Structure−Activity Relationship and Studies on the Molecular Mechanism of Leishmanicidal N,C-Coupled Arylisoquinolinium Salts

ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · FEBRUARY 2009

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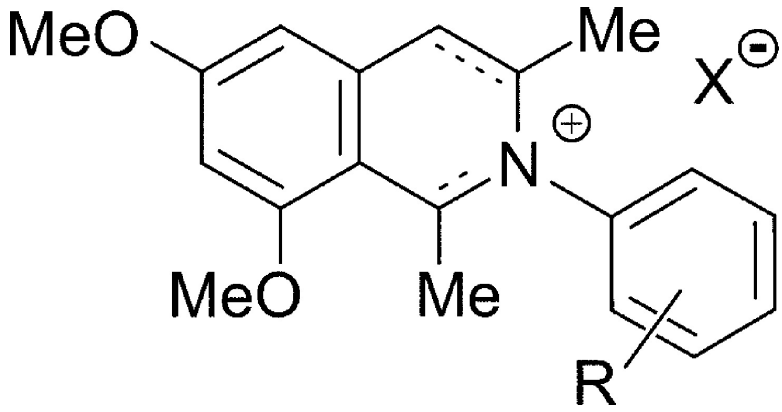
J. Med. Chem., **2009**, 52 (3), 626-636• DOI: 10.1021/jm801084u • Publication Date (Web): 31 December 2008



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Structure−Activity Relationship and Studies on the Molecular Mechanism of Leishmanicidal *N*,*C*-Coupled Arylisoquinolinium Salts

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Received May 14, 2008

Alternative drugs against leishmaniasis are desperately needed. Antimonials, the main chemotherapeutic tool, cause serious side effects and promote chemoresistance. We previously demonstrated that representatives of *N*,*C*-linked arylisoquinolines are promising leishmanicidal drug candidates. We now performed structure−activity relationship studies varying the aryl portion of our lead substrate. The new series of compounds show an enhanced selectivity against *Leishmania major* in comparison to their major host cell, the macrophage. Our results suggest that the arylisoquinolinium salts decrease the macrophage infection rate acting directly on the intracellular parasites. However, the activity of the 4′-*i*-propyl derivative might also involve the modulation of cytokine and nitric oxide production by host macrophages. Additionally, this isoquinoline acts synergistically with amphotericin B and does not interact with drug-metabolizing cytochrome P450 enzymes involved in the metabolism of antileishmanial drugs. The results demonstrate that the newly synthesized structurally simplified *N*,*C*-coupled arylisoquinolinium salts are promising candidates to be considered as leishmanicidal pharmacophores.

Introduction

Chemotherapy against leishmaniasis is unsatisfactory because it is mainly based on antimony agents like sodium stibogluconate and meglumine antimoniate, amphotericin B, miltefosine, and paromomycin (for the chemical structures see Supporting Information). The mode of action of these compounds is poorly understood, and their toxicity causes serious side effects that often result in patients deserting the treatment. According to the World Health Organization (WHO), infections caused by *Leishmania* parasites belong to the most hazardous infectious diseases, the main reasons being the increasing number of cases, where human immunodeficiency virus and *Leishmania* simultaneously infect the patient, and the worldwide escalating frequency of chemoresistance to the standard therapy with antimonial drugs.[1−3] Thus, affordable alternative drugs against leishmaniasis are desperately needed and the search for them is a challenging task.

Naphthylisoquinoline alkaloids constitute an intriguing class of natural products. They are axially chiral secondary metabolites isolated from lianas of the small palaeotropic plant families Ancistrocladaceae and Dioncophyllaceae.[4] These compounds exhibit a very good activity against pathogens causative of several tropical infectious diseases, e.g., *Plasmodium falciparum* and *P. berghei*,[5−7] *Trypanosoma brucei*,[8] and *Leishmania donovani*.[9−11] The study of these pharmacologically interesting natural products reached an essential stage with the discovery of the *N*,*C*-linked arylisoquinolinium salts, a structurally new alkaloid subtype.[12,13] In these compounds, the naphthalene portion is connected with the isoquinoline moiety via a nitrogen−carbon bond causing a positive charge at the nitrogen atom in the isoquinoline portion, whereas usually it is linked via a carbon−carbon biaryl axis.

