International Journal of Antimicrobial Agents 60 (2022) 106612

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Review

Models for cytotoxicity screening of antileishmanial drugs: what has been done so far?

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ARTICLE INFO

Article history:
Received 15 December 2021
Accepted 14 May 2022

Editor: Dr Stephane Ranque

Keywords:
Antileishmanial assay
In vitro cytotoxicity
Leishmania
Macrophage

ABSTRACT

A growing number of studies have demonstrated the in vitro potential of an impressive number of antileishmanial candidates in the past years. However, the lack of uniformity regarding the choice of cell types for cytotoxicity assays may lead to uncomparable and inconclusive data. In vitro assays relying solely on non-phagocytic cell models may not represent a realistic result as the effect of an antileishmanial agent should ideally be presented based on its cytotoxicity profile against reticuloendothelial system cells. In the present review, we have assembled studies published in the scientific literature from 2015 to 2021 that explored leishmanicidal candidates, emphasising the main host cell models used for cytotoxicity assays. The pros and cons of different host cell types as well as primary cells and cell lines are discussed in order to draw attention to the need to establish standardised protocols for preclinical testing when assessing new antileishmanial candidates.

1. Leishmaniasis: general aspects and current chemotherapy

Leishmaniasis is a disease caused by more than 20 species of protozoan parasites belonging to the genus *Leishmania*. It is estimated that 350 million people live in risk areas worldwide and there are approximately two million new cases of leishmaniasis per year [1]. Female sandflies are responsible for transmission of the parasite between invertebrate and mammalian hosts. During the blood meal, amastigotes are ingested by Phlebotominae sandflies and transform into promastigotes in the insect's gut, while some of them accumulate in the stomodeal valve as metacyclic promastigotes. During a new blood meal, parasites are inoculated into the mammalian host and infect mononuclear phagocytes, mainly macrophages. Within these cells, *Leishmania* remains in parasitophorous vacuoles as amastigotes [2–4].

Leishmaniasis disease has different clinical forms, varying from asymptomatic lesions to disfiguring cutaneous leishmaniasis (CL) and fatal visceral leishmaniasis (VL). The latter form occurs due to the dissemination of infected macrophages through the reticuloendothelial system resulting in hepatosplenomegaly, thrombocytopenia, hypoalbuminemia and anaemia [5], often leading to death if the patient remains untreated. In East Africa and India, post-kala-azar dermal leishmaniasis may develop after VL manifestation [4,5]. Dermotropic strains cause CL, characterised by a spectrum of clinical manifestations including localised cutaneous leishmaniasis, diffuse cutaneous leishmaniasis, disseminated leishmaniasis and mucocutaneous leishmaniasis. Diffuse cutaneous leishmaniasis is characterised by the appearance of multiple non-ulcerated papular lesions, while mucocutaneous leishmaniasis causes destructive lesions on the lips, nasal septum and palate [4,6,7].

In general, drugs available for the treatment of leishmaniasis are toxic and require parenteral administration, which may lead to poor patient adherence. In addition to pentavalent antimonials, available in two formulations meglumine antimoniate and sodium stibogluconate, amphotericin B and its liposomal formulation, and miltefosine, there are also other drug options including pentamidine, paromomycin, azoles and allopurinol [8,9]. In the Americas, meglumine antimoniate is the drug of choice for the treatment of VL and CL [10]. Amphotericin B has been successfully used against VL, despite its toxicity when administered in the deoxycholate free form. The liposomal formulation is less toxic and more effective, and although being more expensive, it is considered the drug of choice for VL in many countries [10,11]. Additionally, the use of aromatic diamidine pentamidine has shown diminished effectiveness in the last decades, especially in India [12,13]. Paromomycin, a broad-spectrum aminoglycoside antibiotic, has been used topically for New World CL with very comparable results to those observed for parenteral antimonials [14,15]. The drawbacks of this

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https://doi.org/10.1016/j.ijantimicag.2022.106612
0924-8579/

drug, however, are related to the relevant side effects that involve vestibular, cochlear and renal toxicity [11]. One of the most recent advances in the chemotherapy of leishmaniasis is the use of miltefosine, an orally administered phosphatidylcholine analogue developed as an antitumour compound that successfully replaced pentavalent antimonials in the Indian subcontinent despite disadvantages such as prolonged half-life and teratogenic effects [16].

