Perifosine Mechanisms of Action in *Leishmania* Species

Atteneri López-Arencibia,^{a,b} Carmen Martín-Navarro,^b Ines Sifaoui,^c
María Reyes-Batlle,^b Carolina Wagner,^{b,d} Jacob Lorenzo-Morales,^b
Sutherland K. Maciver,^a José E. Piñero^b

Centre for Integrative Physiology, Biomedical Sciences, Edinburgh Medical School, University of Edinburgh, Edinburgh, United Kingdom^a; University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Tenerife, Canary Islands, Spain^b; Laboratoire Matériaux-Molécules et Applications, IPEST, University of Carthage, La Marsa, Tunisia^c; Cátedra de Parasitología, Escuela de Bioanálisis, Facultad de Medicina, Universidad Central de Venezuela, Venezuela^d

ABSTRACT Here the mechanism by which perifosine induced cell death in *Leishmania donovani* and *Leishmania amazonensis* is described. The drug reduced *Leishmania* mitochondrial membrane potential and decreased cellular ATP levels while increasing phosphatidylserine externalization. Perifosine did not increase membrane permeabilization. We also found that the drug inhibited the phosphorylation of Akt in the parasites. These results highlight the potential use of perifosine as an alternative to miltefosine against *Leishmania*.

KEYWORDS perifosine, *Leishmania*, activity, apoptosis-like

Leishmania species are obligate intracellular parasites that are transmitted to the mammalian host by the bites of infected sand flies. Cutaneous leishmaniasis (CL), the most common form of leishmaniasis, is a group of diseases with a varied spectrum of clinical manifestations that range from small cutaneous nodules to wide mucosal tissue destruction. Visceral leishmaniasis (VL), characterized by prolonged fever and splenomegaly, is the most severe form, and it is often fatal if untreated (1).

The World Health Organization (WHO) estimates that 1.5 million cases of CL and 500,000 cases of VL occur every year in 82 countries. CL is endemic in more than 70 countries worldwide, and 90% of cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, and Syria. VL presents in 65 countries, and the majority (90%) of these cases occur in five countries, Bangladesh, India, Nepal, Sudan, and Brazil. Estimates indicate that there are approximately 350 million people at risk for acquiring leishmaniasis, with 12 million currently infected worldwide (1).

The current available leishmanicidal treatments include pentavalent antimonials, amphotericin B, miltefosine, paromomycin, and pentamidine. These drugs can be administered alone or in combination with each other. However, these drugs are normally highly toxic, even at low doses. Furthermore, most of these treatments require several days of hospitalization because of their intravenous or parenteral manner of administration. The appearance of strains resistant to these active compounds presents a major problem for the current therapeutic measures against these parasites (2). Moreover, since there is no immediate prospect of a vaccine for leishmaniasis (3), there is an urgent need to develop novel leishmanicidal agents.

Several typical markers of mammalian apoptosis have been found in *Leishmania*, suggesting the existence of an apoptosis-like death in these organisms. The markers include cell shrinkage, nuclear chromatin condensation, DNA fragmentation, membrane blebbing, mitochondrial transmembrane potential loss, and phosphatidylserine exposure (4).

Received 4 October 2016 Returned for
modification 17 November 2016 Accepted 8
January 2017

Accepted manuscript posted online 17
January 2017

Citation López-Arencibia A, Martín-Navarro C, Sifaoui I, Reyes-Batlle M, Wagner C, Lorenzo-Morales J, Maciver SK, and Piñero JE. 2017. Perifosine mechanisms of action in *Leishmania* species. *Antimicrob Agents Chemother* 61:e02127-16. <https://doi.org/10.1128/AAC.02127-16>.

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Atteneri López-Arencibia, atlopez@ull.edu.es.