In a recent study,[14] we compared the efficacy of nine *C*,*C*-coupled naphthylisoquinoline alkaloids to impair the growth of *L. major* promastigotes and their host cells with that of five *N*,*C*-linked substances, three of which were structurally simplified analogues of the natural naphthylisoquinolinium salts. We also evaluated the effectiveness of selected substances like the natural products ancistrocladinium A (**1**) and B (**2**) and the synthetically prepared naphthylisoquinolinium salt **3** (Figure 1) in decreasing the infection rate of peritoneal macrophages containing *L. major* parasites. The results demonstrated that representatives of this new coupling type together with its synthetic derivatives are promising candidates to be considered as lead compounds for leishmanicidal drugs. They are effective against intracellular amastigotes at concentrations in the low submicromolar range, while toxicity against various mammalian cells is observed only at higher concentrations. Additionally, we demonstrated that the leishmanicidal effect of the alkaloids **1** and **2** as well as that of the structurally simplified analogue **3** was not associated with the stimulation of cytokine secretion or the production of nitric oxide by infected macrophages, suggesting that the leishmanicidal activity of these compounds is mainly targeted toward the parasite.[14] Encouraged by these promising results, we performed studies on the structure−activity relationship (SAR) focusing on the aryl portion of our lead structure **3**.

In this report, we present the synthesis and an SAR study on structurally simplified analogues of the naphthylisoquinolinium alkaloids leading to the most active agents **4f**, **4i**, and **4m**, which exhibit an enhanced selectivity against *L. major* compared to **3**. *L. major* promastigotes were sensitive to these derivatives in the low micromolar range. The extent of plasma protein binding of the compounds **4f**, **4i**, and **4m** was in a range between 50

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10.1021/jm801084u CCC: $40.75
Published on Web 12/31/2008

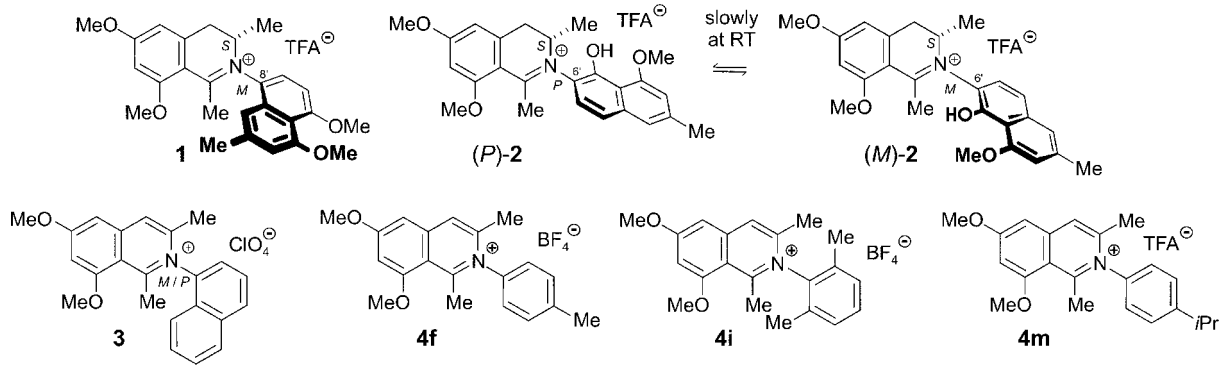


Figure 1. The *N,C*-coupled isoquinolinium alkaloids ancistrocladinium A (**1**) and B (**2**) and their structurally simplified analogues **3**, **4f**, **4i**, and **4m**.

and 70%. This is adequate from the pharmacokinetic point of view for a new lead compound. However, for further developments, it would be fundamental to decrease the extent of plasma protein binding. On the other hand, the arylisoquinolines **4f**, **4i**, and **4m** were toxic against mammalian cell lines at concentrations (≅30 μM, total concentration) that are significantly higher than those effective against parasites. Interestingly, when we assessed the interaction between amphotericin B and the isoquinolines **2**, **3**, **4f**, and **4m** in vitro, using a modified fixed-ratio isobologram method,[15,16] we found that the *i*-propyl derivative **4m** and amphotericin B act in a synergistic manner.

We additionally investigated the drug interaction potential of these substances using in vitro assays.[17] Neither the naphthylisoquinoline **3** nor compounds **4f**, **4i**, or **4m** inhibited significantly the major human drug-metabolizing cytochrome P450 (CYP[a]) enzymes 1A2, 2C8/9/19, or 3A4; however, they were strong inhibitors of CYP2D6, an enzyme not involved in the metabolism of antileishmanial drugs. These results suggest that the potential drug interaction of the arylisoquinolinium salts **3**, **4f**, **4i**, and **4m** may not be of clinical significance if these structurally simplified substances are applied in combination with common leishmanicidal therapeutics.

