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Synthesis and Biological Evaluation of Substituted Quinolines: Potential Treatment of Protozoal and Retroviral Co-infections

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Abstract—We report the synthesis of substituted quinolines and their in vitro biological evaluation against the causal agents of cutaneous leishmaniasis, visceral leishmaniasis, African trypanosomiasis and Chagas' disease. Furthermore, several quinolines have also been tested for their anti-retroviral activity in HIV-1 infected cells. The structure–activity relationships of these new synthetic compounds are discussed and emphasis was placed on the treatment of *leishmania*/HIV co-infections.

Introduction

Parasitic diseases such as leishmaniasis and trypanosomiasis have significant impacts in developing countries, with infections spread over several hundred millions of people and are the cause of a mortality rate of several millions per year. Conventional chemotherapies are often inadequate, toxic or are becoming less effective due to emergence of numerous resistances.[1] There is an urgent need for new drugs for the chemotherapy of human African trypanosomiasis (caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodiense*), Chagas disease[2,3] [caused by *Trypanosoma* (Schizotrypanum) *cruzi*] and leishmaniases (*Leishmania* spp).

Treatments of Human African trypanosomiasis, commonly known as sleeping sickness, (pentamidine, suramin and melarsoprol) often induce serious side effects.

Chemotherapy of Chagas' disease is still unsatisfactory and, since Nifurtimox (a nitrofuran) is no more available, chemotherapies are nowadays exclusively based on nitroimidazoles treatments (e.g., benznidazole, Roche). Although benznidazole has an efficiency in the acute and short-term (up to a few years) chronic phases,[4] no benefits could be definitively evidenced for chronically infected patients.

The leishmaniases[5] constitute a diverse group of diseases that varies from simple cutaneous leishmaniasis to visceral leishmaniasis (VL), a very fatal disease if left untreated. VL, also known as 'kala-azar' is a severe disease and epidemics are generally devastating. More recently, VL has also gained attention as an opportunistic infection in AIDS. Owing to the fact that overlapping geographical distribution of leishmaniases and HIV/AIDS is increasing, *Leishmania*–HIV co-infection has been regarded as an emerging infectious disease.[5,6] For 50 years, the pentavalent antimony compounds sodium stilbogluconate (Pentostam, Glaxo Wellcome, UK) and meglumine antimonate (Glucantime, Aventis, France) have been the first-line treatments of leishmaniases. However, relapse is frequent and resistance to antimony is growing. Amphotericin B and pentamidine, the parenteral alternatives to antimony are considered to cause serious and irreversible toxic effects. New chemotherapeutic methods have been proposed based

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on the use of other non-parenteral agents such as the aminoglycoside, aminosidine (topical application) or oral agents like hexadecylphosphocholine (miltefosine). The latter molecule, an antineoplastic agent, has been identified as the first effective oral treatment in visceral infection, and is currently in phase IV clinical studies.[7]

To develop other alternative drugs, new compounds have been looked for based on empirical screening or ethnopharmacological studies, and 2-substituted quinolines isolated from a Bolivian medicinal plant, *Galipea longiflora* Kr (Rutaceae),[8] have shown efficacy in the experimental treatment of cutaneous leishmaniasis of the New World.[9,10] The activity of these compounds has also been described in the experimental treatment of VL by oral and parenteral routes.[11]

Recently, the effects of oral treatment of 2-*n*-propylquinoline, a major alkaloid isolated from *G. longiflora* Kr (Rutaceae) were evaluated in Balb/c mice chronically infected with *T. cruzi*, and preliminary data indicate that the serological response, evidenced by ELISA (enzyme linked immuno assay), is significantly different from that of the controls and of the benznidazole-treated mice.[12]

In order to get information on the structure–activity relationship in this family, Fakhfakh et al.,[13] have synthesised a library of 2-substituted quinolines in solution from Grignard reagents and a chloride salt of *N*-oxyquinoline. Then 2-alkenylquinolines were prepared either from-1,1-dibromo-2-(2-quinolyl)-ethylene[14] or from 2-quinaldehyde.[15] Finally, 3-substituted,[16] and disubstituted quinolines were also synthesised.[15]

Results and Discussion

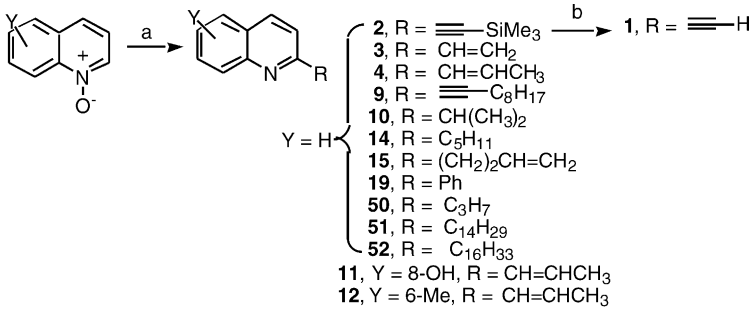
Chemistry

Compounds **2–4**, **9**, **10**, **14**, **15**, **19**, **50–52** were prepared from *N*-oxyquinoline, by addition of isobutyl chloroformate followed by the desired Grignard reagent, to afford the corresponding quinoline in good yields (32–82%), as reported.[13] Compounds **11** and **12** were obtained through the same procedure but starting from *N*-oxy-8-hydroxy- and *N*-oxy-6-methylquinoline, in 23 and 30% yield, respectively. Compound **1** was obtained in 88% yield from **2** by TBAF treatment in THF (Scheme 1).