TABLE 1 Leishmanicidal activities, cytotoxicities, and SIs of the tested alkylphospholipids

Drug	Promastigote stage, IC ₅₀ (μM) (mean ± SD) (IC ₉₀ [μM]) against:		Amastigote stage, IC ₅₀ (μM) against <i>L. amazonensis</i>	SI	Cytotoxicity, CC ₅₀ (μM) against J774A.1 cells
	<i>L. amazonensis</i>	<i>L. donovani</i>			
Perifosine	3.59 ± 0.15 (4.30)	1.98 ± 0.29 (4.52)	2.82 ± 1.64	23.1	65.14
Miltefosine	6.48 ± 0.24 (9.63)	3.32 ± 0.27 (5.15)	3.12 ± 0.30	23.2	72.19

Perifosine was purchased from Cayman Chemical, and miltefosine, used as reference drug, was provided by Aeterna Zentaris. In this study, strains of *Leishmania amazonensis* (MHOM/BR/77/LTB0016) and *Leishmania donovani* (MHOM/IN/90/GE1F8R) were used. The murine macrophage J774A.1 (ATCC TIB-67) cell line was also used to evaluate host cell toxicity.

For the activity assays against the promastigote stage of *Leishmania* spp., a colorimetric assay based on alamarBlue reagent (Invitrogen, Life Technologies) was used as previously described (5).

FIG 1 (A) ATP levels of *L. amazonensis* and *L. donovani* after 24 h of incubation with the IC₉₀s of perifosine and miltefosine. (B) Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) of *L. amazonensis* and *L. donovani* after 24 h of incubation with the IC₉₀ of perifosine and miltefosine. Error bars represent the standard deviation (SD). Each data point indicates the mean of three measurements. (C) Images of *L. amazonensis* incubated with JC-1 dye after 24 h of treatment with the IC₉₀ of perifosine; images of negative controls of parasites without treatment are shown on the upper left and upper right.

FIG 2 (A) Results of phosphatidylserine exposure after 48 h of incubation with the IC_{50} s of perifosine and miltefosine. Error bars represent the standard deviation (SD). Each data point indicates the mean of three measurements. Images of *L. amazonensis* (B) and *L. donovani* (C) captured using a Tali image-based cytometer.

Activity assays against intracellular amastigotes were performed as previously described with minor modifications (6). For the toxicity assay, an LDH cytotoxicity detection kit (Roche Applied Science) was used (7). The results against the promastigote and intracellular amastigote stages are shown in Table 1, as are the cytotoxicities and selectivity indexes (SIs). The 50% inhibitory concentrations (IC_{50} s) are consistent with data obtained in previous studies (5, 8). A study by Glaser et al. (9) showed similar cytotoxicity data for miltefosine.

For measuring changes in the mitochondrial membrane potential ($\Delta\Psi_m$) of *L. amazonensis* and *L. donovani*, the JC-1 mitochondrial membrane potential assay kit (Cayman Chemical) was used. The ratio of the reading at 595 nm to the reading at 535 nm was considered the relative $\Delta\Psi_m$ value (10). The two molecules induced decreases in the $\Delta\Psi_m$ in both parasitic strains; the decrease was greater in the case of *L. amazonensis* (Fig. 1).

ATP levels were measured using the Cell Titer-Glo luminescent cell viability assay (Promega). This assay proved the strong effects of miltefosine and perifosine in decreasing ATP levels (Fig. 1). The apoptosis-like process in *Leishmania* spp. requires ATP to carry out all the involved procedures; however, it seems that ATP levels are

FIG 3 (A) Plasma membrane permeability assay results for *L. amazonensis* and *L. donovani*. Triton X-100 was added to obtain 100% permeabilized cells, and amphotericin B (10 μ M) was used as positive control. (B) Changes in the relative expression of phosphorylated kinases on *L. donovani* after 24 h of incubation with the IC₉₀ of perifosine. Error bars represent the standard deviation (SD). Each data point indicates the mean of three measurements.

maintained by either glycolysis and/or minimal mitochondrial activity to support the parasite metabolism until the conclusion of the apoptosis-like process (11).

The Tali apoptosis kit (annexin V Alexa Fluor 488 and propidium iodide) (Life Technologies) double-staining assay was performed according to manufacturer instructions using a Tali image-based cytometer. Figure 2 shows all the obtained results. Moreover, the differences in the amounts of annexin V observed between the two strains of *Leishmania* (amounts were greater in *L. amazonensis*) might have been due to differences in the amount of polysaccharides (PSs) present in the membrane of the parasites (12).