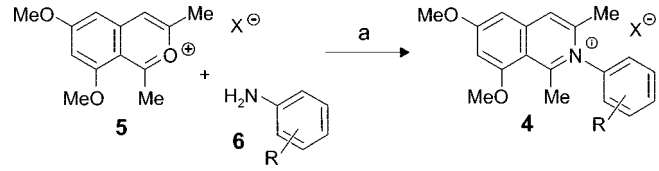
Taken together, our results indicate that the arylisoquinolinium salt **4m** is a promising candidate for further investigation of its potential usefulness as a leishmanicidal drug, especially because combination therapy seems to be fundamental to decrease the cytotoxicity of individual drugs as well as the duration of the therapy and thus could have direct clinical relevance to prevent the emergence of drug resistance.

Results and Discussion

Chemistry. In addition to the previously reported naphthylisoquinolinium salt **3**,[14] we prepared a series of dehydroisoquinolines of type **4** that varied in the aryl portion. These substrates were synthesized in a convergent way by cyclocondensation of the benzopyrylium salt **5**[18] and differently substituted aromatic amines **6** in good to excellent yields (Scheme 1). This synthetic pathway allowed us to rapidly generate SAR and to efficiently scale up key compounds needed for initial studies on the mode of action of this novel class of anti-infective compounds.

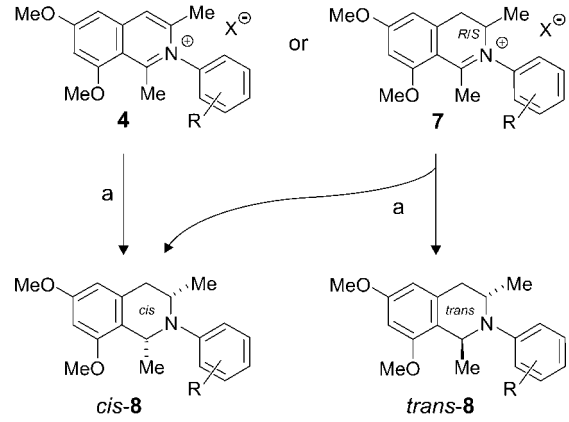
[a] Abbreviations: CYP, drug-metabolizing cytochrome P450 enzymes; DC, dendritic cells; DMSO, dimethylsulfoxide; EC_{50}, concentration that decreases the macrophage infection rate by 50%; FIC, fractional inhibitory concentration; IC_{50}, concentration that inhibits cell proliferation by 50%; IL, interleukin; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; QSAR, quantitative structure−activity relationship; SAR, structure−activity relationship; ΣFICs, sum FICs; TGF-β, transforming growth factor-β, TNF-α, tumor necrosis factor-α.

Scheme 1. Rational 1-Step Synthesis of the *N*-Arylisoquinolinium Salts **4**[a]



[a] Reagents and conditions: (a) HOAc, room temperature.

Scheme 2. Reduction of Isoquinolinium Salts **4** and their Dihydro Analogue **7**[a]

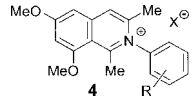


[a] Reagents and conditions: (a) $NaBH_4$, MeOH, H_2O, 0 °C.

The low activity of noncharged *C,C*-coupled naphthylisoquinoline alkaloids against *L. major* promastigotes prompted us to evaluate the influence of the oxidation level of the isoquinoline portion and thus the positive charge at the nitrogen atom in the isoquinoline portion. To this end, selected substrates were further transformed to *N,C*-coupled tetrahydroisoquinolines by treatment of the corresponding isoquinolines or their dihydro analogues with $NaBH_4$ (Scheme 2). The reduction of the fully dehydrogenated isoquinolinium salts **4** with sodium borohydride resulted in the *cis*-configured compounds **8** in high diastereoselectivities (ds > 90). If the dihydro substrates **7** were applied as the educt, by contrast, the attack of the hydride transfer reagent occurred mainly from the opposite side, delivering the *trans* isomer of **8** as the major product (dr = 2:1).

Structure−Activity Relationship. Compounds of type **4** and **8** were tested for their ability to inhibit the proliferation of *L. major* promastigotes in vitro using the Alamar Blue assay.[14,19] In parallel, the toxicity of the compounds was tested using the macrophage cell line J774.1 (Table 1).