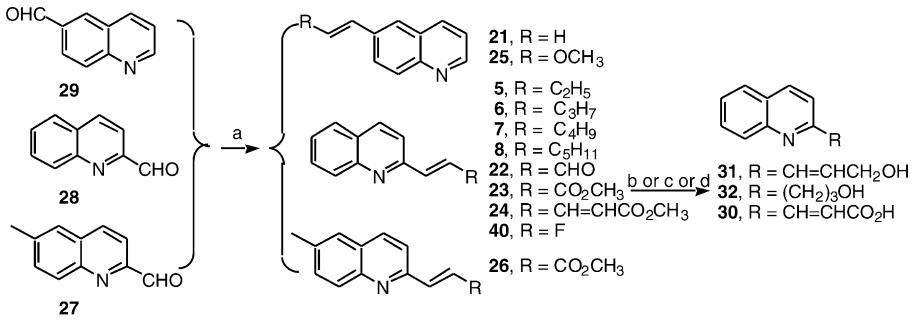
Compounds **5–8**, **22**, and **23** were obtained in good yields (54–84%) by treatment of 2-quinaldehyde **28** by the desired alkyltriphenylphosphonium bromide, in the presence of *t*-BuOK in toluene at 0 °C. Compounds **21**, **25** were obtained in moderate non-optimized yields (21, 15%, respectively) from 6-quinaldehyde **29**, under the same reaction conditions, whereas **24** was obtained from **22** in 63% yield. Compound **26** was obtained in 63% yield from **27** (which was prepared by selenium oxidation of commercially available 2,6-dimethylquinoline) under the same reaction conditions (Scheme 2).

Carboxylic acid **30** was obtained in 60% yield from ester **23**, by KOH treatment, whereas alcohols **31** and **32** were obtained in 49 and 60% yield by DIBAL reduction of **23**, depending on the reaction conditions (stoïchiometric and excess of DIBAL, respectively) (Scheme 2).

Alcohols **35** and **37** were obtained in 53 and 42% yield, from 2-quinaldehyde, by addition of the required



Scheme 1. (a) *i*-BuCOCl, then RMgBr, −78 °C, THF; (b) TBAF, THF.



Scheme 2. (a) Ph_3P=CHR, toluene, 0 °C; (b) DIBAL, −78 °C; (c) DIBAL, excess; (d) KOH, MeOH, H_2O.

Grignard reagent. Compounds **33** and **43** were obtained in 57% yield, from 2-quinaldine, by treatment with pyridine followed by addition of the desired carboxylic anhydride, whereas when 2-quinaldine was treated by butyllithium and then by acetaldehyde, compound **34** was obtained in 60% yield. Under the same reaction conditions (but with 2 equiv of butyllithium), 8-hydroxy-2-methylquinoline gave **36** in 53% yield (Scheme 3).

Compounds **38** and **39** were obtained in 89 and 22% yield, respectively, by treatment of **28** and **29** by PPh_3 and CBr_4. Then when **38** was treated by 1 equiv of *i*-PrMgCl in the presence of a catalytic amount of Fe(acac)$_3$ in a 1/1 mixture of THF/NMP, **42** was obtained in 84% yield, however if a second equiv of a Grignard reagent was added, thus compounds **16** and **17** were obtained in 63 and 47% yield, respectively. Compounds **45** and **46** were obtained in 60 and 72% yield, respectively, when **38** was mixed with the desired alkyne in toluene in the presence of *N*,*N*-diisopropylethylamine and catalytic amounts of $Pd(PPh_3)_4$ and CuI. However, compound **47** was obtained in 20% yield (non-optimized) when **42** was treated by **1** in THF in the presence of pyrrolidine and catalytic amounts of $Pd(PPh_3)_4$ and CuI (Scheme 4).

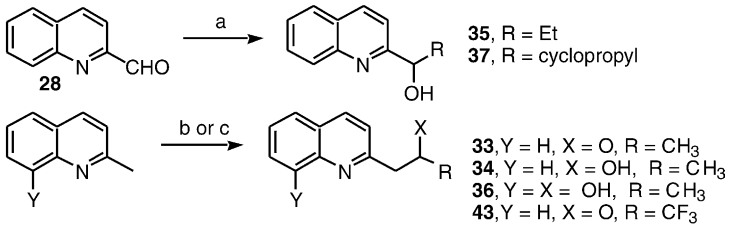
Compound **41** was obtained from **38** in 87% yield by *t*-BuOK treatment (whereas **1** was alternatively obtained from **38** by butyllithium treatment). When **1** in Et_2O was mixed with *E*-dichloroethylene in the presence of piperidine and catalytic amounts of $Pd(PPh_3)_4$ and CuI, compound **44** was obtained in 52% yield (Scheme 5).