Notably, the Drugs for Neglected Diseases *initiative* (DND*i*) has evaluated the impact of clinical trials based on the association of reference drugs [17]. For example, in patients with VL in India, combination therapy has been recommended, for which a single liposomal dose of amphotericin B plus miltefosine or a single dosage of liposomal amphotericin B plus paromomycin proved to be effective [18,19].

Given the absence of available vaccines for humans and regarding the aforementioned obstacles in leishmaniasis chemotherapy, it remains mandatory to evaluate the efficacy of more affordable drugs with lower toxicity. Several approaches have been used in the search for new leishmanicidal compounds, but scientific and non-scientific bottlenecks delay successful pipelines towards the finding of new drugs [17]. Identification of potential antileishmanial molecules is generally initiated by characterisation of their in vitro activity, using direct incubation tests mainly directed against the promastigote stage, followed by assays against the intracellular amastigote form. However, there is still a lack of consensus in the literature on the ideal parameters required to evaluate toxicity profiles for host cells, especially when choosing the most appropriate cell type(s) [9,17].

2. Cytotoxicity assays and the search for new leishmanicidal agents: what has been done for the last years?

In vitro cell culture assays are very common tools with a broad range of applications often used in academic, pharmaceutical and biotechnology companies' laboratories. Several advantages can be listed, such as: (i) quick achievement of quantitative results; (ii) experimental variables that can be relatively well controlled; and (iii) the possibility of performing high-throughput screening assays, allowing systematic series of analyses of large chemical compound libraries in terms of cell toxicity [20–22].

In the context of leishmanial infections, the most common host cell belongs to the mononuclear phagocytic system [23], which is responsible for the establishment and progression of the disease. Therefore, when investigating the potential of a given antileishmanial candidate, it is expected that macrophages and/or monocytes will be the first cell types to be tested regarding its toxicity, i.e. 'cytotoxicity assay'. By using these specific host cell models, initial and crucial information is obtained to infer the concentrations to be evaluated against the intracellular amastigote stage as well as the planning of further assays. Since the 1980s, it has been proposed that ideal antileishmanial experiments should focus on the utilisation of primary isolated macrophages as host cells (mouse bone marrow-derived/peritoneal or human blood monocyte-derived macrophages) or lineages of human-monocyte transformed macrophages [17,24]. It is relevant to point out that in general, phagocytes from all human/non-human primates and rodents are susceptible to *Leishmania* infection, indicating that the choice of these biological sources is appropriate. Even so, several studies have based their investigations on cell types not susceptible to *Leishmania* infection during the steps of in vitro toxicity evaluation.

Based on this scenario, in vitro studies identifying new antileishmanial candidates published from January 2015 to July 2021 were collected from the MEDLINE/PubMed database using the following descriptors simultaneously: '*Leishmania*'; 'cytotoxic'; and 'in vitro'. Only articles in indexed journals and written in English were included in this review, totalling 175 articles (Table 1).

From this systematic review, the 175 studies listed in Table 1 show that research laboratories from different countries have been using 17 cell types, including primary and immortalised cells from distinct mammalian species (*Homo sapiens*, *Mus musculus* and *Cercopithecus aethiops*) for cytotoxicity evaluation, including not only macrophages and monocytes but also endothelial and epithelial cells, fibroblasts and melanocytes. Approximately 63% of the studies used phagocytic cells for cytotoxicity assays, while ~22% used exclusively non-phagocytic cell lineages. Both phagocytic and non-phagocytic cells were utilised in 6 studies [25–30], while 11 employed more than one type of phagocytic cells [31–41] and 3 of them used more than one type of non-phagocytic cells [42–44], in a total of 20 studies with at least two cell types tested for cytotoxicity.

Regarding the absolute number of cells employed in the cytotoxic assays, 46% of the studies used amounts raised to the 5^{th} power, followed by exponent power of 4 (26%), 3 (15%) and 6 (5.1%). Only one study used 10^7 cells and, surprisingly, 15% of these studies did not specify the cell number used in the assays. Only 7 (4.0%) of the 175 reports described using relative number of cells per mL [10^5 (1.7%), 2×10^5 (0.6%), 5×10^5 (1.1%) and 10^6 (0.6%)].

Incubation times ranged from 24 h to 168 h, for which 34%, 33% and 30% of the assays were followed by 48 h, 72 h and 24 h, respectively. Interestingly, 5.1% of the studies did not specify the incubation time. Longer periods of incubation (96 h and 120 h) were performed in 2.3% of the studies. Only in a few reports, time points included 44 h (0.6%) and 'overnight' period (0.6%).

