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Graphical Abstract

Substituted 1,5-naphthyridine derivatives as novel antileishmanial agents. Synthesis and biological evaluation.

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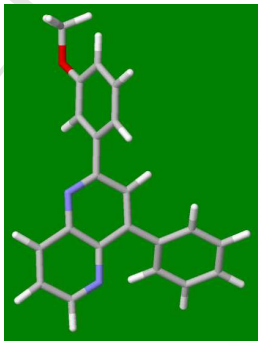

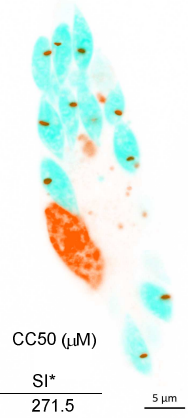

IC50 (µM)			CC50 (µM)
L. infantum Promastigotes	L. infantum Amastigotes	Murine splenocytes	SI*
>100	0.58±0.03	157.45±2.36	271.5

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Keywords: Leishmania, naphthyridines, DNA-topoisomerase

Abbreviations: VL, Visceral Leishmaniasis; TopIB, Type IB DNA Topoisomerase; IRFP, Infra Red Fluorescent Protein; FCS, Fetal Calf Serum; DMSO, Dimethyl sulfoxide; SI, Selectivity Index; CPT, camptothecin; CPTs, camptothecin derivatives; HDAr, hetero-Diels-Alder reaction; TLC, thin layer chromatography; ERC, electrocyclic ring closure.

ABSTRACT. Visceral leishmaniasis is a parasitic disease that affects, among other areas, both sides of the Mediterranean Basin. The drugs classically used in clinical practice are pentavalent antimonials (Sb^V) and amphotericin B, which are nephrotoxic, require parenteral administration, and increasing drug resistance in visceral leishmaniasis has been observed. These circumstances justify the search of new families of compounds to find effective drugs against the disease. Eukaryotic type I DNA topoisomerase (TopIB) has been found essential for the viability of the parasites, and therefore represents a promising target in the development of an antileishmanial therapy. In this search, heterocyclic compounds, such as 1,5-naphthyridines, have been prepared by cycloaddition reaction between *N*-(3-pyridyl)aldimines and acetylenes and their antileishmanial activity on promastigotes and amastigote-infected splenocytes of *Leishmania infantum* has been evaluated.

In addition, the cytotoxic effects of newly synthesized compounds were assessed on host murine splenocytes in order to calculate the corresponding selective indexes (SI). Excellent antileishmanial activity of 1,5-naphthyridine **19**, **21**, **22**, **24** and **27** has been observed with similar activity than the standard drug amphotericin B and higher selective index (SI >100) towards *L. infantum* amastigotes than amphotericin B (SI > 62.5). Special interest shows the 1,5-naphthyridine **22** with an IC_{50} value (0.58 ± 0.03 μM) similar to the standard drug amphotericin B (0.32 ± 0.05 μM) and with the highest selective index (SI = 271.5). In addition, this compound shows remarkable inhibition on leishmanial TopIB. However, despite these interesting results, further studies are needed to disclose other potential targets involved in the antileishmanial effect of these novel compounds.

1. Introduction

Similar to other Neglected Tropical Diseases, the treatment of the three forms of leishmaniasis resides in a limited number of medicines that are either outdated or plenty of side effects [1]. Briefly, first line pentavalent antimony (Sb^{V}) derivatives are metabolically-activated pro-drugs that have to be reduced to toxic trivalent antimony (Sb^{III}). The second line drugs include polyene fungicides, such as amphotericin B (AMB) or the oral anticancer drug miltefosine. AMB, administered either as sodium deoxycholate salt (Fungizone®) or as liposomal suspension (AmBisome®), is nephrotoxic and needs patient hospitalization [2]. For its part, miltefosine is the most remarkable antileishmanial compound introduced in the last decades, except for the potential embriotoxicity reported in pregnant women [3]. Therefore, an efficient, safe, economically affordable and self-administrable drug that accomplishes the Target Product Profile requirements for leishmaniasis requested by Drug for Neglected Diseases initiative (DNDi) is an urgent need (https://www.dndi.org). In addition, new and closer-to-reality disease models are demanded in order to test under High Throughput Screening conditions the myriads of existing and novel small molecules present in different libraries and repositories [4].

The role played by DNA topoisomerases (Top) in DNA physiology has inspired several anticancer and antimicrobial drugs based on the irreversible inhibition of these enzymes [5]. Unlike the type II family, eukaryotic type I Top (TopI) have singular features in *Leishmania* (and other trypanosomatids) that make them a selective target against these pathogens: i) the enzyme has an increased expression during the rapidly invading stages of the parasite, and especially ii) the pathogen's enzyme has an atypical structure that can be exploited in targeted-driven programs of drug design [6]. Several years ago, our research group found that *L. infantum* TopI

(LTopIB) had heterodimeric structure composed of two different protomers, which had to be previously assembled to reconstitute a full active enzyme [7]. Furthermore, it was undoubtedly demonstrated that the large protomer contained the four amino acids of the active site, whereas the small protomer contained a catalytic tyrosine, the latter being a phylogenetically conserved amino acid involved in the introduction of transient phosphodiester bonds to DNA substrate [8].

To date, two classes of TopIB inhibitors have been described: i) interfacial inhibitors or poisons that stabilize the enzyme-DNA complex (cleavage complex), thus delaying the religation step and facilitating the formation of single-strand breaks in DNA, and ii) catalytic inhibitors, which interact either with the substrate DNA or with catalytic domains of the enzyme [9]. Camptothecin (CPT) their hydrosoluble derivatives topotecan and irinotecan [10], and other non-CPT poisons such as indolocarbazoles and indenoisoquinolines [11] belong to the first group. Catalytic inhibitors for their part, reversibly interact with the enzyme and do not induce DNA lesions. Amongst them, many natural products and derivatives have been reported as potential TopI catalytic inhibitors: plant terpenoids like betulinic acid, acetylenic fatty acids isolated from sea animals or plant naphthoquinones. Many of these compounds have shown strong antileishmanial activity [12].

In this study, we assess the antileishmanial effect of a series of 1,5-naphthyridines compounds against both stages – free-living promastigotes and intracellular amastigotes – of *L. infantum*, the aethiological agent responsible for VL in the Old World. For this purpose, an intracellular screening of macrophages isolated from naturally infected BALB/c mice with an infrared-emitting *L. infantum* strain was used [13]. Furthermore, we have studied the potential role played by LTopIB as putative target of these compounds.

Some chemicals showing good human TopIB inhibition, such as CPT (Figure 1), its derivatives and indeno[1,5]naphthyridines, have been studied as LTopIB inhibitors [14]. From a chemical point of view, these derivatives present nitrogenated fused ring heterocycles. This feature may be important towards their effectiveness as TopIB inhibitors. In addition, a wide range of approved drugs [15] and antileishmanial lead compounds revealed the importance of the skeleton of nitrogen-containing heterocycles [16]. Taking this into account, other quasi-flat condensed heterocycles, such as naphthyridine derivatives (Figure 1) [17], might be also adequate candidates as LTopIB inhibitors and overcome some of CPTs inherent limitations.

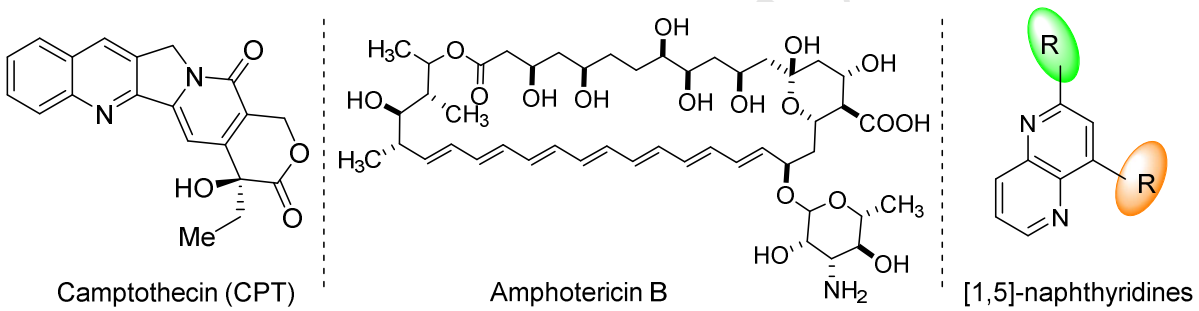


Figure 1. Structure of camptothecin (left), amphotericin B (middle) and newly synthesized 1,5-naphthyridines (right).

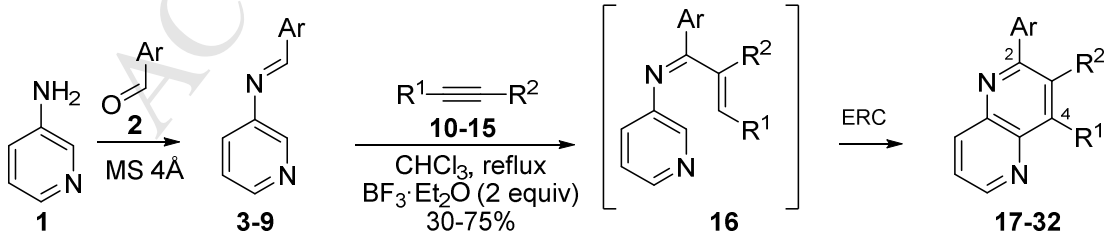
A wide range of six-membered nitrogen-containing heterocyclic compounds play a major role in organic chemistry through their widespread presence in nature and in their consequent biological activity with applications in biochemistry, pharmacology and material science. [15, 18] Many strategies are described in the literature for the synthesis of nitrogenated heterocycles, among which one of the most straightforward is the hetero-Diels-Alder reaction (HDAr). This reaction type is an atom-economic alternative for the carbon-carbon and carbon-heteroatom bond construction [19] and represents an excellent tool for the generation of six-membered rings with a high molecular complexity [20], which may have industrial applications.[21] Among those strategies, the Povarov reaction [22, 23] allows the preparation of nitrogen-containing

heterocyclic compounds in an excellent way. [24] This methodology also represents a direct route to the naphthyridine core structure of interesting biologically active compounds as TopI inhibitors and with antiproliferative activity against several cancer cell lines as reported in our research group.