Compound **13** was obtained in 34% yield when 3-bromoquinoline was treated by propenylmagnesium bromide in a 1/1 mixture of NMP/THF, in the presence of a catalytic amount of Co(acac)$_2$. Whereas **20** was prepared in 46% yield when 3-bromoquinoline was treated at 30 °C by phenylmagnesium bromide in THF in the presence of a catalytic amount of Fe(acac)$_3$. Compounds **18** and **48** were obtained in 98 and 70% yield, respectively, from 3-bromoquinoline, in pyrrolidine, and in the presence of the desired alkyne and catalytic amounts of $Pd(PPh_3)_4$ and CuI (Scheme 6).

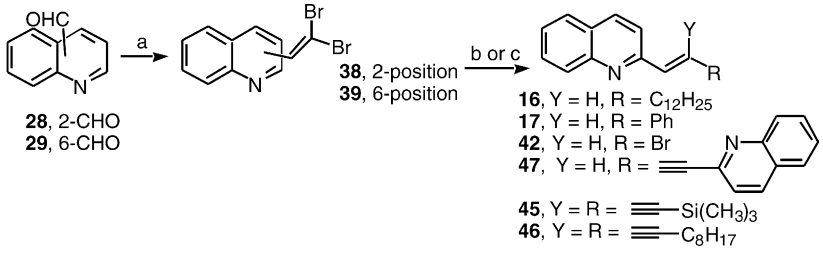
Finally, compound **49** was obtained in 42% yield by heating 2-quinaldine and 2-quinaldehyde **28** in the presence of acetic anhydride (Scheme 7).

Biological results

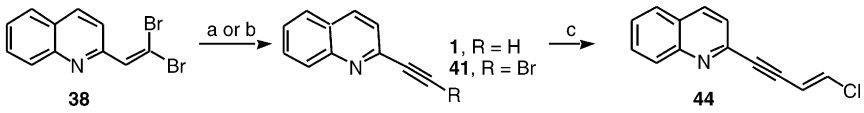
Anti-protozoal, anti-retroviral and cytotoxic activities of various synthetic compounds were presented in four tables. Table 1 shows the activities of 21 unfunctionalized alkenyl and alkynylquinolines (**1**–**21**) against intracellular amastigotes of *L. amazonensis*, *L. infantum*, and



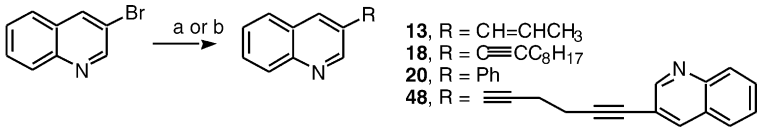
Scheme 3. (a) RMgBr; (b) BuLi, then RCHO; (c) pyridine, then RCO_2COR.



Scheme 4. (a) PPh_3, CBr_4; (b) (i) *i*-PrMgCl, Fe(acec)$_3$; (c) alkyne, $Pd(PPh_3)_4$, CuI.

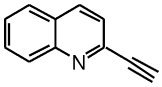
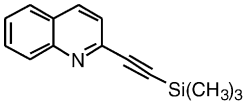
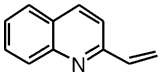
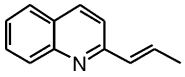
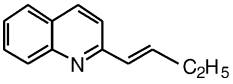
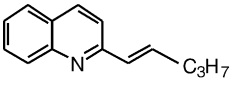
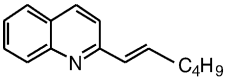
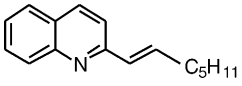
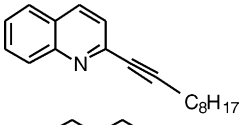
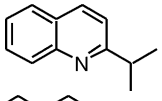
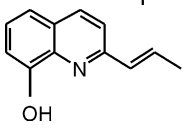
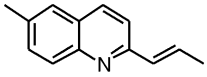
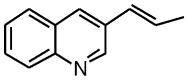
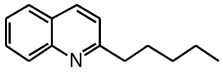
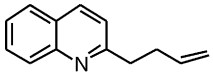
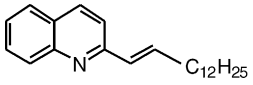
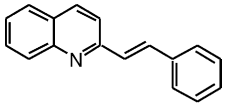


Scheme 5. BuLi (2 equiv); (b) *t*-BuOK (2 equiv); (c) (*E*)-ClCH=CHCl, piperidine, Et_2O, $Pd(PPh_3)_4$, CuI.