Plasma membrane permeability was measured using SYTOX Green (Molecular Probes). The results confirmed that the plasma membrane integrity was not altered in the treated parasites, at least not in the first 6 h of treatment (Fig. 3). This provided us with important data with which to distinguish between apoptosis and necrosis, considering the rupture of the plasma membrane, which is a morphological feature of necrosis (13). Also, this assay might indicate that the target of these molecules is not likely to be primarily the plasma membrane of the parasites (14).

The Human Phospho-Kinase Array 1 proteome profiler (R&D Systems) was used for the phosphorylation analysis of different kinases during the promastigote stage and

was developed by chemiluminescence using ChemiDoc XRS+ image capture (Bio-Rad). The Q-View software (Quansys Biosciences) was used to measure pixel density. All statistical analyses were performed with Sigma Plot 12.0 (Systat Software). After incubation of *L. donovani* with perifosine, one protein presented a difference in its relative expression, a decrease in the phosphorylation levels of Akt (phosphorylation of S473) necessary for its activation (Fig. 3). Miltefosine is an inhibitor of Akt in *Leishmania* (15), and perifosine also inhibits Akt protein in human cancer cells (16). These facts lead us to believe that perifosine has the same Akt-inhibiting capacity in *Leishmania*, which leads to the induction of an apoptosis-like cell death that is known to occur with miltefosine in *L. amazonensis* (17) and *L. donovani* (18).

Previous results obtained in our laboratory on alkylphospholipids showed that perifosine was superior to miltefosine and edelfosine in killing *Leishmania* promastigotes *in vitro* (5), and we also showed that perifosine was superior to other alkylphospholipids in an *in vivo* mouse model (19). We now conclude that perifosine induces an apoptosis-like process in *L. amazonensis* and *L. donovani* at very low doses, which makes perifosine a promising candidate for further study and possible therapeutic use in humans.

ACKNOWLEDGMENTS

This work was supported by the RICET grants (project RD12/0018/0012 of the programme of Redes Temáticas de Investigación Cooperativa, FIS), Spanish Ministry of Health, Madrid, Spain; PI13/00490 “Protozoosis Emergentes por Amebas de Vida Libre: Aislamiento, Caracterización, Nuevas Aproximaciones Terapéuticas y Traslación Clínica de los Resultados” from the Instituto de Salud Carlos III; and Project BIO24 “Principios activos inductores de apoptosis en la quimioterapia de tripanosomosis y leishmaniosis” (project 2016_25) from Obra Social La Caixa-Fundación CajaCanarias. A.L.-A. was funded by a grant from “Fundación Canaria Doctor Manuel Morales” and the Agustín de Betancourt Programme, and J.L.-M. was supported by the Ramón y Cajal Subprogramme from the Spanish Ministry of Science and Innovation (grant RYC-2011-08863).