This manuscript describes the synthesis of 1,5-naphthyridines by cycloaddition reaction between *N*-(3-pyridyl)aldimines and alkynes as well as the antileishmanial effect of new heterocyclic compounds on *L. infantum*, the aethiological agent responsible for VL in humans and dogs in Mediterranean countries. For this purpose an *ex vivo* intracellular screening on macrophages isolated from naturally infected BALB/c mice with an infrared-emitting *L. infantum* strain was used. [13] In addition, the inhibitory effect of these compounds has been studied on recombinant TopIB from both *Leishmania* and human sources. These results point 1,5-naphthyridines as promising antileishmanial drugs.

2. Chemistry

First, the synthesis of target compounds, 1,5-naphthyridines, was accomplished. We started with the preparation of the corresponding *N*-(3-pyridyl)aldimines **3-9** by using 3-aminopyridine **1** and aromatic aldehydes **2** in chloroform as solvent and in the presence of molecular sieves (Scheme 1).



Scheme 1. Preparation of novel 1,5-naphthyridines **17-32**. Reagents and conditions: (A) 3-pyridylamine **1** (1 equiv), aldehyde **2** (1 equiv), molecular sieves 4 Å, chloroform, reflux, 15-48 h. (B) alkyne **10-15**, chloroform, reflux, 15-70h.

Afterwards, the obtained aldimines **3-9** were reacted with acetylenes **10-15** (Chart 1). The reaction without catalyst did not work and the starting products were recovered. However, the use of trifluorboroetherate as Lewis acid catalyst gave us good results: the optimal ones were when 2 equivalents of Lewis acid were used and only 2,4-disubstituted 1,5-naphthyridines **17-31** were regioselectively obtained (Chart 2) when terminal alkynes were used (R^2 = H, Chart 1), while the formation of the other regioisomers, namely 2,3-disubstituted 1,5-naphthyridines was not observed. [27] It is noteworthy that not only terminal alkynes **10-14** (Chart 1) can be used for the synthesis of 1,5-naphthyridines **17-31** but also internal alkyne **15** is appropriate for the formation of corresponding heterocyclic compounds, such as nahthyiridine **32** (Chart 2).

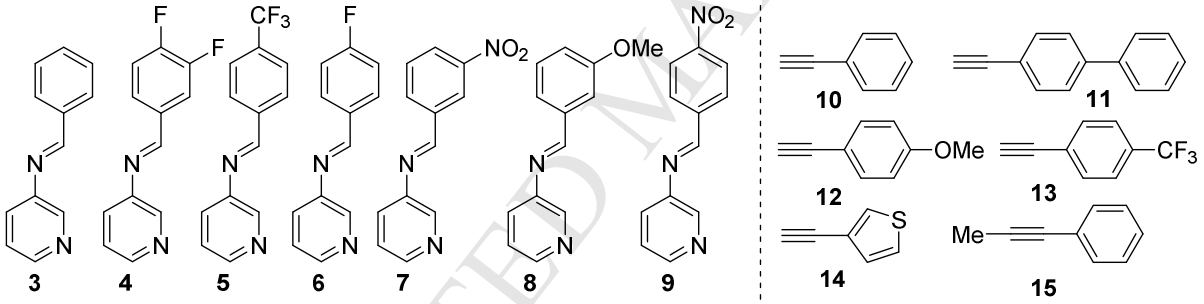


Chart 1. Structures of aldimines **3-9** and acetylenes **10-15** used.

The structure of compounds **17-32** was assigned on basis of NMR spectra and mass spectrometry. For example, when aldimine **5** (Ar = 4-$CF_3C_6H_4$) and 4-methoxyphenyl acetylene **12** (R^1 = 4-$MeOC_6H_4$, R^2 = H) were used the 1,5-naphthyridine **25** (Chart 2) was obtained. Its structure was assigned by means of NMR experiments and confirmed by HRMS. For instance, in the ^{19}F-NMR spectrum of compound **25** one signal was observed at δ_F = - 63.1 ppm and in the ^{1}H-NMR spectrum the corresponding two signals at low field corresponding to two protons of the naphthyridine ring, one double doublet at δ_H = 8.49 ppm with coupling constants $^3J_{HH}$ = 8.5

Hz, $^{4}J_{HH}$ = 1.8 Hz and another double doublet at δ_H = 9.01 with coupling constants $^{3}J_{HH}$ = 4.3 Hz and $^{4}J_{HH}$ = 1.8 Hz. In general for 1,5-naphthyridines **17-32**, in the ^{1}H-NMR spectra, a representatively characteristic signal appears as a singlet approximately at 8.1 ppm for the proton at 3 position of naphthyridine ring. Moreover, in ^{13}C NMR spectra signals for aromatic carbons were observed for 1,5-naphthyridines **17-32**. Among them two signals could be representatively characteristic for these compounds, such as signal of CH aromatic carbon at 8 position which appears at δ = 137.9 – 138.2 ppm range and signal at δ = 150.0 – 151.6 ppm range which corresponds to the CH at 6 position of the naphthyridine ring. Moreover, its structure has been unequivocally confirmed by X-ray analysis. [27]

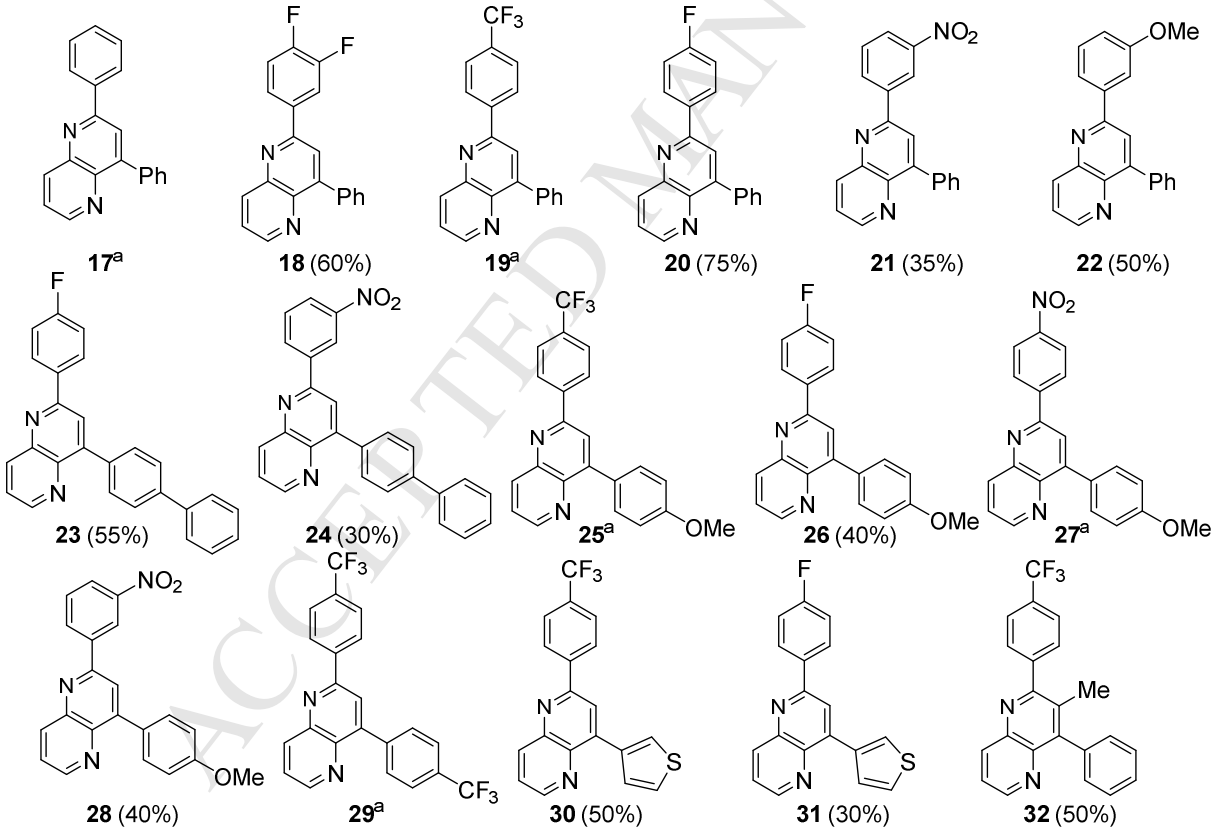


Chart 2. Structures of 1,5-naphthyridines **17-32** obtained ([d] Reference 27).

Formation of 1,5-naphthyridines **17-32** could be explained by a formal [4+2] process of imines **3-9** with alkynes **10-15** and subsequent aromatization under the reaction conditions to afford corresponding naphthyridines **17-32** (Scheme 1). In fact, our previous work based on a combined experimental and computational study of the Povarov reaction between *N*-(3-pyridyl)aldimines and acetylenes with a Lewis acid suggested a stepwise [4+2]-cycloaddition mechanism through a 3-azatriene intermediate **16**. [27] A wide scope of 1,5-naphthyridines **17-32** with electron-donating and electron-withdrawing aromatic substituents, including fluorine substituents, [28] has been achieved, and in order to increase the diversity of these compounds also derivatives with heterocyclic substituents, such as thiophene (R^1 = 3-thienyl), **30** and **31** were also obtained. Biological activity against promastigotes and amastigotes-infected macrophages of *L. infantum*, has been studied for all these 1,5-naphthyridine heterocyclic compounds **17-32** (Chart 2).