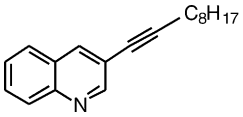
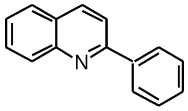
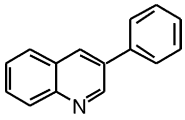
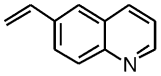
Scheme 6. (a) RMgBr, Co(acac)$_2$ or Fe(acac)$_3$, THF; (b) alkyne, $Pd(PPh_3)_4$, CuI.

Table 1. In vitro cytotoxicity of unfunctionalized alkenyl and alkynylquinolines upon bone marrow-derived macrophages and amastigote or trypomastigote forms of *L. amazonensis*, *L. infantum*, *T. brucei* and *T. cruzi* parasites

Compd		IC_{50} (μM)				
		Cytotoxicity macrophages	*L. amazonensis* amastigotes[a]	*L. infantum* amastigotes[b]	*T. brucei* trypomastigotes[c]	*T. cruzi* amastigotes[d]
1		>41	<5	32	>32	8
2		7	56	8	>32	8
3		40	21	32	13	12
4		74	37	12	16	>32
5		137	34	ND	17	>32
6		>200	63	>32	16	>32
7		>200	>200	16	4	11
8		111	56	28	4	11
9		47	47	ND	>32	>32
10		>200	>200	>32	>32	>32
11		>200	>200	14	>32	19
12		137	68	ND	ND	ND
13		148	148	ND	ND	ND
14		>200	>200	16	13	19
15		>200	>200	30	ND	ND
16		>200	>200	>32	ND	ND
17		>200	<100	11	4	14

(continued)

Table 1 (*continued*)

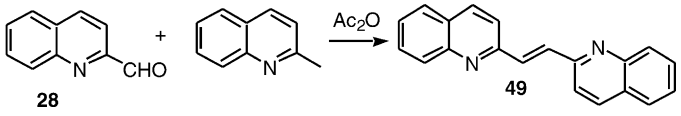
Compd		IC$_{50}$ (μM)				
		Cytotoxicity macrophages	*L. amazonensis* amastigotes[a]	*L. infantum* amastigotes[b]	*T. brucei* trypomastigotes[c]	*T. cruzi* amastigotes[d]
18		>200	>200	ND	ND	ND
19		>200	<100	ND	ND	ND
20		>200	<100	ND	ND	ND
21		>200	>200	ND	ND	ND
	Amphotericin B	25	0.13	ND	ND	ND
	Glucantime®	>200	20	7	ND	ND
	Suramin	ND	ND	ND	0.09	ND
	Nifurtimox	ND	ND	ND	ND	0.4

[a]Thirty-hour contact with parasites.
[b]Five-day contact with parasites.
[c]Four-day contact with parasites.
[d]Twenty four-hour contact with parasites.

T. cruzi and extracellular trypomastigotes of *T. brucei*. Cytotoxicity against host cell macrophages is also represented for all compounds tested. In the case of leishmanicidal activity, compound **1** (2-ethynylquinoline) displayed against *L. amazonensis* amastigotes a lower IC$_{50}$ (around 5 μM) than the reference drug, glucantime® (20 μM) which shows identical activity than compound **3** (2-vinylquinoline). All compounds have a lower activity against *L. infantum* amastigotes than glucantime® (7 μM) excepted for compound **2** (2-trimethylsilylethynylquinoline with IC$_{50}$ = 8 μM), but this compound showed a high cytotoxicity against macrophages. Among these compounds, the alkenylquinolines **7**, **8** and **17** inhibited the growth of trypomastigote forms of *T. brucei* with an IC$_{50}$ around 4 μM. However, these quinolines are 20 times less active than the reference drug, suramin (0.09 μM). Against the amastigote forms of *T. cruzi*, compounds **1** and **2** were the more active ones (IC$_{50}$ = 8 μM), but still much less active than nifurtimox (IC$_{50}$ = 0.4 μM). Increasing the carbon chain length did not result in an increase activity against the parasites.

