2014; Kovats, 2015). Accordingly, differential immunosuppressive effects are potentially attributed to testosterone and progesterone levels, which can differentially downregulate NFκB cell signal pathway, cytokines production, NK cells and macrophages activity (D'Agostino *et al.*, 1999; Snider *et al.*, 2009; Bhatia *et al.*, 2014). Previous studies also indicated that increased levels of these steroidal hormones were associated with a more intense production of Th2/Treg effectors such as IL-4 and IL-10 (Piccinni, 2000; Snider *et al.*, 2009), which are recognized by increased host susceptibility to VL (Shakya *et al.*, 2012*b*). From this physiological perspective, previous studies indicated that male hamsters infected with are more susceptible to *L. donovani* infection, exhibiting higher parasite load compared to female counterparts. Thus, male animals are suggested as a more appropriate preclinical model of leishmaniasis, an aspect also related to lower hormonal variability (Travi *et al.*, 2002; Lockard *et al.*, 2019).

Alongside the characteristics of the animal model, genetic and phenotypic variability of *Leishmania* strains also exerts a marked influence on host–pathogen interaction, time-course of infection and pathological outcomes (Loeuillet *et al.*, 2016; Samarasinghe *et al.*, 2018). From the studies reviewed, VL was especially induced by *L. donovani* MHOM/KE/82/LRC-L445/NLB065 and *L. infantum* MHOM/MA/67/ITMAP263 strains, which proved to be infective and pathogenic. In addition, parasite strains (i.e. *L. donovani* pentavalent antimonial-resistant MHOM/IN/1989/GE1) with recognized pharmacological resistance to classical leishmanicidal drugs (i.e. pentamidine, sodium antimony gluconate, sodium stibogluconate) were used (Haldar *et al.*, 2009; Bhattacharjee *et al.*, 2015). Interestingly, these strains were intentionally used in realistic strategies to identify more efficient chemotherapeutic strategies to overcome drug resistance observed in several cases of VL (Haldar *et al.*, 2009; Bhattacharjee *et al.*, 2015). In preclinical models, the parasite inoculum is additionally relevant and must be carefully delimited. Accordingly, the inoculum size can exert a marked impact on the parasite load, tissue parasitism, immunological sensitization, infection severity and mortality rates of the infected host (Loeuillet *et al.*, 2016). In the studies reviewed, the use of medium (1×10^6) or higher (1×10^8) parasite inoculum was effective in inducing a marked parasitism in target organs (i.e. liver, spleen and bone marrow), which was consistent with the human infection (Rolão *et al.*, 2004; Oliveira *et al.*, 2012). There is evidence that poorly sized

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inoculum (i.e. 10^3 to 10^4 parasites) may be inadequate to induce VL, since Th1 immunological effectors can resolve the infection resolution before relevant pathological manifestations develop (Kaur *et al.*, 2008; Oliveira *et al.*, 2012). Conversely, high inoculum ($\cong 10^7$ parasites) can trigger an exacerbated Th2 immune response, which aggravates tissue parasitism and infection severity (Rolão *et al.*, 2004; Kaur *et al.*, 2008). When evaluating the parasite load induced by different inoculation routes, Kaur *et al.* (2008) concluded that subcutaneous is less efficient than intradermal, intraperitoneal and intracardiac routes to induce liver parasitism. In this sense, intracardiac and intraperitoneal routes were used in 80% of the studies reviewed. These routes were consistent with the development of a more prominent Th2 phenotype, which was suitable to stimulate tissue parasitism and a persistence infection (Mukherjee *et al.*, 2003).

