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Antileishmanial activity and tubulin polymerization inhibition of podophyllotoxin derivatives on *Leishmania infantum*

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ABSTRACT

Leishmania microtubules play an important role not only in cell division, but also in keeping the shape of the parasite and motility of its free-living stages. Microtubules result from the self-assembly of alpha and beta tubulins, two phylogenetically conserved and very abundant eukaryotic proteins in kinetoplastids. The colchicine binding domain has inspired the discovery and development of several drugs currently in clinical use against parasites. However, this domain is less conserved in kinetoplastids and may be selectively targeted by new compounds. This report shows the antileishmanial effect of several series of compounds (53), derived from podophyllotoxin (a natural cyclolignan isolated from rhizomes of *Podophyllum* spp.) and podophyllic aldehyde, on a transgenic, fluorescence-emitting strain of *Leishmania infantum*. These compounds were tested on both promastigotes and amastigote-infected mouse splenocytes, and in mammalian – mouse non-infected splenocytes and liver HepG2 cells – in order to determine selective indexes of the drugs. Results obtained with podophyllotoxin derivatives showed that the hydroxyl group at position C-7α was a structural requisite to kill the parasites. On regards podophyllic aldehyde, derivatives with C9-aldehyde group integrated into a bicyclic heterostructure displayed more potent antileishmanial effects and were relatively safe for host cells. Docking studies of podophyllotoxin and podophyllic aldehyde derivatives showed that these compounds share a similar pattern of interaction at the colchicine site of Leishmania tubulin, thus pointing to a common mechanism of action. However, the results obtained suggested that despite tubulin is a remarkable target against leishmaniasis, there is a poor correlation between inhibition of tubulin polymerization and antileishmanial effect of many of the compounds tested, fact that points to alternative pathways to kill the parasites.

1. Introduction

Visceral leishmaniasis (VL) is a vector-borne zoonotic disease responsible for one of the most neglected diseases linked to the poorest communities of low-income countries. The treatment of this disease is based on chemotherapy, since potential vaccines are in preclinical or early clinical stages of development (Beaumier et al., 2013). Current pharmacopeia against this disease includes the first line antimony derivatives (Sb^V), different formulations of the polyene fungicide amphotericin B (AMB) and the oral alkylphosphocholine miltefosine (Balaña-Fouce et al., 1998; Monge-Maillo and Lopez-Velez, 2013). These compounds have many undesirable side effects that include Sb^V cardiotoxicity (Sundar and Chakravarty, 2010), AMB nephrotoxicity (Croft and Olliaro, 2011) and the developmental toxicity of miltefosine

Abbreviations: NTD, Neglected Tropical Diseases; VL, Visceral Leishmaniasis; AMB, Amphotericin B; AMBdc, AMB deoxicholate; iRFP, Infra Red Fluorescent Protein; FCS, Foetal Calf Serum; SI, Selectivity Index.

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(Bhattacharya et al., 2007). Therefore, the discovery and development of new drugs based on validated targets against VL is an urgent need, especially when Big Pharma companies have waived funds and efforts in research and development against this neglected disease.

Microtubule-targeting (antimitotic) drugs include compounds of diverse structure that can disrupt microtubule function by interacting with tubulin (Dumontet and Sikic, 1999). Microtubules are eukaryotic protein structures resulting from the polymerization of α/β-tubulin heterodimers. Microtubules are responsible for the formation of the mitotic spindle, cell shape, ciliary and flagellar motility and intracellular transport (Hawkins et al., 2010). β-Tubulin is a GTP-hydrolyzing and highly conserved protein that interacts with α-tubulin, both conforming the basic structural subunit of microtubules. Microtubules of trypanosomatids have some unique features. They are constituted by the most abundant protein in these cells, since they are responsible for the subpellicular corset that conforms their specific shape (Seeback et al., 1990; Kohl and Gull, 1998). In addition, they are relatively stable at low temperatures, in contrast to microtubules of many higher organisms, and they are also resistant to many drugs currently used in anthelminthic and anticancer therapies (Werbovetz, 2002). One of these drugs is colchicine, a potent inhibitor of tubulin polymerization in higher eukaryotes (Vindya et al., 2015). In mammals, colchicine binds to a β-tubulin domain located near the α/β-tubulin interface, where it prevents tubulin to adopt a straight linear structure required for further adequate polymerization (Ravelli et al., 2004). Despite β-tubulin is highly conserved from trypanosomes to other higher eukaryotic organisms, up to eleven amino acid substitutions are found in the colchicine-binding site that may explain the resistance of these parasites to such drug (Luis et al., 2013). These differences have been pointed as putative targets to be exploited in the design of new more selective polymerization inhibitors (Kaur et al., 2014).