In terms of culture cell types, primary cells differ from cell lines and both offer advantages and limitations, as detailed in Table 1. Primary cells are expected to be more genotypically stable, given their inability to reproduce without interruption. They also have the advantage of being of natural origin, but donor viability and high variation can lead to multifactorial results [217]. Besides that, the number of cells recovered from a given tissue may be an obstacle when large screenings are performed, especially when dealing with a limited number of animal sources. In this context, the experimental use of animals has been a constant concern among researchers. There has been much debate on the ethical use of in vivo models, in addition to the development of new technologies designed to avoid the excessive use of these animal models [218,219]. On the other hand, the utilisation of seeded non-dividing cells ensures the exact amount of a given molecule for a fixed number of cells during the period of incubation, allowing the comprehension of the compounds' effectiveness [105–107].

Cell lines, instead, have advantages such as virtually everlasting reproduction with facilitated laboratory maintenance and the possibility of genetic manipulation [220]. However, a disadvantage relies on the malignant nature or artificial manipulation to allow indefinite proliferation and cultivation of these cells under controlled conditions that may result in different sensitivities [221] and interactions between the target cell and surrounding cells [217]. Another undesirable aspect is that depending on the type of the immortalised lineage, it may present increased genotypic alteration (e.g. accumulation of chromosomal aberrations) after a prolonged number of cell divisions [222].

The right choice of cell line model is of utmost relevance, especially regarding biological parameters [i.e. adhesion ability, doubling time (DT) and passage number during specific propagation protocols] that must be known when planning the most appropriate incubation period. Ideally, DT, for instance, should be higher than the total incubation period of a given cytotoxicity assay to avoid the dilution factor of the compound followed by the increase in cell number over time (in this case, referred to as 'Advantages' in Table 1). Also, attention should be given to the pres-

Table 1
Cell types employed in cytotoxicity assays aiming at the search for new leishmanicidal compounds recovered from articles published from January 2015 to July 2021. Biological sources, origin, and cell types are described, with emphasis on the advantages and limitations of lineages and primary cells.

Biological source	Origin	Cell type	Advantages	Limitations	Reference
Homo sapiens					
HeLa and Parental cell line (KB) (Hela derivative)	Cervix adenocarcinoma	Epithelial	Ideal for continuous cultivation, suitable for transfection protocols. Used for testing and calibration protocols in ISO 17025 accredited laboratories. #DT∼40h (KB).	Sensitivity differences [45,46]. Relevance for *Leishmania* infection is unknown. DT>24h (Hela).	[42,47–49]
HepG2	Hepatocellular carcinoma	Epithelial-like	Suitable for transfection protocols. Continuous cultivation. DT=48-72h.	Sensitivity differences depending on the Hep cell line source [50]. Relevance for *Leishmania* infection is unknown.	[28,29,43,51–59]
HL-60*	Peripheral blood	Promyeloblast Granulocyte-like cells	It can be stored for years and recovered without effects on cell viability. DT=36-48h.	Variable sensitivity. Differentiation is dependent on the appropriate stimulus [60].	[43]
HT29	Colorectal adenocarcinoma	Epithelial	Suitable for transfection protocols. DT=1-4 days [61].	Differentiation can be modulated by the exposure to different compounds [62]. Relevance for *Leishmania* infection is unknown.	[63]
HUVEC	Umbilical vein/vascular endothelium	Endothelial	Suitable model for studying immune response to infections, wound healing, oxidative stress, and angiogenesis. It has been demonstrated that lymphangiogenesis is associated with CL control [64]. DT∼36h.	Sensitivity differences. DT varies according to the passage number. Relevance for *Leishmania* infection is unknown.	[65]
MCF-7	Mammary gland adenocarcinoma	Epithelial	Suitable for transfection protocols.	Sensitivity differences and estrogen responsiveness [66]. Relevance for *Leishmania* infection is unknown. DT∼24h.	[42,44,67–69]
MRC-5	Lung	Fibroblast	Continuous cultivation. DT>24h-5 days.	Sensitivity differences. It has been demonstrated that these fibroblasts achieve senescence after a given number of passages and cease to replicate [70]. Relevance for *Leishmania* infection is unknown.	[71–78]
PC-3	Prostate adenocarcinoma metastatic derived	Epithelial	Suitable for transfection protocols. DT>24h.	Sensitivity differences. Relevance for *Leishmania* infection is unknown.	[42,44,79]
THP-1*	Monocytic-leukemia	Monocyte	No changes in genotype and phenotype upon 25 passages. It can be stored for years and recovered without effects on cell viability [80]. Classic *Leishmania* host cell type.	Phorbol 12-myristate 13-acetate (PMA), a toxic compound, is required for macrophage-like differentiation and it must be kept in culture for long-period assays [81].	[27,30–33,53,82–94, 203]
U937*	Histiocytic lymphoma	Monocyte	It can be stored for years and recovered without effects on cell viability. Feasible for genetic manipulation and maintenance [65]. Classic *Leishmania* host cell type.	Sensitivity differences. Differentiation requires ethanol or PMA [80].	[34,35,95–99]
Mus musculus					
Bone marrow derived-macrophage (BMDM)*	Bone marrow myeloid progenitor	Macrophage	Primary cells from natural origin. Potential for data to be translated into preclinical results. Classic *Leishmania* host cell type.	Ethical approval requirement. Good yield in the number of cells (∼1-2 × 10^7 BMDM/mouse). It requires ∼1 week to differentiate followed by the addition of specific factors (rGM-CSF or GM-CSF). Possibility of contamination during the differentiation process [100].	[101–107, 136]