3. Biological results and discussion

Activity of 1,5-naphthyridine against L. infantum. To assess the proficiency of new 1,5-naphthyridines against *Leishmania* parasites, an improved protocol consisting in evaluating the effect on both free-living promastigotes and intracellular amastigotes that infect natural host cells were used. This method uses a specially engineered *L. infantum* strain that constitutively express the infrared fluorescent protein (iRFP) called *L. infantum*-iRFP, which provides infrared emission (708 nm) to both viable promastigotes and amastigotes. In this report we used infected macrophages harboring amastigotes that were isolated from BALB/c mice infected 5 to 6 weeks earlier with *L. infantum*-iRFP [13]. This method has several advantages: i) allows the rapid assessment of viable pathogens under drug pressure; ii) the action of the drugs is performed under natural *ex vivo* conditions, which includes the immunological environment that can help to destroy the invading cells within spleen macrophages [29]. Finally, drug cytotoxicity, was

assessed in free-parasite macrophages obtained under the same conditions from spleens dissected -from uninfected BALB/c mice.

Table 1 shows the antileishmanial effect of the new series of compounds with the 1,5-naphthyridine core rings by means of dose-response curves on *L. infantum*-iRFP promastigotes and amastigotes, fitted by nonlinear analysis with the Sigma-Plot statistical package. For both forms the drug effect on the pathogen was expressed as the 50%-reduction of infrared emission (IC_{50}) with respect to negative control (that contains DMSO as drug vehicle). Selectivity indexes of each compound were raised from the ratio between their cytotoxicity on uninfected explants (CC_{50}) *vs*. the IC_{50} values obtained on infected *ex vivo* splenic explants. The leishmanicidal effect of AMB (drug in clinical use) was included as positive control just for comparison purposes.

In the case of fluoro substituted 1,5-naphthyridines **20**, **23**, **26** and **31** (Ar = 4-F-C_6H_4, Table 1, entries 5, 8, 11 and 16) a high degree of the macrophage destruction or low killing capability of the parasite were observed. Interestingly, the values of antiparasitic activity obtained with 1,5-naphthyridines **17-32** derivatives were better on intracellular amastigotes than on free-living promastigotes (Table 1). It is noteworthy the remarkable effect observed for compounds **25**, **29**, **30** and **32** with trifluoromethyl substituents (Ar = 4-CF_3-C_6H_4), where generally the cytotoxicity on free-living promastigotes is higher than 100 μM (Table 1, entries 10, 14, 15 and 17). At the same time the macrophage destruction is not observed for these mentioned compounds, showing in murine splenocytes low cytotoxic values (over 100 μM in almost all these cases), which means a selective killing effect on intracellular parasites.

Table 1: Chemical structure and biological activity of 1,5-naphthyridine compounds (**17-32**) on *L. infantum* iRFP promastigotes and amastigotes (naturally-infecting mouse macrophages). Each value is the mean ± sd of at least three different experiments made by triplicate.

					IC_{50} (μM)		CC_{50} (μM)	
entry	**Compound**	**Ar**	**R^1**	**R^2**	***L. infantum* Promastigotes**	***L. infantum* Amastigotes**	**Murine splenocytes**	**SI***
1		Amphotericin B (AMB)			0.77 ±0.15	0.32 ±0.05	>20	>62.5
2	**17**	C_6H_5	C_6H_5	H	27.2 ± 12.3	8.42±1.91	54,8 ± 6,1	6.5
3	**18**	3,4-F_2-C_6H_3	C_6H_5	H	>100	2.82±0.76	21.7 ± 4.8	7.7
4	**19**	4-CF_3-C_6H_4	C_6H_5	H	37.3 ± 7.1	0.47±0.95	65.7 ± 5.4	139.8
5	**20**	4-F-C_6H_4	C_6H_5	H	>100	7.41±1.26	124 ± 44.3	16.7
6	**21**	3-NO_2-C_6H_4	C_6H_5	H	79.24±2.28	1.02±0.12	145.90±20.83	143.1
7	**22**	3-MeO-C_6H_4	C_6H_5	H	>100	0.58±0.03	157.45±2.36	271.5
8	**23**	4-F-C_6H_4	4-C_6H_5-C_6H_4	H	45.17±5.26	5.04±0.19	152.54±37.49	30.2
9	**24**	3-NO_2-C_6H_4	4-C_6H_5-C_6H_4	H	15.09±0.42	0.29±0.35	30.13±3.87	103.9
10	**25**	4-CF_3-C_6H_4	4-MeO-C_6H_4	H	>100	2.26±0.45	151.6 ± 89.7	67.1
11	**26**	4-F-C_6H_4	4-MeO-C_6H_4	H	97.17±3.5	0.76±0.06	24.16±2.92	31.8
12	**27**	4-NO_2-C_6H_4	4-MeO-C_6H_4	H	20.69±0.95	0.58±0.04	108.65±0.35	187.3
13	**28**	3-NO_2-C_6H_4	4-MeO-C_6H_4	H	45.18±8.47	3.79±0.81	132.71±1.74	35.1
14	**29**	4-CF_3-C_6H_4	4-CF_3-C_6H_4	H	>100	9.47±0.12	178.4 ± 63.2	18.8
15	**30**	4-CF_3-C_6H_4	3-thienyl	H	>100	3.67±0.16	181.4 ± 51.5	49.4
16	**31**	4-F-C_6H_4	3-thienyl	H	66.95±1.51	7.93±22.26	32.51±12.30	4.1
17	**32**	4-CF_3-C_6H_4	C_6H_5	CH_3	>100	2.37±0.12	126.1 ± 39.6	53.2

[a]Antileishmanial effects (IC_{50} ± SD) on promastigotes and amastigotes of *L. infantum*. Cytotoxicity effects (CC_{50} ± SD) on murine splenocytes. *SI: Selective Index.

In addition, when nitro substituted 1,5-naphthyridines **27** (Ar = 4-NO_2-C_6H_4) and **21** and **24** (Ar = 3-NO_2-C_6H_4) were tested high selectivity indexes (SI > 100) were observed (Table 1, entries 6, 9 and 12). For example, when **27** (Ar = 4-NO_2-C_6H_4, R^1 = 4-$MeOC_6H_4$, R^2 = H) was tested no macrophage destruction was observed with an IC_{50} of murine splenocytes of 108.65±0.35 μM and a promastigotes killing capability of IC_{50} = 0.58±0.04, which results in a very high selectivity index (SI = 187.3). Even trifluoromethyl substituted 1,5-naphthyridine **19** (Ar = 4-CF_3-C_6H_4, R^1 = C_6H_5, R^2 = H) shows an excellent selectivity index (SI = 139.8). These results along with the previous ones may suggest that the presence of electron-withdrawing substituents such as trifluoromethyl or nitro groups in the aromatic ring of position 2 in 1,5-naphthyridines **19**, **21**, **24**, **25**, **27**, **29**, **30** and **32** increases the antileishmanial effect.

Interestingly, the inclusion of an electron-donating subtituent in the phenyl substituent at position 2 of the 1,5-naphthyridine core, very much improved both the antileishmanial effect (amastigote) and the corresponding SI values while macrophage destruction was minimum. In such a way, the compound **22** (Ar = 3-MeO-C_6H_4, R^1 = C_6H_5, R^2 = H) showed the highest SI value of this family (SI = 271.5; Table 1, entry 7).

Inhibition of leishmanial and human TopIB. Previous results reported by the authors showed that the antileishmanial effect of a series of tetrahydroindeno-1,5-naphthyridines and indeno-1,5-naphthyridines was due in part to their selective inhibition on LTopIB [14]. With similar aims we performed conventional DNA relaxation assays with the new series of compounds. Therefore, in order to study the effect of 1,5-naphthyridines on TopIB from human and *Leishmania* sources, conventional relaxation assays were carried out.

These assays are based on the ability of these compounds to prevent the relaxation of circular supercoiled DNA mediated by TopI. For this purpose, recombinant *LTopIB* and *hTopIB* genes expressed in a TopIB-deficient *Saccharomyces cerevisiae* platform and purified as described elsewhere [7] were used. The effect of these compounds was compared with the inhibitory effect of CPT that was used as positive control in these experiments [14,17].

To assess the effect of 1,5-naphthyridines on TopIB enzymes, we performed previous studies with or without a 15-min preincubation step (Figure 2) of purified TopIB with a fixed concentration of compound **17** (as representative compound of the whole series) both at 4ºC and 37ºC. However, since CPT does not bind the enzyme in the absence of DNA, the preincubation step was unnecessary [25]. Preincubation temperature plays an important role in TopIB inhibition of compound **17** and in the rest of the compounds, as well, since only when this compound was in contact with TopIB at 37 ºC, the inhibition was effective. The concentration of the drug was set at 100 μM and CPT was used as a positive control at the same concentration. Relaxation assay was triggered by addition of 0.5 μg supercoiled pSK DNA substrate and subsequent incubation for increasing time periods ranging from (1-16 min) (see Supplementary Material, Fig. 1.SM). Reactions were stopped adding SDS and topoisomers were resolved by agarose gel electrophoresis at the conditions established in Material and Methods section.