From the 14 studies investigating the *in vivo* models of VL described, 7 studies also evaluated drug combinations *in vitro*. Most studies were consistent in reporting a potent leishmanicidal effect *in vitro* (Kar *et al.*, 1993; Mutiso *et al.*, 2011; Bhattacharjee *et al.*, 2015; Mwololo *et al.*, 2015; Hendrickx *et al.*, 2017; Joice *et al.*, 2017), and only 1 study indicated cytotoxicity on host cells (Rebello *et al.*, 2019). According to Huang *et al.* (2019), *in vitro* studies are relevant to investigate potential drug interactions. Thus, the combination of drugs with additive and especially synergistic effects generally results in better therapeutic outcomes compared to monotherapy in preclinical models (Huang *et al.*, 2019). In this perspective, 23 drugs administered in 40 different combinations were identified from all studies reviewed. These combinations were especially based on the following drugs: (i) BSO + SSG (Carter *et al.*, 2003); (ii) PV6 + SAG (Haldar *et al.*, 2009); (iii) DIM + ART (Mutiso *et al.*, 2011); (iv) Pam3Cys + MTF (Shakya *et al.*, 2012*a*); (v) TUF + MTF (Shakya *et al.*, 2012*b*); (vi) GA + SAG (Bhattacharjee *et al.*, 2015); (vii) DIM + CHQ (Mwololo *et al.*, 2015); (viii) CAL-101 + AMB (Khadem *et al.*, 2017); (ix) DB766 + POS or DB766 + KET (Joice *et al.*, 2017); (x) MTF + LPV (Rebello *et al.*, 2019); (xi) PAR + MEG (Oliva *et al.*, 1998); (xii) SSG + TAA, ALO + TAA, PET + TAA and SSG + ALO (Kar *et al.*, 1993); (xii) AMB + ALL (Corral *et al.*, 2014) and (xiv) MTF + PAR (Hendrickx *et al.*, 2017).

Based on the results of parasite control compared to antiparasitic monotherapy, superior therapeutic responses were obtained from the following combination strategies: (i) BSO (34 mg kg^{-1}) + SSG (74 mg SBv kg^{-1}) compared to SSG (282 mg SBv kg^{-1}) (Carter *et al.*, 2003); (ii) PV6 (0.5 μmol 30 g^{-1}) + SAG (50 mg kg^{-1}) compared to SAG (250 mg kg^{-1}) (Haldar *et al.*, 2009); (iii) Pam3Cys (100 μg) + MTF (5 mg kg^{-1}) compared to MTF (20 mg kg^{-1}) (Shakya *et al.*, 2012*a*); (iv) L-TUF (60 μg) + MTF (5 mg kg^{-1}) compared to MTF (20 mg kg^{-1}) (Shakya *et al.*, 2012*b*); (v) GA (50 mg kg^{-1}) + SAG (250 mg kg^{-1}) compared to SAG (250 mg kg^{-1}) (Bhattacharjee *et al.*, 2015); (vi) CAL-101 (0.05 mg) + AMB (0.1 mg kg^{-1}) compared to AMB (0.1 mg kg^{-1}) (Khadem *et al.*, 2017); (vii) DB766 (45 mg kg^{-1}) + KET (18 mg kg^{-1}) compared to MTF (10 mg kg^{-1}) (Joice *et al.*, 2017); (viii) LPV (493.2 mg kg^{-1}) + MTF (7.2 mg kg^{-1}) and LPV (246.6 mg kg^{-1}) + MTF (7.2 mg kg^{-1}) compared to MTF (15.4 mg kg^{-1}) (Rebello *et al.*, 2019); (ix) PAR (3.5 mg kg^{-1}) + MEG (20 mg Sb kg^{-1}) compared to MEG (30 mg Sb kg^{-1}); (x) SSG (50 mg Sb kg^{-1}) + TAA (400 mg) or PET (8 mg kg^{-1}) + TAA (200 mg) or PET (8 mg kg^{-1}) + TAA (400 mg) and ALO (15 mg kg^{-1}) + TAA (400 mg) compared to monotherapy using SSG (100 mg Sb kg^{-1}), SSG (50 mg Sb kg^{-1}), PET (8 mg kg^{-1}) or ALO (15 mg kg^{-1}) (Kar *et al.*, 1993); (xi) AMB (1 mg kg^{-1}) + ALL (5 mg kg^{-1}) compared to AMB (5 mg kg^{-1}) (Corral *et al.*, 2014); and (xii) MTF (20 mg kg^{-1}) + PAR (350 mg kg^{-1}) or MTF (10 mg kg^{-1}) + PAR (180 mg kg^{-1}) compared to MTF (40 mg kg^{-1}) (Hendrickx *et al.*, 2017).