Several drugs have been used to target trypanosomatids microtubules with low activity against mammalian counterparts. These drugs can act either by inhibiting or promoting tubulin polymerization. Dinitroaniline herbicides have shown activity against both *Leishmania* and *Trypanosoma* (Chan and Fong, 1990; Chan et al., 1991, 1993a, 1993b; Traub-Cseko et al., 2001), and tubulin has been implicated as being the target of these compounds (Chan and Fong, 1990; Chan et al., 1993b; Traub-Cseko et al., 2001). One interesting natural compound in cancer chemotherapy is podophyllotoxin (Fig. 1), an antineoplastic and antiviral cytotoxic cyclolignan isolated from *Podophyllum* spp. (Berberidaceae) and several species of other genera and families. From a mechanistic point of view, it has been demonstrated that podophyllotoxin and most of its related 4′-methoxy congeners act by inhibiting tubulin polymerization through interaction at the colchicine-binding site (Ravelli et al., 2004), while another not yet completely defined apoptosis mechanism has been proposed for some of those compounds included in other study (Castro et al., 2010).

This article describes for the first time the evaluation of the killing effect of several series of semisynthetic podophyllotoxin derivatives on *Leishmania infantum*, the aetiological agent responsible for VL in humans and dogs in the Old World. For this purpose, an intracellular screening on macrophages isolated from naturally infected BALB/c mice with an infrared-emitting *L. infantum* strain was used. This method has the remarkable advantage of using host-infected cells under natural conditions, where splenocytes are still playing a role in the immunological response (Reguera et al., 2014). Furthermore, we have studied the potential role played by leishmanial tubulin as putative target of these compounds.

2. Material and methods

2.1. Chemistry

The chemical structures of the starting compounds podophyllotoxin and podophyllic aldehyde (Fig. 1) and the derivatives evaluated in this work are compiled in Tables (1–4). Compounds were prepared according to previous reports (Castro et al., 2004, 2010, 2012; Abad et al., 2012) and to unpublished procedures (chemical data not shown here). Their structures were respectively confirmed or assigned by either direct comparison with authentic samples, or through complete analysis of One- and Two-Dimensional ^{1}H and ^{13}C nuclear magnetic resonance (1D- and 2D-NMR, respectively), infrared (IR) and mass (MS) spectra for the new compounds.

2.2. Ethic statement

The animal research described in this manuscript complies with Spanish Act (RD 53/2013) and European Union Legislation (2010/63/UE). The protocols used here were approved by the Animal Care Committee of the University of León (Spain), project license number (PI12/00104).

2.3. Promastigote cultures and in vitro assays

Leishmania infantum BCN150 iRFP (henceforth referred as *L. infantum-iRFP*) is a genetically modified strain that constitutively incorporates the infrared *iRFP* encoding gen for infrared detection (Calvo-Álvarez et al., 2012) Promastigotes were cultured in M199 medium supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 6.9, 10 mM glutamine,

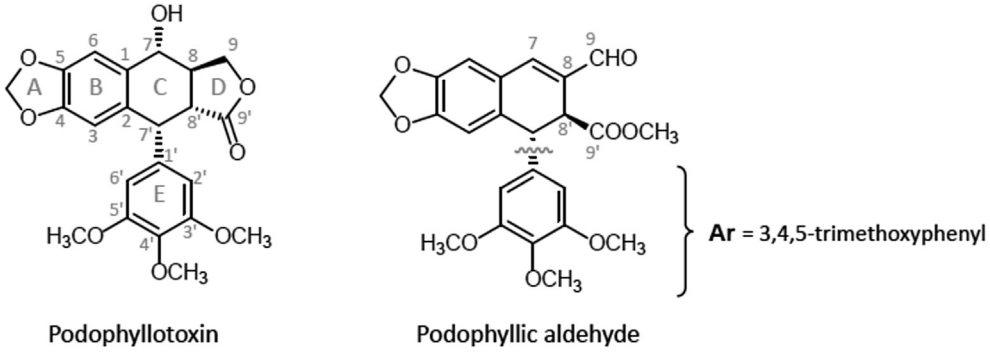

Fig. 1. Structures and numbering of positions and rings of podophyllotoxin (2a, left) and podophyllic aldehyde (14, right).