Once established the optimal assay conditions, the IC_{50} of each compound was calculated by means of dose-response curves. Table 2 shows the inhibition of both LTopIB and hTopIB mediated by 1,5-naphthyridines calculated in terms of the conversion of supercoiled DNA to its relaxed form, as determined by quantifying the bands representing relaxed or supercoiled DNA in agarose gels according to the method

described in material and methods. Equal concentrations of DMSO were added to each reaction in order to assess the potential negative effect of the vehicle in the experiments.

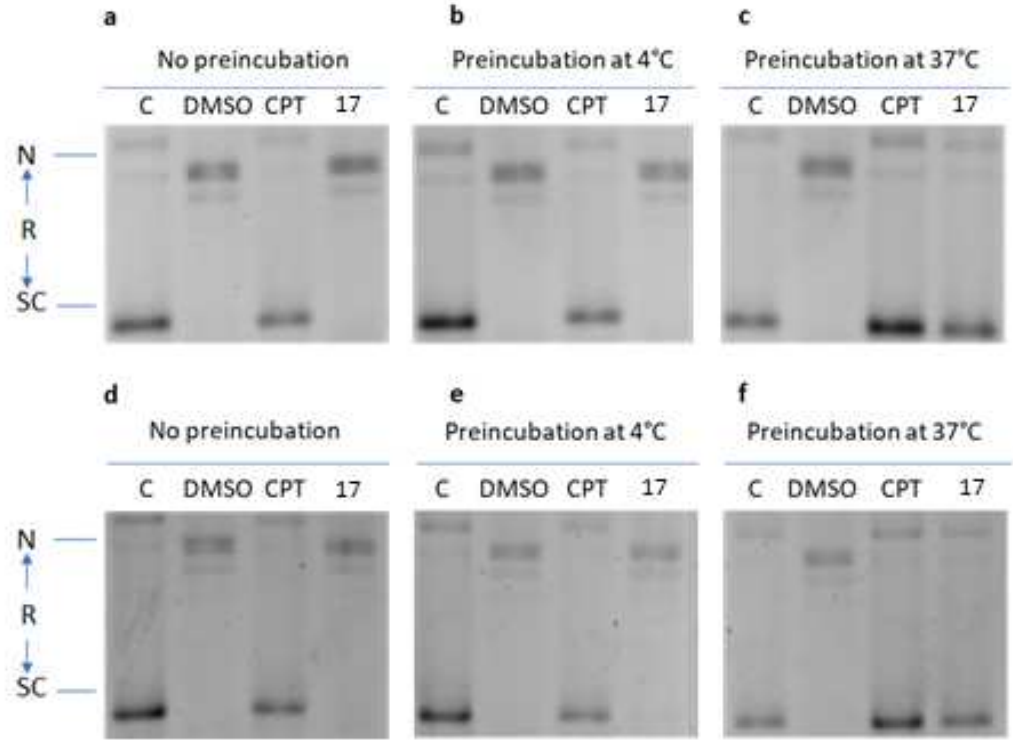


Figure 2. Representative agarose gels showing the role of preincubation time in the inhibition of the relaxation activity of TopIB. Compound **17** was assayed on LdTopIB (top panel) and on hTopIB (bottom panel) without (figures a and d) and with 10 min preincubation at 4°C (figures b and e) and at 37 °C (figures c and f), respectively. The relative position of the negatively supercoiled DNA substrate is indicated by SC, N is the nicked DNA. Reactions were stopped with 1% (w/v) SDS. Lanes (C) contain 0.5 µg of pSK plasmid DNA. Lanes (DMSO) show the activity of the enzymes in absence of drugs but with 2.5% (v/v) DMSO. Lanes (CPT) show the inhibitory effect of 100 µM CPT. Lanes (6a) show the effect of 100 µM of compound **17**.

Table 2 gathers the IC_{50} values of a total of 16 compounds tested on both LTopIB and hTopIB enzymes. According to the results only the compound **17** (Ar = R^1 = C_6H_5, R^2 = H) showed significant ability to prevent the relaxation of supercoiled DNA by conventional TopIB assay (IC_{50} = 24.42 µM). However, this compound was not selective inhibiting the leishmanial enzyme since it prevented hTopIB-mediated DNA relaxation at even lower concentrations (IC_{50} = 11.24 µM).

Table 2. Inhibition of relaxation activity of LTopIB and hTopIB by 1,5-naphthyridines **17-32**. Each value is the mean ± sd of at least three different experiments made by triplicate.

entry	Compound	Ar	R^1	R^2	Leishmania TopIB IC_{50} (μM)	Human TopIB IC_{50} (μM)
1	**CPT**				no inhibition	
2	**17**	C_6H_5	C_6H_5	H	24.42 ± 1.69	11.24±0.95
3	**18**	3,4-F_2-C_6H_3	C_6H_5	H	76.57 ± 1.82	73.11±1.11
4	**19**	4-CF_3-C_6H_4	C_6H_5	H	69.46 ± 0.38	72.68±0.94
5	**20**	4-F-C_6H_4	C_6H_5	H	81.94 ± 4.70	72.77±1.08
6	**21**	3-NO_2-C_6H_4	C_6H_5	H	No inhibition	No inhibition
7	**22**	3-MeO-C_6H_4	C_6H_5	H	53.04 ± 0.91	82.03±0.97
8	**23**	4-F-C_6H_4	4-C_6H_5-C_6H_4	H	58.05 ± 0.94	83.25±6.50
9	**24**	3-NO_2-C_6H_4	4-C_6H_5-C_6H_4	H	78.73 ± 0.94	67.53±10.34
10	**25**	4-CF_3-C_6H_4	4-MeO-C_6H_4	H	No inhibition	No inhibition
11	**26**	4-F-C_6H_4	4-MeO-C_6H_4	H	No inhibition	No inhibition
12	**27**	4-NO_2-C_6H_4	4-MeO-C_6H_4	H	No inhibition	No inhibition
13	**28**	3-NO_2-C_6H_4	4-MeO-C_6H_4	H	No inhibition	No inhibition
14	**29**	4-CF_3-C_6H_4	4-CF_3-C_6H_4	H	79.24 ± 0.73	50.93±3.85
15	**30**	4-CF_3-C_6H_4	3-thienyl	H	62.81 ± 2.46	82.08±1.30
16	**31**	4-F-C_6H_4	3-thienyl	H	49.26 ± 0.02	43.85±3.72
17	**32**	4-CF_3-C_6H_4	C_6H_5	CH_3	75.69 ± 3.17	70.61±4.09

[a]Compounds were preincubated with the enzyme for 15 min and then 0.5 μg supercoiled DNA was added. For time/course experiments and for dose/response experiments IC_{50} values are expressed as mean ± sd of three different experiments by triplicate.

A similar picture was found with compounds **20** (Ar = 4-F-C_6H_4, R^1 = C_6H_5, R^2 = H), **18** (Ar = 3,4-F_2-C_6H_4, R^1 = C_6H_5, R^2 = H) and **19** (Ar = 4-CF_3-C_6H_4, R^1 = C_6H_5, R^2 = H), which displayed from one to two fluorine heteroatoms and a trifluoromethyl group [28] in the 2-aryl substituent, respectively. In these cases, the introduction of fluorine atoms to the phenyl substituent did not improve the inhibitory effect on any TopIB, despite compound **19** (Ar = 4-CF_3-C_6H_4, R^1 = C_6H_5, R^2 = H) had an interesting 139.8 SI over the parasites. Only compounds **22** (Ar = 3-MeO-C_6H_4, R^1 = C_6H_5, R^2 = H) and **23** (Ar = 4-F-C_6H_4, R^1 = 4-C_6H_5-C_6H_4, R^2 = H) showed relative selectivity over LTopIB, but their IC_{50} values are far from the antileishmanial effect showed on intracellular amastigotes and their corresponding SIs.

When an electron-donating methoxy group was introduced in R^1 in position 4 of the bicyclic ring those compounds were frankly inactive on both TopIB (compounds **25**, **26**, **27** and **28**), despite some of them displayed interesting Selective Indexes on the bioassays. However, compound **22** containing a methoxy group in the 2 position of the naphthyridine ring resulted more selective towards LTopIB. Finally, the 1,5-naphthyridine derivatives with a thienyl group in R^1 (compounds **30** and **31**) recovered in part the inhibitory effect on TopIB. Interestingly, compound **30** (Ar = 4- $CF_3C_6H_4$, R^1 = 3-thienyl, R^2 = H) was more selective in inhibiting LTopIB than other compounds of the series and retained an interesting SI of 49.4 in killing leishmania amastigotes.

4. Conclusions

In conclusion, a wide range of substituted 1,5-naphthyridines has been prepared, by using the Povarov reaction, and their antileishmanial activity on promastigotes and amastigote-infected splenocytes of *L. infantum* has been evaluated as well as their inhibition of LTopIB and hTopIB enzymatic activity. The synthesis of these nitrogenated heterocycles **17-32** involves the Aza-Diels-Alder reaction of functionalized imines **3-9**, obtained from 3-aminopyridine **1** with aldehydes **2**, and acetylenes **10-15**.