Table 1. In Vitro Activities of Synthetic Analogues of Arylisoquinolines **4** and the Standard Amphotericin B against *L. major* Promastigotes and J774.1 Macrophages



4

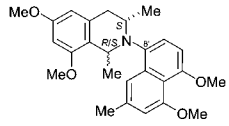
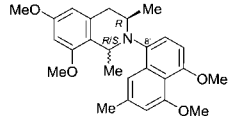
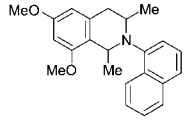
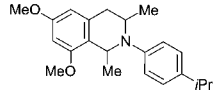
compound		X	*L. major*[a]	J774.1 macrophages[a]	index[b]
4a	1'-naphthyl	BF_4	1.63 ± 0.007	4.29 ± 0.48	2.63
4b	2'-naphthyl	BF_4	3.37 ± 4.23	8.13 ± 12.00	2.41
4c	1'-anthracenyl	TFA	1.85 ± 0.80	1.69 ± 1.94	<1
4d	2'-anthracenyl	BF_4	0.67 ± 0.003	0.23 ± 0.19	<1
4e	5'-anthracenyl	TFA	0.72 ± 0.005	2.95 ± 0.95	<1
4f	4'-methyl phenyl	BF_4	2.64 ± 1.51[1]	14.6 ± 2.76[1]	5.55
4g	2',4'-dimethyl phenyl	BF_4	3.28 ± 0.49	4.07 ± 1.26	1.24
4h	2',3'-dimethyl phenyl	BF_4	11.6 ± 8.68	10.5 ± 6.19	<1
4i	2',6'-dimethyl phenyl	BF_4	3.49 ± 0.60[2]	27.3 ± 11.60[2]	7.82
4j	2',4',6'-trimethyl phenyl	BF_4	1.49 ± 16	4.20 ± 1.38	2.82
4k	4'-ethyl phenyl	BF_4	5.53 ± 6.22	4.50 ± 1.06	<1
4l	4'-*n*-propyl phenyl	BF_4	1.63 ± 0.89	5.36 ± 2.09	1.14
4m	4'-*i*-propyl phenyl	TFA	2.00 ± 0.66[3]	13.10 ± 6.59[3]	6.55
4n	2',4'-di-*i*-propyl phenyl	BF_4	0.54 ± 0.75	2.25 ± 0.39	4.17
4o	4'-*n*-butyl phenyl	BF_4	2.69 ± 0.11	4.47 ± 1.47	<1
4p	4'-*t*-butyl phenyl	BF_4	2.67 ± 0.023	4.28 ± 0.64	1.60
4q	4'-methoxy phenyl	BF_4	>100	>100	n.d
4r	4'-bromo phenyl	BF_4	19.7 ± 1.74	2.97 ± 0.25	<1
4s	4'-chloro phenyl	BF_4	18.9 ± 5.85	3.05 ± 0.21	<1
4t	4'-hydroxy phenyl	TFA	15.9 ± 20.43	>100	n.d.
4u	4'-carboxy phenyl	BF_4	>100	>100	n.d.
4v	4'-nitrile phenyl	TFA	>100	>100	n.d.
4w	6'-quinolinyl	TFA	>100	>100	n.d.
4x	5'-benzthiozolyl	BF_4	>100	63.2 ± 22.5	n.d.
4y	1'-anthraquinonyl	BF_4	>100	>100	n.d.
amphotericin B			5.07 ± 0.05	>70	> 14

[a] The data represent IC_{50} values ± standard deviations and are expressed in μM. Experiments with parasites and macrophages were performed in parallel. [b] The index was calculated as the ratio of the IC_{50} value for macrophages to the IC_{50} to decrease the proliferation of *L. major* promastigotes. n.d.: not determined.[1−3] The activity values described for compounds **4f**, **4i**, and **4m** against *L. major* and macrophages are significantly different with $p < 0.05$.

At first the SAR of the aryl portion was explored by maintaining the isoquinoline portion. The results in Table 1 show that compounds **4a**−**g**, **4j**, and **4i**−**p** exhibited a very good activity against the pathogen *L. major* with submicromolar to micromolar IC_{50} (the concentration that inhibits 50% cell proliferation) values, which were comparable to that of the parent compound **3** ($IC_{50} = 2.91$ μM)[14] and that of amphotericin B evaluated under the same conditions ($IC_{50} = 5.07$ μM). The sensitivity of *Leishmania* promastigotes to amphotericin B was 10- to 25-fold lower than previously reported in the literature.[19] However, it is important to note that intracellular amastigotes, the parasite stage residing in host macrophages, were sensitive to amphotericin B within the same range as previously described[14] (see the section on the macrophage infection rate below). Notably, all active substances described here bear a lipophilic, weakly electron-donating aryl portion displayed either by a nonsubstituted aromatic ring system or by alkyl groups that are located *ortho* or *para* to the heterobiaryl axis at the phenyl ring. The importance of this structural feature was further demonstrated by the synthesis and the testing of the corresponding derivatives **4q**−**y** equipped with electron-withdrawing or hydrophilic electron-donating substituents, e.g., halogen, carboxy, or methoxy. They displayed a very poor or even no inhibition of the parasite growth.