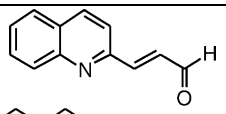
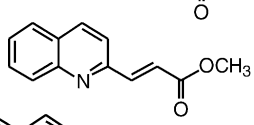
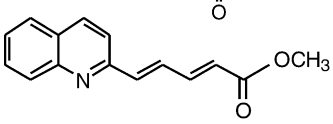
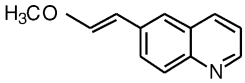
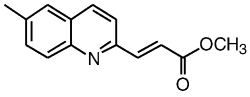
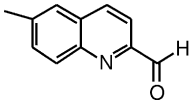
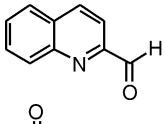
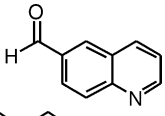
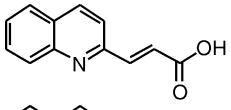
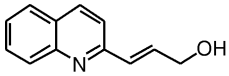
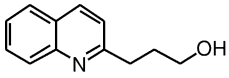
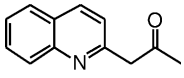
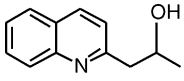
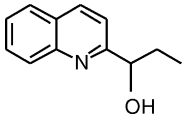
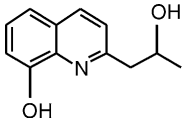
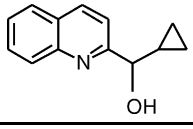
From Table 2, presenting the data obtained from 16 functionalized quinolines (compounds **22**–**37**), some observations can be made on the structure–activity relationship. Compounds **22** and **31** have a significant activity on the inhibition of *L. amazonensis* and *L. infantum* amastigote proliferation in macrophages (both compounds have an IC$_{50}$ of 4 μM and 2 μM, respectively). These results show the importance of the substitution, at the 2 position, by an α-β-unsaturated aldehyde or an allylic alcohol, and the presence of a double bond on the lateral chain, which induces an increase activity against the intracellular amastigotes of *L. amazonensis* and *L. infantum*. However, the unsaturated ester (compound **23**), less electron attractor than compound **22**, induced a lost of activity but was less cytotoxic. Among the functionalized quinolines, compounds **22** and **31** were also found the most efficient ones for inhibiting the amastigote forms of *T. cruzi*, with an IC$_{50}$ around 5 μM. Compound **35** (1-quinol-2-yl-propan-1-ol) was the most efficient one against trypomastigotes of *T. brucei* with IC$_{50}$ = 3 μM. On the other hand, compounds **31**, **34**, **36** and **37,** which all possess an hydroxyl group on the lateral chain in position 2 or 3, showed weaker activity against *T. brucei*.

In Table 3, 12 halogeneous and miscellaneous functionalized quinolines were tested for their anti-leishmanial and anti-trypanosomal activities. Compounds **38** (1,1-dibromo-2-(quinolyl)-1-ethene and **42** [1-bromo-2-(2-quinolyl)ethylene] displayed notably high activity against amastigotes of *L. infantum* (IC$_{50}$ = 2 μM) and



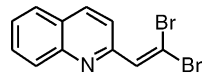
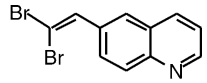
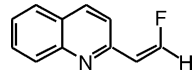
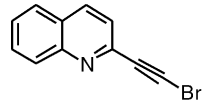
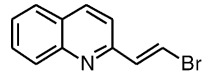
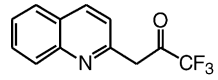
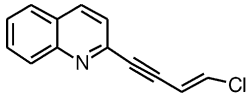
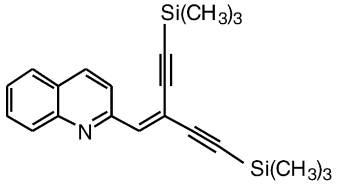
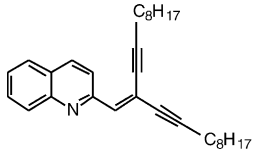
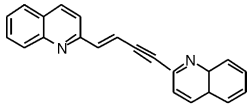
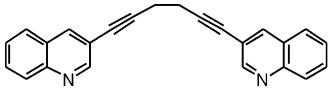
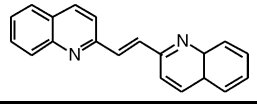
Scheme 7.

Table 2. In vitro cytotoxicity of functionalized quinolines upon bone marrow-derived macrophages and amastigote or trypomastigote forms of *L. amazonensis*, *L. infantum*, *T. brucei* and *T. cruzi* parasites

Compd		IC_{50} (μM)[a]				
		Cytotoxicity macrophages	*L. amazonensis* amastigotes	*L. infantum* amastigotes	*T. brucei* trypomastigotes	*T. cruzi* amastigotes
22		9	4	2	19	5
23		> 200	59	26	16	15
24		105	47	> 32	> 32	26
25		117	117	ND	ND	ND
26		> 200	> 200	8	> 32	25
27		> 200	> 200	> 32	> 32	> 32
28		> 200	> 200	ND	ND	ND
29		> 200	> 200	ND	ND	ND
30		63	63	> 32	> 32	> 32
31		34	4	2	> 32	4
32		> 200	67	ND	ND	ND
33		> 200	> 200	> 32	10	> 32
34		> 200	134	ND	16	> 32
35		> 200	> 200	16	3	> 32
36		> 200	> 200	ND	16	16
37		> 200	> 200	> 32	14	> 32

[a]For lengths of treatments and data for reference drugs, see Table 1.