In general, 1,5-naphthyridines **17-32** have shown higher antiparasitic activity on intracellular amastigotes than on free-living promastigotes. The presence of electron-withdrawing substituents increases the antileishmanial effect. For example, high selectivity indexes (SI > 100) were observed for nitroaryl substituted 1,5-naphthyridines **27** (Ar = 4-NO_2-C_6H_4) and **21** and **24** (Ar = 3-NO_2-C_6H_4). In this sense, it is noteworthy that when compound **27** (Ar = 4-NO_2-C_6H_4, R^1 = 4-$MeOC_6H_4$, R^2 = H) was tested no macrophage destruction was observed with an IC_{50} of murine splenocytes of 108.65±0.35 μM and a promastigotes killing capability of IC_{50} = 0.58±0.04, resulting in

a very high selectivity index (SI = 187.3). Also trifluoromethyl substituted 1,5-naphthyridine **19** (Ar = 4-CF_3-C_6H_4, R^1 = C_6H_5, R^2 = H) showed an excellent selectivity index (SI = 139.8). However, among all new derivatives, 1,5-naphthyridine **22** (Ar = 3-MeO-C_6H_4, R^1 = C_6H_5, R^2 = H) containing a methoxy group in the aryl in position 2 of naphthyridine resulted the most interesting compound to emerge from these derivatives, due to its high *in vitro* antileishmanial activity and low toxicity showing the highest SI value of this family (SI = 271.5).

Few of these compounds had selective inhibitory effects on LTopIB-mediated relaxation assays compatible to their strong biological in vivo and ex vivo effects. This is the case of **22**, the only compound of the series containing an electron-donating methoxy group in the aryl group of position 2 of naphthyridines that combines certain selective effect over the leishmanial enzyme and has the highest SI of the series (SI = 271.5). Despite the good biological activity of **22** is hardly attributable to LTopIB inhibition, this compound can be considered a good candidate and do offer possibilities for further studies. Whereas, the presence of the 4-MeO-C_6H_5 group in R^1 in the position 4 of naphthyridines **25-28** originated a series of 1,5-naphthyridines with no TopIB activity.

We can conclude, in the light of the current data, that the significant antileishmanial effect displayed by these compounds is poorly related to LTopIB inhibition, which just might play a secondary role in their biological efficacy. Nevertheless, the strong antileishmanial activity and the relative safety of many of the current compounds provide a promising basis for further development of biologically active naphthyridines.

5. Experimental protocols

5.1 Chemistry

5.1.1. General experimental information

All reagents from commercial suppliers were used without further purification. All solvents were freshly distilled before use from appropriate drying agents. All other reagents were recrystallized or distilled when necessary. Reactions were performed under a dry nitrogen atmosphere. Analytical TLCs were performed with silica gel 60 F_{254} plates. Visualization was accomplished by UV light. Column chromatography was carried out using silica gel 60 (230-400 mesh ASTM). Melting points were determined with a digital melting point apparatus without correction. NMR spectra were obtained on a 300 MHz and on a 400 MHz spectrometers and recorded at 25 ºC. Chemical shifts for ^{1}H NMR spectra are reported in ppm downfield from TMS, chemical shifts for ^{13}C NMR spectra are recorded in ppm relative to internal chloroform (δ = 77.2 ppm for ^{13}C), chemical shifts for ^{19}F NMR are reported in ppm downfield from fluorotrichloromethane ($CFCl_3$). Coupling constants (*J*) are reported in Hertz. The terms m, s, d, t, q refer to multiplet, singlet, doublet, triplet, quartet. ^{13}C NMR, and ^{19}F NMR were broadband decoupled from hydrogen nuclei. High resolution mass spectra (HRMS) was measured by positive-ion electrospray ionization (EI) method using a mass spectrometer Q-TOF. Aldimines **3**-**5** and **9** [25] and aldimines **6**, **8** [14] were prepared as previously described. Compounds **17**, **19**, **25**, **27** and **29** were prepared as previously described. [27]

5.1.2. Compounds Purity Analysis

All synthesized compounds were analyzed by HPLC to determine their purity. The analyses were performed on Agilent 1260 infinity HPLC system (C-18 column, Hypersil, BDS, 5μm, 0.4 mm×25 mm) at room temperature. All the tested compounds were dissolved in dichloromethane, and 5μL of the sample was loaded onto the column. Ethanol and heptane were used as mobile phase, and the flow rate was set at 1.0 mL/min. The maximal absorbance at the range of 190−400 nm was used as the detection wavelength. The purity of all the tested compounds (compounds **17-32**) is >95%, which meets the purity requirement by the Journal.

5.1.2.1. ***N-[(3-Nitrophenyl)methylene]-3-pyridinamine*** **(7).** To a solution of 3-aminopyridine **1** (10 mmol, 0.941 g) in $CHCl_3$ (30 mL) 3-nitrobenzaldehyde (10 mmol, 1.412 g) was added. The mixture was stirred under nitrogen at room temperature for 15 h. The reaction product is unstable during distillation and/or chromatography and was used *in situ* for further reactions. ^{1}H RMN of crude reaction mixture (300 MHz, $CDCl_3$) δ: 7.35 (ddd, $^5J_{HH}$ = 0.7 Hz, $^3J_{HH}$ = 4.8 Hz, $^3J_{HH}$ = 8.0 Hz, 1 H), 7.56 (ddd, $^4J_{HH}$ = 1.5 Hz, $^4J_{HH}$ = 2.5 Hz, $^3J_{HH}$ = 8.0 Hz, 1 H), 7.68 (dd, $^3J_{HH}$ = 7 Hz, $^3J_{HH}$ = 8.1 Hz, 1 H), 8.25 (ddd, $^4J_{HH}$ = 2.4 Hz, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 8.3 Hz, 1 H), 8.34 (ddd, $^4J_{HH}$ = 1.1 Hz, $^4J_{HH}$ = 2.2 Hz, $^3J_{HH}$ = 8.2 Hz, 1 H), 8.51-8.53 (m, 2 H), 8.56 (s, 1 H, HC=N), 8.75 (dd, $^4J_{HH}$ = 1.9 Hz, $^3J_{HH}$ = 3.9 Hz, 1 H) ppm; ^{13}C {^{1}H} NMR of crude reaction mixture (75 MHz, $CDCl_3$) δ: 123.7 (HC), 123.9 (HC), 126.2 (HC), 127.4 (HC), 127.9 (HC), 130.1 (HC), 133.9 (HC), 134.4 (HC), 137.4 (C), 142.7 (HC), 146.8 (C), 148.1 (HC), 148.8 (C), 159.1 (HC=N) ppm.

5.1.2.2. **General procedure for the synthesis of naphthyridines 17-32.** To a solution of of the *in situ* prepared aldimines **3-9** (5 mmol) in chloroform the corresponding

acetylenes **10-15** (7 mmol) and $BF_3 \cdot Et_2O$ (10 mmol, 1.230 mL) were added and the mixture was stirred at the opportune temperature until TLC and ^1H NMR spectroscopy indicated the disappearance of aldimine. The reaction mixture was washed with 2M aqueous solution of NaOH (25 mL) and water (25 mL), extracted with dichloromethane (2 x 25 mL), and dried over anhydrous $MgSO_4$. The removal of the solvent under vacuum afforded and oil that was purified by silica gel flash column chromatography using a gradient elution of 10-40% ethyl acetate in hexane to afford products **17-32**.

5.1.2.2.1. ***2,4-Diphenyl-1,5-naphthyridine*** **(17)**. The compound was prepared and characterized as previously described. [27] Purity 99.95 % (EtOH/Heptane = 10/90, Rt = 4.741 min).

5.1.2.2.2. ***2-(3,4-Difluorophenyl)-4-phenyl-1,5-naphthyridine*** **(18).** The general procedure was followed using imine **4** prepared *in situ* and phenylacetylene **10** (7 mmol, 0.768 mL) and the reaction mixture was stirred at refluxing chloroform for 24 h. Compound **18** was obtained (0.954 g, 60 %) as a white solid; mp 221-222 ºC (ethyl acetate/hexane). ^1H NMR (400 MHz, $CDCl_3$) δ: 7.20-7.29 (m, 1 H), 7.41-7.52 (m, 3 H), 7.61 (dd, $^3J_{HH}$ = 8.5 Hz, $^3J_{HH}$ = 4.1 Hz, 1 H), 7.72-7.75 (m, 2 H,), 7.84-7.89 (m, 1 H), 7.96 (s, 1 H), 8.1 (ddd, $^3J_{HH}$ = 11.3 Hz, $^3J_{HH}$ = 7.7 Hz, $^3J_{HH}$ = 2.2 Hz, 1 H), 8.41 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.9 Hz, 1 H), 8.92 (dd, $^3J_{HH}$ = 4.1 Hz, $^4J_{HH}$ = 1.9 Hz, 1H) ppm. ^{13}C {^1H} NMR (100 MHz, $CDCl_3$) δ: 117.3 (d, $^2J_{CF}$ = 67.8 Hz, HC), 117.5 (d, $^2J_{CF}$ = 67.8 Hz, HC), 121.7 (HC), 123.8 (dd, $^3J_{CF}$ = 6.8 Hz, $^4J_{CF}$ = 3.7 Hz, HC), 124.8 (HC), 128.6 (2 HC), 129.2 (HC), 130.7 (2 HC), 136.2 (dd, $^3J_{CF}$ = 5.6 Hz, $^4J_{CF}$ = 3.7 Hz, C), 137.0 (C), 137.9 (HC), 141.4 (C), 144.5 (C), 149.6 (C), 151.0 (HC), 151.1 (dd, $^1J_{CF}$ = 247.4 Hz, $^2J_{CF}$ = 12.1 Hz, FC), 151.8 (dd, $^1J_{CF}$ = 250.4 Hz, $^2J_{CF}$ = 10.1 Hz, FC), 155.4 (C) ppm. ^{19}F NMR (282 MHz, $CDCl_3$) δ: - 137.3 to -137.1 (m), -136.6 to -136.5 (m)

ppm. HRMS (EI): calculated for $C_{20}H_{12}N_2F_2$ $[M]^+$ 318.0989 found 318.0992. Purity 99.91 % (EtOH/Heptane = 10/90, Rt = 5.146 min).