The sterical demand of the aryl portion, e.g., naphthyl vs anthracenyl, or of the size of the alkyl groups, e.g. methyl vs propyl, seems not to influence the activity against *L. major* promastigotes significantly but plays an important role for the toxicity of the compounds. In comparison, compound **4d** having an anthracenyl portion was about 5-fold more active against the parasite than its naphthalene analogue **4b** but at the same time up to 35-fold more toxic against macrophages, while the phenyl analogue, tested in our recent study,[14] inhibited the growth of the macrophages at concentrations even 190-fold

Table 2. In Vitro Activity of Tetrahydroisoquinolines **8** against *L. major* Promastigotes and J774.1 Macrophages

compound		*cis*/*trans* ratio	*L. major*[a]	J774.1 macrophages[a]
8a		1:2	64.8 ± 10.80	>100
8b		1:2	66.7 ± 8.06	>100
8c		>10:1	65.6 ± 6.10	>100
8d		>10:1	49.9 ± 5.96	74.7 ± 0.48

[a] The data represent IC_{50} values ± standard deviations and are expressed in μM. Experiments with parasites and macrophages were performed in parallel.

higher than that of **4d**. Interestingly, the substrates **4w**−**y**, possessing huge but more hydrophilic aryl moieties, e.g., quinolinyl, benzthiazolyl, or anthraquinoyl, were not cytotoxic, indicating that the lipophilicity but not the bulkiness of the aryl portion is the decisive factor for the toxicity of the *N*,*C*-coupled arylisoquinolinium salts. This result was further corroborated by the analogues having either a higher number of substituents, e.g., trimethyl, or bearing a longer carbon side chain at the phenyl ring, leading to more lipophilic and thus more toxic compounds than the monosubstituted arylisoquinolinium salts.

The *N*,*C*-linked tetrahydroisoquinolines **8**, having a tertiary nitrogen atom at the heterocycle, displayed weak or no leishmanicidal activities (Table 2), while their corresponding dehydro or dihydro[14] analogues showed good to very good activities against proliferation of *L. major* promastigotes (Table 1). This finding can most likely be explained by the difference in the hybridization of the nitrogen atoms in **4** as compared to **8** (sp^2 vs sp^3). This does not only lead to an electrostatically diverse scaffold of the substrates due to the positively charged compounds **4** in contrast to **8** but also to large differences in the molecular shapes of **4** and **8**. Their conformational arrays are mainly induced by the different orientations of the two molecular portions. In the case of the positively charged salts **4**, the isoquinolinium and the aryl portions are linked via a heterobiaryl axis leading to an almost orthogonal alignment of the two halves, while in the tetrahydroisoquinolines **8**, the nitrogen atom adopts a tetrahedral conformation.

Because the *C*,*C*-coupled naphthylisoquinoline alkaloids, which also feature an almost orthogonal assembly of the two molecular halves and whose molecular shapes are thus comparable to those of compounds **4**, exhibit only low effectiveness against *L. major,* as was demonstrated in our recent study,[14] the activities of compounds **4** against *Leishmania* parasites herein seem to depend on their cationic structure.

Altogether, the most promising substrates evaluated in the SAR studies are the methyl and *i*-propyl isoquinolinium derivatives **4f**, **4i**, and **4m** (Table 1). They are effective against *L. major* promastigotes at concentrations in the low micromolar range, while they impair the growth of J774.1 macrophages at concentrations that are at least 6-fold higher. The data also suggest that the efficacy of these compounds against *L. major* is similar to that reported for amphotericin B in the experiments described herein, although they are more toxic against the macrophage cell line J774.1.

Table 3. Cytotoxicity of Selected Synthetic Analogues of the Arylisoquinolines and Amphotericin B against Dendritic cells (DC), NIH 3T3 Fibroblasts, and Peritoneal Macrophages

compound	DC[a]	NIH 3T3[a]	peritoneal macrophages
amphotericin B	nd	nd	>70
4f	24.0 ± 8.00	49.8 ± 1.10	12.77 ± 3.07
4i	8.76 ± 2.58	37.6 ± 13.8	9.67 ± 2.21
4m	7.60 ± 2.78	11.1 ± 5.83	8.81 ± 2.87

[a] The data represent IC_{50} values ± standard deviations and are expressed in μM. Experiments with parasites and macrophages were performed in parallel. nd: not determined.