Table 3. In vitro cytotoxicity of halogenous and miscellaneous functionalized quinolines upon bone marrow-derived macrophages and amastigote or trypomastigote forms of *L. amazonensis*, *L. infantum*, *T. brucei* and *T. cruzi* parasites

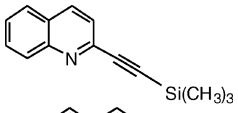
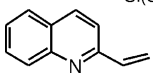
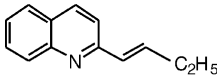
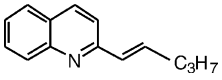
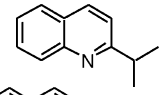
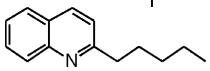
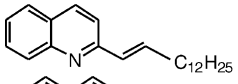
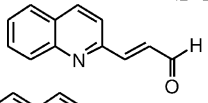
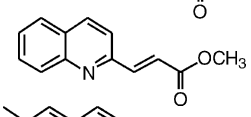
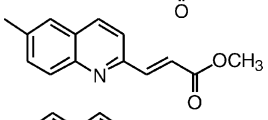
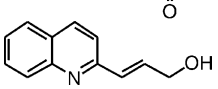
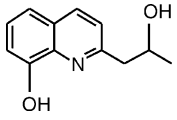
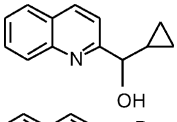
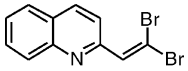
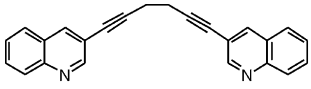
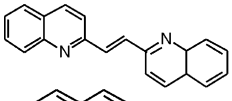
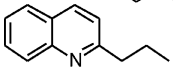
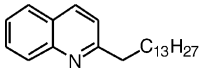
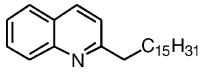
Compd		IC_{50} (μM)[a]				
		Cytotoxicity macrophages	*L. amazonensis* amastigotes	*L. infantum* amastigotes	*T. brucei* trypomastigotes	*T. cruzi* amastigotes
38		80	159	2	4	>32
39		>200	>200	ND	ND	ND
40		100	100	ND	ND	ND
41		54	54	>32	>32	>32
42		13	3	2	1	0.15
43		>200	>200	ND	ND	ND
44		>200	59	ND	ND	ND
45		72	72	ND	ND	ND
46		>200	>200	ND	ND	ND
47		>200	>200	17	ND	ND
48		>200	>200	17	13	>32
49		>200	>200	8	24	>32

[a]For lengths of treatments and data for reference drugs, see Table 1.

trypomastigotes of *T. brucei* (IC_{50} = 4 and 1 μM, respectively). The 1,1-dibromoalkene (compound **38**) was significantly less cytotoxic towards macrophages (IC_{50} = 80 μM) than 1-monobromo compound **42** (IC_{50} = 13 μM). Compound **42** showed also a good anti-*L. amazonensis* activity with an IC_{50} value of 3 μM against amastigotes. Looking at the regression of parasitophorous vacuoles and the overall decrease in parasite number, compound **42** showed complete clearance of amastigotes (at 3 μM) (Fig. 1).

Otherwise, this same compound **42** was more active against the amastigote forms of *T. cruzi* (IC_{50} = 0.15 μM) than the reference drug, nifurtimox (IC_{50} = 0.4 μM, Table 1). Indeed, compound **42** is the 2-substituted quinoline which holds the most potent anti-protozoal activity described in this study. All compounds within the group of bisquinolines (**47**–**49**) or compounds **39**–**41** and **43**–**44** from the halogenated product family were either inactive against *L. amazonensis* or possessed the same or higher level of cytotoxicity against host cells.

Table 4. Inhibition of HIV-1 replication in CEM4fx cells by mono or polysubstituted quinolines

Compd		Antiviral activity IC_{50} (μM)[a]	Cytotoxicity on CEM4fx Cells TC_{50} (μM)[b]
2		0.5	1.8±0.2
3		7.2	9.4±4
5		6.0	5.5±0.4
6		7.9	8.5±1.2
10		4.6	5.9±3
14		2.9	7.5±2.5
16		>100	>100
22		0.5	1.0±0.2
23		>100	>100
26		32.2	30.6±5
31		3.6	1.7±0.3
36		2.7	4.5±0.9
37		9.8	20.4±6
38		3.3	5.3±0.6
48		135	168±51
49		>300	>300
50		78	84±21
51		108	110
52		>300	>300
Ref.	L-731,988	1.2±0.3	>100 μM

[a]Experimental data of 3 days treatment of three independent experiments were averaged and fitted on a sigmoïdal dose–response model in order to evaluate a TC_{50} for each compound. SE are standard deviations of best-fit values.
[b]Cytotoxicity of most compounds masked the true antiviral activity of most compounds thus preventing fitting experimental data to a significant pharmacological model. Therefore, the antiviral activity was merely estimated by graphical determination of the IC_{50} corresponding to the drug concentration for which 50% of viral production was observed as compared to the non-treated control.

Finally, among the 49 compounds tested for anti-protozoal activities, 19 were evaluated for their in vitro antiviral activity, looking for the inhibition of HIV-1 replication in CEM4fx cells (Table 4). Compounds **2** and **22** exhibited submicromolar antiviral activity (IC_{50} around 0.5 μM) and compounds **14**, **31**, **36** and **38** showed IC_{50} in the micromolar range. We observed that two of these compounds possess unsaturated side chain with three carbon atoms (**22** and **31**), whereas compound **14** is an alkylquinoline.