Interestingly, combination strategies based on MTF and antimony-based drugs (SSG, SAG and MEG) were more frequently used in all studies analysed. In general, this is a rational strategy considering a more favourable time and cost of treatment, reducing the dose (use of subdoses) and toxicity of the combined drugs, which can act through complementary ways to overcome the parasite's pharmacological resistance (Olliaro *et al.*, 2005). Despite the drug combination exhibits a theoretical potential to improve the host response to VL treatment, to find an effective dosage schedule is still a challenging task, which is not always successful. Thus, worse results than those obtained for AMB or MTF monotherapy were reported in 7 studies (Mutiso *et al.*, 2011; Shakya *et al.*, 2012*a*, 2012*b*; Mwololo *et al.*, 2015; Hendrickx *et al.*, 2017; Joice *et al.*, 2017; Rebello *et al.*, 2019). Therapeutic schemes such as PAR (3.5 mg kg^{-1}) + MEG (30 mg Sb kg^{-1}) for dog model (Oliva *et al.*, 1998), DIM (12.5 mg kg^{-1}) + ART (12.5 mg kg^{-1}) in mice (Mutiso *et al.*, 2011), and DIM (12.5 mg kg^{-1}) + CHQ (12.5 mg kg^{-1}) also applied in mice (Mwololo *et al.*, 2015) showed lower or the same effectivity achieved from the monotherapy with the reference drugs MEG, PAR or the experimental drugs used alone, such as DIM, ART or CHQ.

Considering the studies investigating the hamster model, 8 different drugs used in 11 therapeutic combinations were identified. Among these combinations, 7 were successful in reducing parasitism and increasing chemotherapy effectiveness when administered in subdoses (Kar *et al.*, 1993; Corral *et al.*, 2014; Hendrickx *et al.*, 2017). In these studies, it was reported that drug combination was also effective in inhibiting parasite multiplication, transformation and infectivity (Kar *et al.*, 1993), as well as achieving parasitological cure (Kar *et al.*, 1993; Corral *et al.*, 2014) without cross-resistance *in vivo* or *in vitro* after repeated exposures to combined treatment (Hendrickx *et al.*, 2017). This is a remarkable finding, especially considering that monotherapy strategies currently prescribed to treat VL have been associated with parasite cross-resistance to alternative or second-line leishmanicidal drugs, especially in endemic areas (Rijal *et al.*, 2013). Thus, the combination of drugs becomes relevant as a safe and effective alternative to reduce the dose and toxicity of the treatment, ensuring the desirable antiparasitic efficacy (Sundar *et al.*, 2011; Hendrickx *et al.*, 2017). In this since, MEG and PAR coadministration was effective in reducing parasite load in the liver and bone marrow in naturally infected dogs, although this combination did not induce parasitological cure (Oliva *et al.*, 1998). However, by attenuating disease severity, MEG plus PAR cannot be disregarded as an alternative combined treatment for natural canine leishmaniasis (Oliva *et al.*, 1998). Although this experimental model has been little used, naturally infected dogs of varying breeds are excellent tools to investigate the therapeutic efficacy of drug combinations for VL. Accordingly, the variable genetic and immunological background modulates disease evolution and increases the heterogeneity of the research outcomes, characteristics that bring this model closer to the epidemiological reality associated with the domestic cycle of VL transmission (Quinnell *et al.*, 2003; de Vasconcelos *et al.*, 2019).

In addition to the hamster model, 17 drugs administered in 28 different combinations were identified in VL mice models. In general, most studies obtained better therapeutic effects from drug combinations than monotherapy. Accordingly, the beneficial effects achieved from combined therapy were mainly associated with the improvement of the following parameters: half-life or drug retention (Bhattacharjee *et al.*, 2015; Joice *et al.*, 2017), subdoses efficacy (Carter *et al.*, 2003; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017; Joice *et al.*, 2017; Rebello *et al.*, 2019), immunomodulation (Shakya *et al.*, 2012*a*, 2012*b*; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017) and activation of oxidative defences (Carter *et al.*, 2003; Shakya *et al.*, 2012*a*, 2012*b*). In addition,