(*continued on next page*)

Table 1 (*continued*)

Biological source	Origin	Cell type	Advantages	Limitations	Reference
J774A.1*	Reticulum cell sarcoma	Macrophage/ Monocyte	It can be stored for years and recovered without effects on cell viability. Feasible for genetic manipulation and maintenance. Continuous cultivation and a classic *Leishmania* host cell type.	Continuous cultivation can lead to loss of the ability to generate oxidative burst [108]. Morphology may difficult *Leishmania* visualization for *in vitro* infection assay. DT∼17h.	[25,26,32–34, 36–41,54,109–135]
L929 and NCTC clone 929	Subcutaneous connective tissue	Fibroblast	Continuous cultivation. It can be used for cytotoxicity tests since it is grown on a large scale to provide GM-CSF enriched-supernatants used in the BMDMs differentiation protocol. This cell line is a suitable transfection host. DT∼30h.	Relevance for *Leishmania* infection is unknown.	[30,136–142]
LLC-1	Lewis lung carcinoma	Epithelial	It is widely used for the study of anticancer agents' mechanisms of action. DT∼21h.	Adherent and suspension culture. Relevance for *Leishmania* infection is unknown.	[143]
Peritoneal macrophage*	Peritoneal cavity resident phagocytes	Macrophage	Primary cells from mice. Data show potential to be translated into preclinical results. Classic *Leishmania* host cell type.	Short lifetime requires immediate use. Ethical approval requirement. Number of cells may be very limited ($\sim$ 0.5-1 $\times$ 10^6 cells/mouse). Yield can be enhanced after thioglycollate injection, but this process leads to the activation of immune cells [144].	[31,36–40,145–185]
RAW 264.7*	Abelson murine leukemia virus-induced tumor	Macrophage	It can be stored for years and recovered without effects on cell viability. Continuous cultivation. Feasible for genetic manipulation and maintenance. Classic *Leishmania* host cell type.	Sensitivity differences. DT∼15h.	[35,41,186–202, 204,205]
Cercopithecus aethiops					
VERO	Kidney	Epithelial	Very popular cell line that is easily maintained. DT∼24h.	Unable to secrete interferon-γ. They may show contact inhibition depending on the strain [206,207]. Infection by *L. infantum* has been described [208].	[25–27,209–216]

[#] DT, Doubling Time. It indicates the time required for doubling a cell population in size and/or number (ATCC 2021 [https://www.atcc.org]).
[*] Phagocytic cells used for cytotoxicity evaluation.

ence of stimulants [e.g. phorbol myristate acetate (PMA) utilised for THP-1 monocyte differentiation] incubated in suitable concentration ranges to avoid interference with the compound tested and optimal differentiation [81,223]. The use of THP-1 cells has been frequently employed in the search for new anti-*Leishmania* agents. The research articles presented herewith reported the addition of PMA in a variable range [10 ng/mL to 100 ng/mL (Table 1)], diverging from the findings of Park et al. for which PMA at 5 ng/mL was sufficient to induce stable differentiation without undesirable changes in gene expression [81].