The results presented herein confirm the crucial role of the side chain length for antileishmanial or trypanosomal activity. In general, the most active quinolines were those with a three carbon alkenyl side chain, containing reactive electrophilic functions such as a carbonyl (compound **22**), an hydroxyl (compound **31**) or an halogen (compound **42**). Although compounds **22** and **31** showed a toxicity on HIV-1 host cells but not on macrophages, they possessed significant in vitro activity against HIV-1 replication and they were the most active quinolines against *Leishmania* parasites. The mechanism by which these quinolines are active against parasites or HIV-1 is unknown. It could be due to either a direct action of these molecules on molecular targets specific of the pathogen or an indirect action involving the intervention of host-specific immunoregulatory molecules like TNF-α, a molecule which has been shown to play an important role in the pathogenesis of *L. infantum* and HIV-1 infections.[17,18]

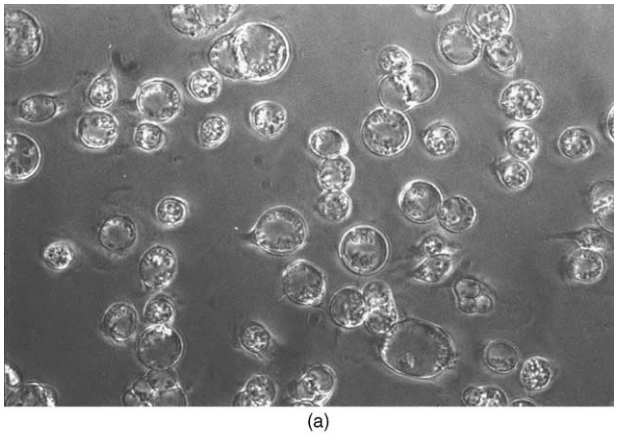


Figure 1. The effect of compound **42** on *L. amazonensis*-infected macrophages. Bone marrow-derived macrophages were infected with *L. amazonensis* amastigotes. After 24 h of infection and for an additional 30-h period, infected cells were exposed either to DMSO (a) or to compound **42** (b, 3 μM). (a) Amastigotes are lodged within large PV and 100% of macrophages are infected. (b) PV and parasites are no more detectable.

Interestingly, several quinolines (**2**, **3**, **36**, **48**) were found to exhibit significant activity against HTLV-1 transformed cells.[19] Indeed, at 10 μM, all these compounds inhibited more than 80% of the virus replication. Further studies are necessary to evaluate the efficiency of these compounds for the treatment of adult-T-cell leukemia/lymphoma (ATLL), a severe HTLV-I-associated disease.[20,21]

The results obtained in the present study are interesting and represent a screening of new quinolines with anti-protozoal and/or anti HIV-1 activities. Our results indicate that two compounds (**22** and **31**) show the best activities against intracellular amastigotes forms of *L. amazonensis* and *L. infantum* and also against the replication of HIV-1. The activities described herein represent a major advance in the search for the treatment of co-infections protozoal/retrovirus,[22] and particularly *Leishmania*/HIV, but these findings must be validated in experimental in vivo studies.

Experimental

Chemistry

The compounds were prepared through the methods published.[13–16] Full experimental procedures with spectroscopic data of the 52 compounds can be obtained as supplementary material, which can be found in the online version of this paper.

In vitro activity against intracellular *L. amazonensis* amastigotes

Female BALB/c mice aged 2–4 months are obtained from the breeding center of the Pasteur Institute. *L. amazonensis* strain LV79 (MPRO/BR/1972/M1841) was propagated in BALB/c mice. *L. amazonensis* amastigotes were isolated from lesions and purified as described earlier[1] Bone marrow plugs from tibias and femurs of BALB/c mice were suspended in RPMI 1640 medium (Seromed) supplemented with 10% heat-inactivated fetal calf serum (FCS, Dutscher, Brumath, France), 50 μg/mL of streptomycin, 50 IU/mL of penicillin (culture medium) and with 15% L-929 fibroblast-conditioned medium. Cells were then distributed in bacteriologic Petri dishes (Greiner, Germany) and were incubated at 37 °C in a 5% CO_2 atmosphere. Five days later, adherent macrophages were washed with Dulbecco's phosphate buffered solution (PBS) and taken off by treatment for 20 min at 37 °C with Ca^{2+}-and Mg^{2+}-free Dulbecco's PBS containing 2 mg/mL of glucose. Recovered macrophages were suspended in culture medium and they were then deposited in flat-bottom 96-well plates (Tanner, Switzerland) at a density of 4×10^4 cells/well. Twenty-four hours after replating, macrophages were infected at a multiplicity of five amastigotes per host cell and were incubated at 34 °C, which is the permissive

temperature for the survival and multiplication of LV79 strain amastigotes. In most instances, more than 95% of the macrophages were found to be infected.