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parasitological cure was achieved in mice models treated with CAL-101 (0.05 mg) + AMB (0.1 mg kg^{-1}) (Khadem *et al.*, 2017), and LPV (493.2 mg kg^{-1}) + MTF (7.2 mg kg^{-1}) (Rebello *et al.*, 2019). More than two-thirds of the studies reviewed reported low toxicity of the new drug combinations, downregulation of Th2/Treg cytokines (i.e. IL-4/IL-10) and upregulation of antiparasitic effectors, especially ROS and RNS and Th1 cytokines (i.e. IFN-γ, IL-12 and TNF). Thus, success in VL treatment was especially associated with the development of a more effective Th1 phenotype, which potentiated the ROS and RNS production and the elimination of intracellular amastigotes by macrophages (Kaur *et al.*, 2008; Khadem *et al.*, 2017). Considering the analysed pathological outcomes, it becomes evident that the reviewed studies direct drug combinations to stimulate the Th1 phenotype, a rational strategy considering that this phenotype is associated with greater host resistance against *Leishmania* spp. infection (Haldar *et al.*, 2009; Mutiso *et al.*, 2011; Shakya *et al.*, 2012*a*, 2012*b*; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017). In fact, the immunomodulatory effects of drug combinations seem to be a convergent mechanism associated with the improvement of leishmanicidal defences in mice (Musa *et al.*, 2010; Shakya *et al.*, 2012*a*, 2012*b*). Thus, current evidence is consistent in demonstrating that curing VL depends simultaneously on the direct ability to kill the parasite and a protective immunological profile, aspects that were simultaneously stimulated in different pharmacological combinations used such as AMB + CAL (Khadem *et al.*, 2017), MTF + TUF (Shakya *et al.*, 2012*b*), MTF + PAM3Cys (Shakya *et al.*, 2012*a*) and SAG + GA (Bhattacharjee *et al.*, 2015).

Considering a critical interpretation of the evidence, the objective assessment of methodological quality indicated important elements of bias in the reviewed studies. Even considering the specificities of each research design in the context of the bias analysis, no study fulfilled all methodological criteria, with an average of 43.57% fulfilled criteria. In addition, the studies presented variable methodological score without a temporal influence (year of publication), indicating that elements of bias are systematically replicated in this area of parasitological research, despite methodological advances and the greater availability of more sensitive and specific analytical tools. Surprisingly, over half of the essential criteria to be reported in *in vivo* animal studies were neglected. There is no doubt that under-reported aspects such as randomization, allocation concealment and complete description of research results undermine the reproducibility, internal and external validity of the reviewed studies, limiting the reliability research evidence. Methodological bias analysis corroborated the low quality of research reports, showing high or unknown risk of bias for most studies and categories evaluated. In general, the description of baseline characteristics (characterization of models and experimental conditions) represented the criteria best performed by studies with murine models. However, studies using dogs had obvious limitations in describing these baseline characteristics. Considering that naturally infected dogs were used, the difficulty to delimit the aspects related to race, sex, weight, age and time of infection is justifiable, representing a methodological limitation inherent to this preclinical model. However, it is imperative that experimental models artificially constructed to simulate human VL overcome the bias factors previously identified from current scientific evidence. In this sense, by mapping the risk of bias in all investigated studies, this review provides objective support to delimit further studies with greater methodological rigor, providing unequivocal evidence on the relevance and effectiveness of drug combinations for VL treatment.

From this systematic review, we identified that despite exhibiting some risk of methodological bias, current evidence indicates that the combination of drugs with different mechanisms of action is potentially relevant to treat VL. In general, combinations based on MTF + CAL-101, MTF + LPV, AMB + ALL, AMB + PAR, TAA + SSG, TAA + ALO and TAA + PET achieved better therapeutic effects compared to monotherapy with the currently prescribed reference leishmanicidal drugs, such as SAG, MEG and SSG. From a mechanistic point of view, the improved therapeutic effects induced by the pharmacological associations were achieved by the combination of direct parasitic toxicity and the upregulation of immunological effectors linked to the Th1 protective phenotype, which increases the host's natural defences against infection by *Leishmania* spp. By acting in an additive or synergistic way, drug combinations can improve the management of VL, reducing the costs, doses, time and adverse effects associated with the treatment of this infection. Thus, drug combination offers a realistic opportunity to overcome parasitic resistance to leishmanicidal chemotherapy, alleviating infection severity and increasing the parasitological cure rates in *Leishmania* spp.-infected hosts. However, combinations based on SSG + ALO, PAR + MEG, DB766 + POS, DB766 + KET, DIM + CHQ, MTF + Pam3Cys, MTF + TUF, PV6 + SAG and DIM + ART did not show additional therapeutic benefits compared to monotherapy with AMB and MTF. Thus, drugs used in combination strategies must be carefully and rationally delimited, as they can trigger similar or even worse effects than monotherapy, characterizing relative or absolute contraindications to treat VL.

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