In summary, the study on this novel class of antileishmanial compounds described here implies that the good antileishmanial properties of *N*,*C*-coupled isoquinolinium salts do not only depend on the oxidation state of the isoquinoline portion, and thus on the overall shape and on the electrostatic properties of the substrates, but also strongly rely on the substitution pattern of the aryl portion of these compounds. These further hints at the structural features needed for antileishmanial activity help to improve our model for the activity- and toxicity-guided quantitative structure−activity relationship (QSAR) investigations that are in progress.

Cytotoxicity of Selected Arylisoquinolines (Compounds 4f, 4i, and 4m) against Different Cell Lines. The cytotoxicity of the most promising substances **4f**, **4i**, and **4m** (Figure 1) was further evaluated with different cell lines. The compounds inhibited the growth of dendritic cells as well as the survival of NIH 3T3 fibroblasts and of peritoneal macrophages at concentrations at least 3-fold higher than the concentration needed for a 50% inhibition of the growth of *L. major* promastigotes (Table 3). Together, these results demonstrated that the simplified analogues **4f**, **4i**, and **4m** of the *N*,*C*-coupled isoquinolinium salts remained selective against *Leishmania* promastigotes compared to different mammalian cells.

Effect of *N*,*C*-Linked Arylisoquinolines 4f, 4i, and 4m on the Infection Rate of Macrophages. To further analyze the antileishmanial activity of **4f**, **4i**, and **4m**, freshly isolated peritoneal macrophages were infected with *L. major* for 24 h

Table 4. Infection Rate of Selected *N,C*-Coupled Arylisoquinolines and Amphotericin B

compound	infection rate[a]	index[c]
amphotericin B	0.075 [0.070] (CI[b]: 0.040−0.139, r^2 0.999)	>1000
4f	0.092 [0.036] (CI[b]: 0.040−1.99, r^2 0.923)	138
4i	0.241 [0.104] (CI[b]: 0.125−0.461, r^2 0.963)	40
4m	0.158 [0.070] (CI[b]: 0.08−0.312, r^2 0.978)	56

[a] The data represent EC_{50} values and are expressed in μM and in μg/mL within rectangular brackets. [b] CI: confidence interval. [c] The index was calculated as the ratio of the IC_{50} value for peritoneal macrophages (Table 3) to the EC_{50} to decrease the infection rate.

and then treated with increasing concentrations of the compounds **4f**, **4i**, and **4m** for 48 h. Table 4 presents a summary of the data for macrophage survival and decrease in infection rate. Untreated macrophages had infection rates of 20−30% (consistent within each experiment), which were normalized to 100% for further analysis of the results. Treatment of infected macrophages with compounds **4f**, **4i**, and **4m** resulted in a dose-dependent decrease in infection rate. A 50% decrease (EC_{50}) was obtained at concentrations of 0.092 μM (confidence interval 0.040 to 1.99 μM, r^2 0.923), 0.241 μM (confidence interval 0.125 to 0.461 μM, r^2 0.963) and 0.158 μM (confidence interval 0.080 to 0.312 μM, r^2 0.978) for **4f**, **4i**, and **4m**, respectively (Table 4). Similar to what was previously demonstrated,[14] simultaneous addition of the arylisoquinolines **4f**, **4i**, or **4m** and the macrophage activator interferon-γ (IFN-γ) did not further modify the EC_{50} (data not shown). The EC_{50} for amphotericin B was 0.075 (confidence interval 0.0397 to 0.139 μM, r^2 0.999), similar to what had been previously described.[14,20]

These results indicate that a significant decrease in macrophage infection rate is obtained at concentrations of compounds **4f**, **4i**, and **4m** that are 9- to 30-fold lower than those needed to inhibit the growth of *L. major* promastigotes. The efficacy of these active agents against intracellular amastigotes is comparable to that of amphotericin B. Notably, although the EC_{50} against amastigotes was not significantly different from the one obtained with the naphthylisoquinoline **3**, the toxicity against different cell lines and freshly isolated cells from BALB/c mice is at least 40-fold, and in some cases even 500-fold, lower than that against intracellular amastigotes. These data confirm our previous finding that *N,C*-coupled isoquinolines are selective against the parasites and in general do not stimulate the killing mechanisms of macrophages.