For all drugs, stock solutions were prepared in DMSO at a concentration of 500 μg/mL. Two-fold serial dilutions were made from 250 μg/mL in culture medium supplemented with 0.5% DMSO final. Twenty-four hours after infection, freshly prepared drugs are added to the infected cultures in triplicate. The first final drug concentration is 50 μg/mL and the final DMSO concentration is 0.1%. This DMSO concentration was proven to have no effect on control cultures.

Thirty hours after drug addition, infected cultures were examined using an inverted phase contrast Zeiss microscope (magnification of 400). Toxic effects in the macrophages were evidenced by the change in morphological features i.e., loss of refringency, vacuolation of cytoplasm or loss of cytoplasmic material.

Leishmanicidal effects of drugs are detectable looking at the regression of parasitophorous vacuoles and the overall decrease in parasite number. For some drugs, complete clearance of amastigotes was achieved.

In vitro activity against intracellular *L. infantum* amastigotes

L. infantum (strain MHOM/MA/BE/67) was maintained in female Golden hamsters (Iffa Credo, France) by passage every 6–8 weeks. Peritoneal macrophages were harvested from female BALB/c mice by peritoneal washing 24 h after injection of a 2% soluble starch solution (Merck, France). After two washes in PBS the exudate cells were dispensed into 16-well Lab-tek tissue culture slides (Nunc Inc. IL) at 4×10^4/well in a volume of 200 μL of RPMI-1640 medium (Sigma-Aldrich, France) plus 10% FCS. After 24 h, amastigotes of *L. infantum* (derived from the spleen of an infected hamster) were added at an infection ratio of 10/1 together with 2-fold serial dilutions of the drugs to be tested and the cultures were incubated at 37 °C in 5% CO_2–95% air. Five days later, cells were fixed by methanol and stained with Giemsa. Leishmanicidal activity of the drugs was determined by counting the percentage of infected macrophages in treated and untreated cultures. *N*-methylglucamine, antimonate (Aventis, France) was used as a control drug.

In vitro activity against intracellular *T. cruzi* amastigotes

Primary peritoneal macrophages were seeded in 96-well microtiter plates at 3×10^4 cells/well/100 μL in RPMI 1640 medium with 10% FCS and 2 mM L-glutamine. After 24 h, 10^5 trypomastigotes of *T. cruzi* (Tulahuen strain) were added in 100 μL per well with 2-fold serial dilutions of the drugs, in triplicate. The cultures were incubated at 37 °C in 5% CO_2–95% air for 4 days. The IC_{50} were determined spectrophotometrically at 570 nm following addition of the substrate Chlorophenolred-β-D-galactopyranoside (CPRG)/Nonidet during 4 h from the dose response curve.

In vitro activity against *T. brucei* trypomastigotes

Bloodstream forms of *T. brucei* (strain S427) were cultivated in HMI-9 medium and set out in a 96-well microplate at a density of 2×10^4 trypomastigotes per 100 μL of medium per well. Trypanosomes were incubated at 37 °C in the presence of various drug concentrations for 48 h, in triplicate. Alamar Blue® (20 μL) was then added to each well, and the plates incubated for a further 24 h period. Drug effects on parasite multiplication were estimated by monitoring the absorbance of AlamarBlue™ at two wavelengths 570 and 600 nm. The IC_{50}, values were calculated from the dose–response curves.

Antiviral assays

The lymphocytes cell line CEM4fx was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum. Hela-CD4+-ßGal (P4) cells were grown in DMEM with 10% fetal calf serum and 0.5 mg/mL geneticin. Cell-free viral supernatants were obtained by trans-fection of P4 cells with HIV-1 PLN4-3 genomic clone.[23] CEM4fx cells were plated in triplicate on a 96-well plates (100 μL) and infected with cell-free virus. Viral supernatants were removed 2 h after infection and drugs dissolved in DMSO were added in fresh medium. Infected cells were grown in the presence of drugs for 3 days. Supernatants were then collected at $t=72$ h and used to infect P4 cells. P4 cultures were incubated for 24 h and subsequently lyzed in a phosphate buffer containing 50 mM 2-mercaptoethanol, 10 mM $MgSO_4$, 25 mM EDTA, 0.125% NP40. 20 μL of lyzate was incubated with 100 μL of CPRG-containing buffer. The red staining intensity was quantified on a multiscan photometer at 570 nm. CEM4fx cell viability was estimated by the MTT (Sigma) assay after 3 days treatment with drugs (20 μL). A solution of (7.5 mg/mL) in phosphate buffer was added to each well of microtiter trays. Plates were further incubated at 37 °C in a CO_2 incubator for 4 h. Solubilization of formazan crystals was achieved by adding 100 mL of 10% SDS, 10 mM HCI. Absorbance was read in a multiscan photometer at 570 nm. Experiments were performed in triplicate and averaged. The anti-integrase compound L731,988 was used as a positive control for antiviral activity.

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