5.1.2.2.3. ***4-Phenyl-2-(4-(trifluoromethyl)phenyl)-1,5-naphthyridine*** (**19**). The compound was prepared and characterized as previously described [27]. Purity 98.84 % (EtOH/Heptane = 10/90, Rt = 5.228 min).

5.1.2.2.4. ***2-(4-Fluorophenyl)-4-phenyl-1,5-naphthyridine*** **(20).** The general procedure was followed using imine **6** (5 mmol, 1.006 g) and phenylacetylene **10** (7 5 mmol, 0.768 ml) and the reaction mixture was stirred at refluxing chloroform for 36 h. Compound **20** was obtained (1.125 g, 75%) as a white solid ; mp 164-165 ºC (ethyl acetate/hexane). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.16 (t, $^3J_{HH}$ = 7.8 Hz, 2 H), 7.42-7.52 (m, 3 H), 7.59 (dd, $^3J_{HH}$ = 8.5 Hz, $^3J_{HH}$ = 4.5 Hz, 1 H), 7.73-7.76 (m, 2 H,), 8.08 (s, 1 H), 8.12-8.17 (m, 2 H), 8.44 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H), 8.92 (dd, $^3J_{HH}$ = 4.1 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H) ppm. ^{13}C {^{1}H} NMR(75 MHz, $CDCl_3$) δ: 116.1 (d, $^2J_{CF}$ = 22.0 Hz, 2 HC), 122.1 (HC), 124.7 (HC), 128.5 (2 HC), 129.0 (HC), 129.7 (d, $^3J_{CF}$ = 8.5 Hz, 2 HC), 130.6 (2 HC), 135.1 (C), 137.1 (C), 137.7 (HC), 141.3 (C), 144.4 (C), 149.4 (C), 150.7 (HC), 156.7 (C), 164.2 (d, $^1J_{CF}$ = 251.0 Hz, FC) ppm. ^{19}F NMR ($CDCl_3$) δ: - 112.0 ppm. HRMS (EI): calculated for $C_{20}H_{13}FN_2$ $[M]^+$ 300.1096 found 300.1075. Purity 96.62 % (EtOH/Heptane = 10/90, Rt = 5.792 min).

5.1.2.2.5. ***2-(3-Nitrophenyl)-4-phenyl-1,5-naphthyridine*** (**21**). The general procedure was followed using imine **7** (5 mmol, 1.136 g) and phenylacetylene **10** (7 mmol, 0.768 ml) and the reaction mixture was stirred at chloroform reflux for 15 h. Compound **21** was obtained (0.572 g, 35%) as a white solid; m.p. 170-171 (ethyl acetate/hexane).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.58-7.61 (m, 3 H), 7.70-7.76 (m, 2 H), 7.82-7.85 (m, 2 H), 8.16 (s, 1 H), 8.34-8.37 (m, 1 H), 8.54 (dd, $^3J_{HH}$ = 8.7 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H) 8.58-8.60

(m, 1H), 9.04 (dd, $^{3}J_{HH}$ = 4.2 Hz, $^{4}J_{HH}$ = 1.8 Hz, 1 H), 9.11 (dd, $^{3}J_{HH}$ = 4.3 Hz, $^{4}J_{HH}$ = 1.8 Hz, 1 H) ppm. ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$) δ: 121.8 (HC), 122.7 (HC), 124.4 (HC), 125.0 (HC), 128.6 (2 HC), 129.2 (HC), 130.1 (HC), 130.6 (2 HC), 133.5 (HC), 136.8 (C), 138.1 (HC), 140.8 (C), 141.6 (C), 144.6 (C), 150.0 (C), 151.4 (HC), 155.0 (C) ppm. HRMS (EI): calculated for $C_{20}H_{13}N_3O_2$ $[M]^+$ 327.1008 found 327.1018. Purity 99.95 % (EtOH/Heptane = 10/90, Rt = 7.840 min).

5.1.2.2.6. ***2-(3-Methoxyphenyl)- 4-phenyl-1,5-naphthyridine*** **(22).** The general procedure was followed using imine **8** (5 mmol, 1.062 g) and phenylacetylene **10** (7 mmol, 0.768 ml) and the reaction mixture was stirred at refluxing chloroform for 20 h. Compound **22** was obtained (0.783 g, 50%) as yellowish oil after purification by flash chromatography; Rf: 0.67 (50:50 ethyl acetate/hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.95 (s, 3 H, OCH_3), 7.06 (ddd, $^{3}J_{HH}$ = 8.1 Hz, $^{4}J_{HH}$ = 2.6 Hz, $^{4}J_{HH}$ = 0.7 Hz, 1 H), 7.44-7.59 (m, 4 H), 7.66 (dd, $^{3}J_{HH}$ = 8.5 Hz, $^{3}J_{HH}$ = 4.3 Hz, 1 H), 7.75-7.86 (m, 4 H), 8.11 (s, 1 H), 8.52 (dd, $^{3}J_{HH}$ = 8.4 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1 H), 8.99 (dd, $^{3}J_{HH}$ = 4.2 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1 H) ppm. ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) δ: 55.5 (OCH_3), 112.9 (HC), 115.8 (HC), 120.2 (HC), 122.4 (HC), 124.6 (HC), 128.4 (2 HC), 128.8 (HC), 130.0 (HC), 130.5 (2 HC), 137.2 (C), 137.8 (HC), 140.5 (C), 141.5 (C), 144.4 (C), 149.0 (C), 150.5 (HC), 157.6 (C), 160.3 (C) ppm. HRMS (EI): calculated for $C_{21}H_{16}N_2O$ $[M]^+$ 312.1263 found 312.1269. Purity 98.85 % (EtOH/Heptane = 10/90, Rt = 7.741 min).

5.1.2.2.7. ***4-[(1,1'-Biphenyl)-4-yl]-2-(4-fluorophenyl)-1,5-naphthyridine*** **(23).** The general procedure was followed using imine **6** (5 mmol, 1.006 g) and 4-ethynylbiphenyl **11** (7 mmol, 1.248 g) and the reaction mixture was heated at refluxing chloroform for 36 h. Compound **23** was obtained (1.034 g, 55%) as a white solid; m.p. 206-207 (ethyl acetate/hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.22-7.27 (m, 2 H), 7.38-7.42 (m, 1 H), 7.47-7.52 (m, 2 H), 7.67-7.71 (m, 3 H), 7.79 (d, $^{3}J_{HH}$ = 8.6 Hz, 2 H), 7.92 (d, $^{3}J_{HH}$ = 8.4

Hz, 2 H), 8.12 (s, 1 H), 8.23 (dd, $^{3}J_{HH}$ = 8.8 Hz, $^{3}J_{HH}$ = 5.3 Hz, 2 H), 8.51 (dd, $^{3}J_{HH}$ = 8.2 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1 H), 9.01 (dd, $^{3}J_{HH}$ = 4.1 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1 H) ppm. ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) δ: 116.1 (d, $^{2}J_{CF}$ = 21.9 Hz, 2 HC), 121.9 (HC), 124.7 (HC), 127.3 (2 HC), 127.4 (2 HC), 127.7 (HC), 129.0 (2 HC), 129.7 (d, $^{3}J_{CF}$ = 8.5 Hz, 2 HC), 131.0 (2 HC), 135.4 (C), 136.1 (C), 137.9 (HC), 140.9 (C), 141.4 (C), 142.0 (C), 144.5 (C). 148.9 (C), 150.7 (HC), 156.8 (C), 164.2 (d, $^{1}J_{CF}$ = 250.1 Hz, FC) ppm. ^{19}F NMR ($CDCl_3$) δ: - 112.1 to -112.3 (m) ppm. HRMS (EI): calculated for $C_{26}H_{17}FN_2$ $[M]^+$ 376.1376 found 376.1383. Purity 99.98 % (EtOH/Heptane = 10/90, Rt = 6.735 min).

5.1.2.2.8. ***4-[(1,1'-Biphenyl)-4-yl]-2-(3-nitrophenyl)-1,5-naphthyridine*** **(24).** The general procedure was followed using imine **7** (5 mmol, 1.136 g) and 4-ethynylbiphenyl **11** (7 mmol, 1.248 g) and the reaction mixture was stirred at chloroform reflux for 24 h. Compound **24** was obtained (0.604 g, 30%) as a yellowish solid; m.p. 197-198 (ethyl acetate/hexane) .^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.38-7.43 (m, 1 H), 7.48-7.52 (m, 2 H), 7.60-7.64 (m, 2 H), 7.68-7.77 (m, 3 H), 7.80-7.83 (m, 2H), 7.93-7.95 (m, 2 H), 8.22 (s, 1 H), 8.37 (dd, $^{3}J_{HH}$ = 8.4 Hz, $^{4}J_{HH}$ = 2.3 Hz, 1 H), 8.57 (dd, $^{3}J_{HH}$ = 8.4 Hz, $^{4}J_{HH}$ = 1.8 Hz, 1 H), 8.60-8.62 (m, 1 H), 9.07 (dd, $^{3}J_{HH}$ = 4.1 Hz, $^{4}J_{HH}$ = 1.8 Hz, 1 H), 9.13 (t, $^{4}J_{HH}$ = 1.8 Hz, 1H) ppm. ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) δ: 121.6 (HC), 122.8 (HC), 124.5 (HC), 125.0 (HC), 127.4 (4 HC), 127.8 (HC), 129.1 (2 HC), 130.1 (HC), 131.1 (2 HC), 133.5 (HC), 135.7 (C), 138.0 (HC), 140.7 (C), 140.8 (C), 141.6 (C), 142.2 (C), 144.6 (C), 149.1 (C), 149.7 (C), 151.5 (HC), 155.1 (C) ppm. HRMS (CI): calculated for $C_{26}H_{17}N_3O_2$ $[M]^+$ 403.1321 found 403.1334. Purity 97.64 % (EtOH/Heptane = 10/90, Rt = 8.657 min).