Because the pharmacokinetic behavior of a drug also governs the efficacy, the extent of plasma protein binding was exemplarily determined for the compounds **4f**, **4i**, **4k**, **4m**, **4n**, and **4x**, using the method of automated continuous ultrafiltration developed and validated in our group.[21] One of the compounds, **4x**, did not induce a significant decrease in macrophage infection rate. The percentages of binding were 55.9 ± 9.1% for **4f**, 46.2 ± 7.2% for **4i**, 52.5 ± 7.4% for **4k**, 70.3 ± 7.6% for **4m**, 58.4 ± 6.5% for **4n**, and 32.9 ± 8.0% for **4x**, indicating that only half of the drug is available for the treatment of a leishmanial infection and that there is no correlation between plasma protein binding and the antileishmanial activity of the compounds. However, the extent of plasma protein binding is within an acceptable range. As an example, two new antibiotics, namely moxifloxacin and telithromycin (see Supporting Information), bind to a similar extent to human serum albumin.[22] The most active compound in the series of the *N,C*-coupled arylisoquinolines, however, showed a very high extent of plasma protein binding. Further optimization of the compounds should aim at a decrease of the plasma protein binding.

Modulation of Macrophage Cytokine Secretion by N,C-Coupled Arylisoquinolinium Salts 3, 4f, 4i, and 4m. To further analyze the leishmanicidal activity of the isoquinolines **3**, **4f**, **4i**, and **4m**, we evaluated the levels of interleukin-1 beta (IL-1β), IL-6, IL-10, IL-12, transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α) in culture supernatants of noninfected or infected cells treated either with compounds **3**, **4f**, **4i**, or **4m** in the absence or presence of the potent macrophage activators IFN-γ or lipopolysaccharide (LPS). Compound **3** was used as an internal control of the experiment. As described previously,[14] its effect is not associated with the stimulation of cytokine secretion or the production of nitric oxide by infected macrophages. The results demonstrate that noninfected untreated cells secreted 0.035 ± 0.014 ng/mL of IL-1β, 2.014 ± 1.159 ng/mL of IL- 6, 0.101 ± 0.080 ng/mL of IL-10, 0.417 ± 0.014 ng/mL of IL-12, and 0.330 ± 0.108 ng/m*l* of TGF-β. Treatment with compounds **3**, **4f**, **4i**, or **4m** did not cause significant changes in the levels of IL-1β, IL-6, or TNF-α; these compounds strongly reduced the release of IL-6 and TNF-α in response to the macrophage stimulators LPS or IFN-γ (data not shown). These are pro-inflammatory cytokines, which are secreted in early stages of *L. donovani* and *L. major* infection[23,24] and seem to be involved in the early recruitment of inflammatory cells to the site of infection.[25] Furthermore, the host defense against *L. major* infection depends on the IL-12-driven expansion of T helper 1 cells.[26,27] However, arylisoquinolinium salts strongly inhibit IL-12 secretion by infected macrophages. Indeed, these results indicate that the studied isoquinolines do not stimulate macrophages to secrete pro-inflammatory cytokines but rather interfere with their cytokine response to potent stimulators.[14]

The results also demonstrate that noninfected untreated cells secreted 0.101 ± 0.080 ng/mL of IL-10, and 0.330 ± 0.108 ng/mL of TGF-β. However, the levels of TGF-β (Figure 2) increased significantly ($p < 0.001$) after incubation with compounds **3** or **4m** (with or without IFN-γ), while the substrates **4i** and **4m**, but not **3** and **4f**, decreased IL-10 levels ($p < 0.001$) significantly (Figure 2).

The results obtained with the 4′-*i*-propyl derivative **4m** strongly suggest that the action of some arylisoquinolines may help to limit the extent of the inflammatory response and to restore homeostasis of the host cell. *Leishmania* infection induces TGF-β production and a delayed nitric oxide production,[25] consistent with a TGF-β inhibition of microbicidal action. TGF-β is a cytokine with dual pro- and anti-inflammatory functions.[28] To play its role, TGF-β should act through the induction of IL-10, which has been categorized as a T helper 2 cytokine.[28] Infections with *L. donovani*[28,29] and *L. major*[30] increase IL-10 levels, which in turn facilitates the intracellular survival of the protozoa, orchestrates several immunomodulatory pathways,[25] and plays a significant role in disease initiation and progression.[31] The isoquinoline **4m** increased the secretion of TGF-β and decreased the secretion of the pro-inflammatory cytokine IL-10, whereas it is very difficult to interpret the leishmanicidal effect of compound **4f** in view of the unusual increase in IL-10 levels observed in the simultaneous presence of compound **4f** and IFN-γ. This result is even more interesting with respect to the fact that compound **4f** does not modulate the secretion of TGF-β and, therefore, does not seem to play a role in the feedback loop that exists between these two cytokines.