One of the most challenging obstacles that affects both lineages and primary cell cultures is related to micro-organism contamination, especially mycoplasma colonisation. Mycoplasma is a prokaryotic organism, a member of the Mollicutes group with over 100 species known. These organisms are implicated in cell culture persistent contamination, being difficult to detect and eliminate [224]. It is estimated that ∼20% of cultures are contaminated by mycoplasma, a fact that may be explained by misconduct or lack of basic care while handling cell cultures. Fortunately, many diagnostic techniques are available for research groups in routine cell culture, including conventional PCR, quantitative PCR (qPCR), fluorescent in situ hybridisation (FISH) assay and enzyme-linked immunosorbent assay (ELISA), among others [224,225]. Uphoff and Drexler showed that antibiotics such as fluoroquinolones, fluoroquinolones associated with macrolide, and pleuromutilin associated with tetracycline are effective anti-mycoplasma agents [226]. However, attention must be given in this case to avoid the interference of these molecules with the cytotoxicity study model and possible synergism/antagonism effects with other co-incubated candidates.

3. Cytotoxicity assessment methods

Cytotoxicity is expressed in terms of a certain drug concentration that inhibits cell viability by 50%, referred to as the 50% cytotoxic concentration (CC_{50}). The most common methods used for cytotoxicity assessment of leishmanicidal drugs include colorimetric assays with tetrazolium salts [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) and 2-para-(iodophenyl)-3(nitrophenyl)-5(phenyl) tetrazolium chloride tetrazolium salt (INT)], resazurin-based solution employed in the lactate dehydrogenase (LDH) or alamarBlue assay, and the trypan blue exclusion assay.

The classic MTT assay involves its incubation in cell cultures that have been previously exposed to the test drug. Following internalisation by viable cells, MTT is reduced in the presence of an electron-coupling reagent to formazan. Further cell lysis is performed to quantify absorbance values [227], yielding satisfactory results in terms of sensitivity and accuracy. In terms of the LDH assay, quantification is possible due to the fact that this cytosolic enzyme catalyses the conversion of lactate to pyruvate. LDH, released into the cell culture supernatant, is measured with an enzymatic assay, which results in the conversion of INT into a coloured formazan product that is quantified spectrophotometrically. The amount of LDH present in the culture medium correlates with the number of dead cells [228]. For the alamarBlue assay, viable cell mitochondrial enzymes convert resazurin (blue) to resorufin (pink) that is detected spectrophotometrically or by fluorescence [229]. When comparing protocol steps, assays with alamarBlue can be relatively faster as they do not require a cell lysis step with detergent or dimethyl sulfoxide, as required for MTT testing, for example. The trypan blue exclusion assay is the simplest and cheapest method to quantify cell viability. In this case, cells are diluted in trypan blue and the amount of viable cells is determined by counting cells that have not acquired blue staining. A major drawback is that trypan blue is not suitable for measuring effects that do not affect the plasma membrane [230].

By evaluating the studies published in the period, 66% used the MTT assay for different cell types. Next, the alamarBlue assay was the most employed method (17%), followed by the trypan blue assay (4.5%). Interestingly, the lack of uniformity in choosing the most appropriate test is evident from studies examined in this review. One could speculate that this variable may generate different interpretations when extrapolating cytotoxicity mechanisms, either involving membrane lysis (i.e. trypan blue exclusion assay) or viability interference (i.e. MTT/LDH assay), depending on the principle of the test used. Curiously, more than one method has been used in four research articles that reported cytotoxicity results for THP-1, HepG2, J774, peritoneal macrophages and Vero cells with the MTT, XTT, resazurin, crystal violet and/or sulforhodamine B assay [27,28,31,36].

4. Choice of cytotoxicity models and implications for antileishmanial screenings

Cytotoxicity tests are of utmost importance to advance the path for the discovery of new drugs against leishmaniasis. However, evaluating cytotoxicity exclusively in cells that do not have any biological relevance in terms of *Leishmania* infection may show altered and unrealistic results. For example, the use of tumour cells is advantageous for laboratory maintenance and handling, but genetic alterations may be seen over time. In addition, cell lines and cultivation protocols vary between research groups. HeLa cells pose as a relevant model in this case. It has been found that this human epithelial cell line derived from cervix adenocarcinoma shows genotypic and phenotypic modifications in different laboratories [231]. Together, these factors prevent proper independent reproducibility of generated results.