5.1.2.2.9. ***4-(4-Methoxyphenyl)-2-(4-(trifluoromethyl)phenyl)-1,5-naphthyridine*** (**25**). The compound was prepared and characterized as previously described [27]. Purity 99.92 % (EtOH/Heptane = 10/90, Rt = 6.601 min).

5.1.2.2.10. ***2-(4-Fluorophenyl)-4-(4-methoxyphenyl)-1,5-naphthyridine*** **(26).** The general procedure was followed using imine **6** (5 mmol, 1.006 g) and 4-methoxyphenylacetylene **12** (7 mmol, 0.908 ml) and the reaction mixture was heated at refluxing chloroform for 24 h. Compound **26** was obtained (0.495 g, 30%) as a white; m.p. 174-175 (ethyl acetate/hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.90 (s, 3 H, OCH_3), 7.08-7.12 (m, 2 H), 7.20-7.25 (m, 2 H), 7.65 (dd, $^{3}J_{HH}$ = 8.6 Hz, $^{3}J_{HH}$ = 4.3 Hz, 1 H), 7.74-7.83 (m, 2 H), 8.04 (s, 1H), 8.18-8.22 (m, 2 H), 8.47 (dd, $^{3}J_{HH}$ = 8.6 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1 H), 8.98 (dd, $^{3}J_{HH}$ = 4.3 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1 H) ppm. ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$) δ = 55.6 (OCH_3), 114.1 (2 HC), 116.0 (d, $^{2}J_{CF}$ = 22.0 Hz, 2 HC), 121.6 (HC), 124.5 (HC), 129.4 (C), 129.7 (q, $^{3}J_{CF}$ = 8.4 Hz, 2 HC), 132.0 (2 HC), 135.5 (C), 137.8 (HC), 141.5 (C), 144.6 (C), 148.8 (C), 150.4 (HC), 156.8 (C), 160.4 (C), 164.3 (d, $^{1}J_{CF}$ = 248.4 Hz, CF) ppm. ^{19}F NMR ($CDCl_3$) δ: - 112.0 to - 112.1 (m) ppm. HRMS (EI): calculated for $C_{21}H_{15}FN_2O$ $[M]^+$ 330.1168 found 330.1176. Purity 97.25 % (EtOH/Heptane = 10/90, Rt = 6.566 min).

5.1.2.2.11. ***2-(4-Nitrophenyl)-4-(4-methoxyphenyl)-1,5-naphthyridine*** (**27**). The compound was prepared and characterized as previously described [27]. Purity 96.92 % (EtOH/Heptane = 10/90, Rt = 15.835 min).

5.1.2.2.12. ***2-[(3-(Nitrophenyl)-4-(4-methoxyphenyl)]-1,5-naphthyridine*** **(28).** The general procedure was followed using imine **7** (5 mmol, 1.136 g) and 4-methoxyphenylacetylene **12** (7mmol, 0.908 ml) and the reaction mixture was stirred at refluxing chloroform for 70 h. Compound **28** was obtained (0.714 g, 40%) as a white

solid; m.p. 193-194 (ethyl acetate/hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.92 (s, 3 H, OCH_3), 7.12 (d, $^{3}J_{HH}$ = 8.8 Hz, 2 H), 7.69-7.76 (m, 2 H), 7.83 (d, $^{3}J_{HH}$ = 8.8 Hz, 2 H), 8.14 (s, 1H), 8.35 (ddd, $^{3}J_{HH}$ = 8.1 Hz, $^{4}J_{HH}$ = 2.2 Hz, $^{4}J_{HH}$ = 1.1 Hz, 1 H), 8.53 (dd, $^{3}J_{HH}$ = 8.5 Hz, $^{4}J_{HH}$ = 1.8 Hz, 1 H), 8.59 (ddd, $^{3}J_{HH}$ = 8.0 Hz, $^{4}J_{HH}$ = 1.7 Hz, $^{4}J_{HH}$ = 1.0 Hz, 1 H), 9.03 (dd, $^{3}J_{HH}$ = 8.6 Hz, $^{3}J_{HH}$ = 4.0 Hz, 1H), 9.10 (t, $^{4}J_{HH}$ = 1.7 Hz, 1H) ppm. ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$) δ: 55.6 (OCH_3), 114.2 (2 HC), 121.3 (HC), 122.7 (HC), 124.4 (HC), 124.9 (HC), 128.9 (C), 130.1(HC), 132.9 (2 HC), 133.5 (HC), 138.0 (HC), 140.8 (C), 141.7 (C), 144.6 (C), 149.1(C), 149.6 (C), 151.2 (HC), 155.0 (C), 160.6 (C) ppm. HRMS (EI): calculated for $C_{22}H_{18}F_3N_2O_2$ $[M]^+$ 342.4005 found 342.4009. Purity 96.99 % (EtOH/Heptane = 10/90, Rt = 11.464 min).

5.1.2.2.13. ***2,4-Bis-(4-Trifluoromethyl)phenyl-1,5-naphthyridine*** (**29**). The compound was prepared and characterized as previously described [27]. Purity 99.85 % (EtOH/Heptane = 10/90, Rt = 5.316 min).

5.1.2.2.14. ***4-(Thiophen-3-yl)-2-(4-(trifluoromethyl)phenyl)-1,5-naphthyridine*** **(30)**. The general procedure was followed using imine **5** (5 mmol, 1.250 g) and 3-ethynyltiophene **14** (7 mmol, 0.693 ml) and the reaction mixture was stirred at refluxing chloroform for 40 h. Compound **30** was obtained (0.890 g, 50%) as a yellowish solid; m.p.195-196 (ethyl acetate/hexane). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.43 (dd, $^{3}J_{HH}$ = 5.0 Hz, $^{3}J_{HH}$ = 3.0 Hz, 1 H), 7.61 (dd, $^{3}J_{HH}$ = 8.5 Hz, $^{3}J_{HH}$ = 4.1 Hz, 1 H), 7.71-7.74 (m, 3 H), 8.14 (s, 1H,), 8.24-8.25 (m, 3 H), 8.41 (dd, $^{3}J_{HH}$ = 8.5 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1H), 8.95 (dd, $^{3}J_{HH}$ = 4.1 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1H) ppm. ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$) δ: 120.7 (HC), 123.9 (q, $^{1}J_{CF}$ = 271.8 Hz, CF_3), 124.7 (HC), 125.7 (HC), 126.0 (q, $^{3}J_{CF}$ = 3.8 Hz, 2 HC), 128.1 (2 HC), 128.3 (HC), 129.1 (HC), 131.6 (q, $^{2}J_{CF}$ = 32.9 Hz C-CF_3), 137.0 (C), 138.1 (HC), 141.2 (C), 142.5 (C), 143.2 (C), 144.7 (C), 150.8 (HC), 156.5 (C) ppm.

^{19}F NMR ($CDCl_3$) δ: - 63.0 ppm. HRMS (EI): calculated for $C_{19}H_{11}F_3N_2S$ $[M]^+$ 356.0595 found 356.0592. Purity 99.67 % (EtOH/Heptane = 10/90, Rt = 5.695 min).

5.1.2.2.15. ***2-(4-Fluorophenyl)-4-(thiophen-3-yl)-1,5-naphthyridine*** **(31)**. The general procedure was followed using imine **6** (5 mmol, 1.006 g) and 3-ethynyltiophene **14** (7 mmol, 0.693 ml) and the reaction mixture was heated at refluxing chloroform for 48 h. Compound **31** was obtained (0.459 g, 30%) as a white solid; m.p. 106-107 (ethyl acetate/hexane). ^{1}H NMR(300 MHz, $CDCl_3$) δ: 7.21-7.25 (m, 2 H), 7.49-7.52 (m, 1 H), 7.64-7.69 (m, 1 H), 7.78-7.80 (m, 1 H), 8.16-8.22 (m, 3 H), 8.29-8.31 (m, 1 H), 8.46 (dd, $^3J_{HH}$ = 8.6 Hz, $^4J_{HH}$ = 1.8 Hz, 1H, H), 9.00 (dd, $^3J_{HH}$ = 4.0 Hz, $^4J_{HH}$ = 1.8 Hz, 1H, H) ppm. ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$) δ: 116.0 (d, $^2J_{CF}$ = 21.7 Hz, 2 HC), 120.6 (HC), 124.6 (HC), 125.6 (HC), 128.0 (HC), 129.1 (HC), 129.6 (d, $^3J_{CF}$ = 8.9 Hz, 2 HC), 135.5 (C), 137.1 (C), 137.9 (HC), 141.1 (C), 143.0 (C), 144.6 (C), 150.3 (HC), 157.0 (C), 164.0 d, $^1J_{CF}$ = 252.3 Hz, C) ppm. ^{19}F NMR ($CDCl_3$) δ: -112.1 to -112.0 ppm. HRMS (EI): calculated for $C_{18}H_{11}FN_2S$ $[M]^+$ 306.0627 found 306.0633. Purity 99.97 % (EtOH/Heptane = 10/90, Rt = 5.679 min).