REFERENCES

- World Health Organization. 2016. Leishmaniasis: background information. <http://www.who.int/leishmaniasis/en/>.
- Croft SL, Sundar S, Fairlamb AH. 2006. Drug resistance in leishmaniasis. *Clin Microbiol Rev* 19:111–126. <https://doi.org/10.1128/CMR.19.1.111-126.2006>.
- Singh B, Sundar S. 2012. Leishmaniasis: vaccine candidates and perspectives. *Vaccine* 30:3834–3842. <https://doi.org/10.1016/j.vaccine.2012.03.068>.
- Bruchhaus I, Roeder T, Rennenberg A, Heussler VT. 2007. Protozoan parasites: programmed cell death as a mechanism of parasitism. *Trends Parasitol* 23:376–383. <https://doi.org/10.1016/j.pt.2007.06.004>.
- Cabrera-Serra MG, Lorenzo-Morales J, Romero M, Valladares B, Piñero JE. 2007. *In vitro* activity of perifosine: a novel alkylphospholipid against the promastigote stage of *Leishmania* species. *Parasitol Res* 100:1155–1157. <https://doi.org/10.1007/s00436-006-0408-4>.
- Jain SK, Sahu R, Walker LA, Tekwani BL. 2012. A parasite rescue and transformation assay for antileishmanial screening against intracellular *Leishmania donovani* amastigotes in THP1 human acute monocytic leukemia cell line. *J Vis Exp* 30:pil 4054.
- Lorenzo-Morales J, Martín-Navarro CM, López-Arencibia A, Santana-Morales MA, Afonso-Lehmann RN, Maciver SK, Valladares B, Martínez-Carretero E. 2010. Therapeutic potential of a combination of two gene-specific small interfering RNAs against clinical strains of *Acanthamoeba*. *Antimicrob Agents Chemother* 54:5151–5155. <https://doi.org/10.1128/AAC.00329-10>.
- Escobar P, Matu S, Marques C, Croft SL. 2002. Sensitivities of *Leishmania* species to hexadecylphosphocholine (miltefosine), ET-18-OCH₃ (edelfosine) and amphotericin B. *Acta Trop* 81:151–157. [https://doi.org/10.1016/S0001-706X\(01\)00197-8](https://doi.org/10.1016/S0001-706X(01)00197-8).
- Glaser J, Schultheis M, Hazra S, Hazra B, Moll H, Schurig U, Holzgrabe U. 2014. Antileishmanial lead structures from nature: analysis of structure-activity relationships of a compound library derived from caffeic acid bornyl ester. *Molecules* 19:1394–1410. <https://doi.org/10.3390/molecules19021394>.
- Sifaoui I, López-Arencibia A, Martín-Navarro CM, Ticona JC, Reyes-Batlle M, Mejri M, Jiménez AI, Lopez-Bazzocchi I, Valladares B, Lorenzo-Morales J, Abderabba M, Piñero JE. 2014. *In vitro* effects of triterpenic acids from olive leaf extracts on the mitochondrial membrane potential of promastigote stage of *Leishmania* spp. *Phytomedicine* 21:1689–1694. <https://doi.org/10.1016/j.phymed.2014.08.004>.
- Mukherjee SB, Das M, Sudhandiran G, Shaha C. 2002. Increase in cytosolic Ca²⁺ levels through the activation of non-selective cation channels induced by oxidative stress causes mitochondrial depolarization leading to apoptosis-like death in *Leishmania donovani* promastigotes. *J Biol Chem* 277:24717–24727. <https://doi.org/10.1074/jbc.M201961200>.
- López-Arencibia A, García-Velázquez D, Martín-Navarro CM, Sifaoui I, Reyes-Batlle M, Lorenzo-Morales J, Gutiérrez-Ravelo Á, Piñero JE. 2015. *In vitro* activities of hexaazatrinaphthylenes against *Leishmania* spp. *Antimicrob Agents Chemother* 59:2867–2874. <https://doi.org/10.1128/AAC.00226-15>.
- Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nuñez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G; Nomenclature Committee on Cell Death. 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ* 16:3–11. <https://doi.org/10.1038/cdd.2008.150>.
- Bou DD, Tempone AG, Pinto EG, Lago JH, Sartorelli P. 2014. Antiparasitic activity and effect of casearins isolated from *Casearia sylvestris* on *Leishmania* and *Trypanosoma cruzi* plasma membrane. *Phytomedicine* 21: 676–681. <https://doi.org/10.1016/j.phymed.2014.01.004>.
- Dorlo TP, Balasegaram M, Beijnen JH, de Vries PJ. 2012. Miltefosine: a

- review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J Antimicrob Chemother* 67:2576–2597. <https://doi.org/10.1093/jac/dks275>.
16. Fensterle J, Aicher B, Seipelt I, Teifel M, Engel J. 2014. Current view on the mechanism of action of perifosine in cancer. *Anticancer Agents Med Chem* 14:629–635. <https://doi.org/10.2174/1871520614666140309225912>.
17. Marinho Fde A, Gonçalves KC, Oliveira SS, Oliveira AC, Bellio M, d'Avila-Levy CM, Santos AL, Branquinha MH. 2011. Miltefosine induces programmed cell death in *Leishmania amazonensis* promastigotes. *Mem Inst Oswaldo Cruz* 106:507–509. <https://doi.org/10.1590/S0074-02762011000400021>.
18. Verma NK, Dey CS. 2004. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrob Agents Chemother* 48:3010–3015. <https://doi.org/10.1128/AAC.48.8.3010-3015.2004>.
19. Cabrera-Serra MG, Valladares B, Piñero JE. 2008. *In vivo* activity of perifosine against *Leishmania amazonensis*. *Acta Trop* 108:20–25. <https://doi.org/10.1016/j.actatropica.2008.08.005>.