Besides the fact that several studies have demonstrated the interaction between *Leishmania* and non-phagocytic cells in vitro [e.g. Chinese Hamster ovarian (CHO) cells, Vero cells, 3T3-L1 fibroblast-differentiated adipocytes] [208,232–234], there is still a gap in the comprehension of the roles of non-phagocytic cells during in vivo infections, which may be explained by the limited number of exploratory studies in the field. Although the use of other non-phagocytic cell models for cytotoxic activity may contribute to the design of preclinical assays with ADMET (absorption, distribution, metabolism, excretion and toxicity) modelling, we emphasise the inclusion of phagocytes in the cytotoxic analysis as being fundamental when considering the *Leishmania* infection model, which will bring even more robust data once the selectivity index (SI = host cell 50% cytotoxic concentration/*Leishmania* half-maximal effective concentration (CC_{50}/EC_{50})] is determined. Indeed, the comparison of compound efficacy and cytotoxic potential would be greatly benefited by presenting the SI for equivalent cell models. As the data obtained with different cell models are not exclusive and can increase knowledge on the toxicity of a given candidate, it is interesting to recommend the inclusion of a complete cytotoxic activity panel covering different cell types, but with emphasis on cells from the reticuloendothelial system, lineages or primary macrophages/monocytes that should be chosen when determining SIs.

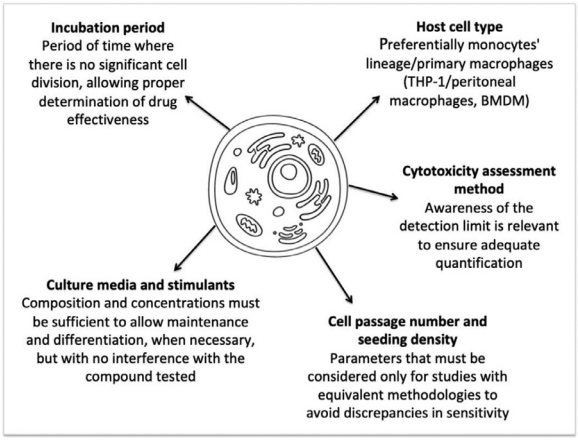

Fig. 1. Biological and technical parameters to be considered when establishing a protocol for cytotoxicity evaluation of antileishmanial candidates. BMDM, bone marrow-derived macrophages.

Notably, the Japanese Global Health Innovative Technology (GHIT) Fund has proposed criteria for new drug candidates by establishing that selectivity of a given hit should be ≥10-fold the cytotoxicity range. Regarding antileishmanial agents, a good candidate should present an EC_{50} lower than 10 μM against intracellular amastigotes [235]. Several consortium studies in partnership with the DND*i* have employed both lineages (THP-1 and RAW 264.7 macrophages) and bone marrow-derived macrophages for in vitro laboratory routine [236,237], criteria on which we are in common agreement.

Taking all into consideration, we propose that the parameters discussed in this review, and summarised in Fig. 1, must be followed in order to assure comparable results among different research groups and to promote a more homogeneous body of evidence toward the development of new anti-*Leishmania* agents.

5. Conclusions

Although we recognise that the in vitro assay focused on the cytotoxic parameter brings a series of limitations, such as preventing the assessment of the best routes for administering a given drug and aspects related to metabolisation and toxicity, the substantial amount of studies in the literature that rely exclusively on cell models as the only parameters of toxicity is still remarkable. For initial tests, this approach may still prove to be useful for the selection of candidates from large libraries, but it is undeniable that the proper choice of the model can significantly contribute to advancing the interlaboratory comparison of data and the search for hits with the potential to be directed to in vivo experiments. We reinforce the need for collective protocol guidelines to be followed by different laboratories, taking into account that human and/or rodent phagocytic cells should be routinely applied

in in vitro toxicity assays when searching for novel leishmanicidal molecules.

Ethical approval

Not required.

Sequence information

Not applicable.

Declaration of Competing Interest

None declared.

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [#2014/21129-4 (DCM), #2016/21171-6 (ACC)] and FAEPEX-PRP/Unicamp [#519.292 (DCM)]. ACC has also, in part, received funding from UK Research and Innovation via the Global Challenges Research Fund under grant agreement 'A Global Network for Neglected Tropical Diseases' [#MR/P027989/1]. MBCB and EMC are currently supported by CAPES-DS and FAPESP doctorate scholarships [#88887.487057/2020-00 and #2019/22175-3, respectively]. The authors also thank the Canva's free image bank available at www.canva.com.

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