5.1.2.2.16. ***4-Phenyl-3-methyl-2(4-trifluoromethylphenyl)-1,5-naphthyridine*** **(32).** The general procedure was followed using imine **5** (5 mmol, 1.250 g) and propyl-1-yl-benzene **15** (7 mmol, 0.802 ml) and the reaction mixture was heated at refluxing chloroform for 58 h. Compound **32** was obtained (0.910 g, 50%) as a yellowish solid; m.p. 189-190 (ethyl acetate/hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.25 (CH_3), 7.38-7.40 (m, 2 H), 7.48-7.52 (m, 1 H), 7.55-7.61 (m, 3 H), 7.79 (q, $^3J_{HH}$ = 8.5 Hz, 4 H), 8.45 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H), 8.95 (dd, $^3J_{HH}$ = 4.1 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H) ppm. ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) δ: 19.1 (CH_3), 123.8 (HC), 124.7 (q, $^1J_{CF}$ = 275.0 Hz, CF_3), 125.5 (q, $^3J_{CF}$ = 3.7 Hz, 2 HC), 128.1 (HC), 128.4 (2 HC), 129.5 (2 HC), 130.1 (2 HC), 130.5 (C), 130.6 (q, $^2J_{CF}$ = 32.5 Hz, C-CF_3), 136.6 (C), 137.3 (HC),

141.8 (C), 142.2 (C), 144.6 (C), 149.3 (C), 151.3 (HC), 160.3 (C) ppm. ^{19}F NMR ($CDCl_3$) δ: - 63.0 ppm. HRMS (EI): calculated for $C_{22}H_{15}F_3N_2$ $[M]^+$ 364.1187 found 364.1189. Purity 97.43 % (EtOH/Heptane = 10/90, Rt = 5.507 min).

5.2. Biology

All used protocols described in this work were approved by the Animal Care Committee of University of Leon, project license PI12/00104. It complies with European Union Legislation (2010/63/UE) and Spanish Act (RD 53/2013).

5.2.1. ***L. infantum*** **strain and culture conditions.** A genetically modified *L. infantum* BCN150 strain that constitutively produces the near infrared fluorescent protein iRFP (*L. infantum*-iRFP) as readable reporter [13] was used in the experiments. Promastigotes are routinely cultured in M199 medium supplemented with 25 mM HEPES (pH 6.9), 10 mM glutamine, 7.6 mM hemin, 0.1 mM adenosine, 0.01 mM folic acid, 1× RPMI 1640 vitamin mix (Sigma, Aldrich), 10% (v/v) heat-inactivated foetal calf serum (FCS) and antibiotic cocktail (50 U/mL penicillin and 50 μg/mL streptomycin). To test the leishmanicidal effect of compounds on promastigotes, 10^6 cells/mL were seeded in black 96-well plates with clear bottom. Stock solutions of each naphthyridine were prepared in DMSO and stepwise (three-fold) diluted in M199 media and growing concentrations of each compound (0.07 to 300 mM) were added to each well. Final concentration of DMSO in each well was never higher than 0.1% (v/v). The viability of promastigotes was monitored recording the infrared emitted fluorescence of living promastigotes in an Odyssey facility (λ exc. 684 nm; λ em. 708 nm) (LI-COR, USA). AMB deoxycholate was used as positive control, whereas 0.1 (v/v) DMSO – the maximum concentration of solvent used in the experiments – was included as negative control. All compounds and controls were assayed by triplicate.

5.2.2. **Intracellular *ex vivo* infections.** The effect of 1,5-naphthyridines on intracellular amastigotes of *L. infantum* was performed in *ex vivo* splenic explants prepared from infected mice according to [13]. Female BALB/c mice were infected intravenous with 108 metacyclic promastigotes of *L. infantum* iRFP. Briefly, after 5 weeks post-infection, animals were euthanized, and their spleens were aseptically recovered to obtain a primary culture of infected splenic explant. Spleens were washed in cold phosphate-buffered saline (PBS) and cut in small pieces. To obtain a single cell suspension, the tissue was incubated with 5 mL of collagenase D (Roche®) at 2 mg/mL prepared in buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2) for 20 min at 37 °C. The cell suspension and remaining tissue fragments were gently passed through 100 mm mesh to remove the tissue fragments. After washing 3x by centrifugation (500 × g for 7 min at 4 °C) with PBS, splenocytes were seeded in black 384-wells clear bottom plates in RPMI supplemented with 20% (v/v) FCS, 1 mM sodium pyruvate, 1x RPMI vitamins, 10 mM HEPES and 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were counted and diluted at different cell densities in order to fix the fluorescence level per well. Different concentrations (in a concentration range between 10 and 0.07 μM) of naphthyrydines were added to the cultures for an extended period of 72 h. Amastigote viability was assessed by recording the fluorescence emission of infected splenocytes at 708 nm in an Odyssey (Li-Cor) infrared imaging system.

5.2.3. **Cytotoxicity assessment and selectivity index (SI) determination.** The cytotoxicity of substituted 1,5–naphthyridines was assessed on mouse uninfected splenocytes. The splenocytes used to determine cytotoxicity were obtained and cultured as described above. Cells were seeded in 96-well plates in the presence of different concentrations (in a concentration range of 100 to 0.8 μM) of the assayed compounds

for 72 h at 37 °C. Alamar Blue staining method (Invitrogen) was used to determine the viability of the cultures, following the manufacturer's instructions. Cytotoxic concentration 50 (CC_{50}) was determined by dose-response curves using Sigma Plot program. Selectivity Indexes (SI) for the compounds were determined as the ratio between the CC_{50} values for non-infected mouse splenocytes and the IC_{50} values for amastigotes in infected splenocytes.

5.2.4. **Production of recombinant TopIB enzymes.** Cloning, expression and purification of LTopIB and hTopIB open reading frames were carried out according to previously published protocols [7]. Briefly, Saccharomyces cerevisiae EKY3 strain deficient in TopIB activity [MAT α ura3-52 his3Δ200 leu2 Δ1 trp1 Δ63 top1 Δ:TRP1], was transformed with the pESC-URA vector, which carries both LTopIB or hTopIB ORFs. Single colonies were incubated overnight in SC-uracil media supplemented with 2% dextrose (w/v). The induction of GAL1/GAL10 promoters was performed with 2% galactose (w/v) for 6h. Yeasts were harvested, washed with cold 1 x TEEG buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 10% glycerol) and resuspended in 15 mL of the same buffer supplemented with 0.2 M KCl and protease inhibitors cocktail (Roche Farma SA, Spain). Yeasts were disrupted by vortexing in a glass bead-beater at 4 ºC. Protein extracts were obtained by centrifugation at 15,000 x g for 45 min in cold and precipitated with ammonium sulphate added to 75% saturation at 4°C. Enzymes were further purified by ion-exchange (P-11 phosphocellulose) and hydrophobic interaction (phenyl-Sepharose) using an Akta FPLC device (GE Healthcare).

5.2.5. **DNA relaxation assays**. Relaxation activity were detected by incubating recombinant LTopIB or hTopIB enzymes with negatively supercoiled plasmid DNA. The reaction mixture contained 0.5 µg of circular supercoiled pSK DNA, 10 mM Tris-HCl buffer pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mg/mL bovine serum albumin, 50

mM KCl, and 1 unit of the corresponding enzyme in a final volume of 20 μL. Reaction mixtures were incubated at different times in a water bath at 37 ºC. Enzyme reactions were stopped by the addition of 1% SDS (w/v) (final concentration). DNA topoisomers were resolved by agarose gel 1% (w/v) electrophoresis prepared in 0.1 M Tris borate EDTA buffer (pH 8.0) at 20 V/cm O/N. Gels were visualized with UV illumination after ethidium bromide (0.5 μg/mL) staining and the images of gels were acquired with a G-BOX (Syngene UK).

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List of Captions

Figure 1. Structure of camptothecin (left), amphotericin B (middle) and newly synthesized 1,5-naphthyridines (right).

Scheme 1. Preparation of novel 1,5-naphthyridines **17-32**. Reagents and conditions: (A) 3-pyridylamine **1** (1 equiv), aldehyde **2** (1 equiv), molecular sieves 4 Å, chloroform, reflux, 15-48 h. (B) alkyne **10-15**, chloroform, reflux, 15-70h.

Chart 1. Structures of aldimines **3-9** and acetylenes **10-15** used.

Chart 2. Structures of 1,5-naphthyridines **17-32** obtained ([a] Reference 27).

Table 1: Chemical structure and biological activity of 1,5-naphthyridine compounds (**17-32**) on *L. infantum* iRFP promastigotes and amastigotes (naturally-infecting mouse macrophages). Each value is the mean ± sd of at least three different experiments made by triplicate.

Figure 2. Representative agarose gels showing the role of preincubation time in the inhibition of the relaxation activity of TopIB. Compound **17** was assayed on LdTopIB (top panel) and on hTopIB (bottom panel) without (figures a and d) and with 10 min preincubation at 4°C (figures b and e) and at 37 °C (figures c and f), respectively. The relative position of the negatively supercoiled DNA substrate is indicated by SC, N is the nicked DNA. Reactions were stopped with 1% (w/v) SDS. Lanes (C) contain 0.5 µg of pSK plasmid DNA. Lanes (DMSO) show the activity of the enzymes in absence of drugs but with 2.5% (v/v) DMSO. Lanes (CPT) show the inhibitory effect of 100 µM CPT. Lanes (6a) show the effect of 100 µM of compound **17**.

Table 2. Inhibition of relaxation activity of LTopIB and hTopIB by 1,5-naphthyridines **17**-**32**. Each value is the mean ± sd of at least three different experiments made by triplicate.

Substituted 1,5-naphthyridine derivatives as novel antileishmanial agents. Synthesis and biological evaluation.

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HIGHLIGHTS.

1. Several 1,5-naphthyridines were prepared with a high diversity of substituents.
2. Synthesized compounds show inhibitory effects against LTopI and hTopI mediated relaxation comparable to those observed for the natural inhibitor, camptothecin (CPT).
3. Some of prepared compounds present significant antileishmanial activity comparable to the activity of amphotericin B.
4. Some of prepared compounds show excellent selectivity indexes (SI).