To analyze whether the decrease in macrophage infection rate observed in the presence of compounds **3**, **4f**, **4i**, or **4m** correlates with the capacity of macrophages to secrete nitric oxide, we evaluated the nitrite production in noninfected and

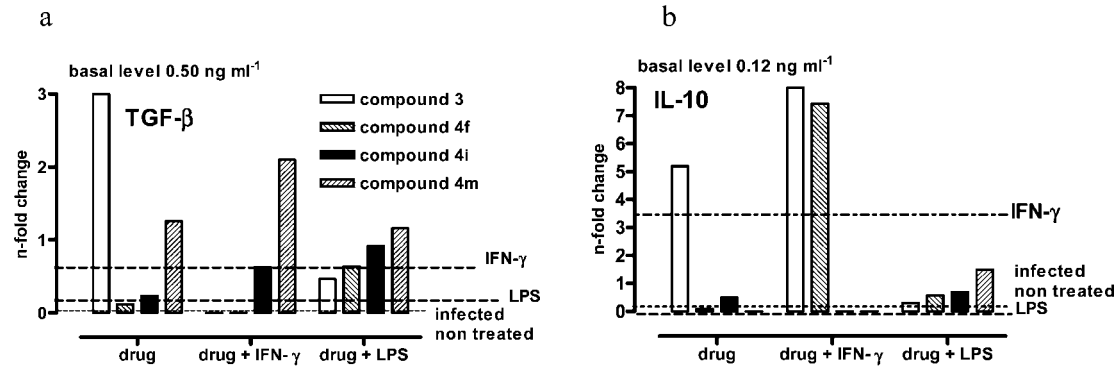


Figure 2. TGF-β and IL-10 secretion by macrophages. Macrophages were infected with stationary-phase *L. major* promastigotes for 24 h and treated with the compounds in the absence or presence of IFN-γ or LPS. *n*-Fold change (infected/noninfected) of TGF-β levels in supernatants of infected macrophages cultured in the presence of compounds **3**, **4f**, **4i**, or **4m** in the absence or presence of IFN-γ or LPS (a); *n*-fold change (infected/noninfected) of IL-10 levels in supernatants of infected macrophages cultured in the presence of compounds **3**, **4f**, **4i**, or **4m** in the absence or presence of IFN-γ or LPS (b). The horizontal lines indicate changes in cytokine production induced by IFN-γ or LPS alone. $p < 0.001$, $p < 0.05$. A ratio of 1 indicates no change.

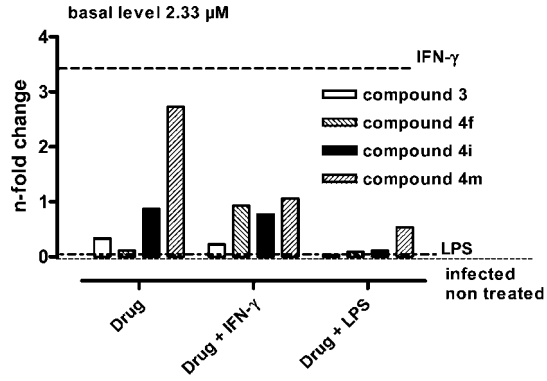


Figure 3. Nitric oxide production by macrophages. Macrophages were infected with stationary-phase *L. major* promastigotes for 24 h. Cells were then washed and incubated for further 48 h with the compounds in the absence or presence of IFN-γ or LPS. The culture supernatants were then collected for determination of the nitrite concentrations. *n*-Fold change (infected/noninfected) of the micromolar concentration of nitrite in supernatants of infected macrophages cultured in the presence of compounds **3**, **4f**, **4i**, or **4m** in the absence or presence of IFN-γ or LPS. A ratio of 1 indicates no change.

infected cells. The nitric oxide release was measured after 48 h of incubation of infected macrophages with the compounds (Figure 3). Noninfected cells treated with compounds **3**, **4f**, **4i**, or **4m** secreted nitric oxide levels that were similar to those of noninfected untreated cells. These levels did not change in cells incubated with IFN-γ or with compounds **3**, **4f**, **4i**, or **4m** plus IFN-γ (data not shown). Infection with *Leishmania* promastigotes decreased the nitric oxide production (Figure 3). The treatment with LPS, or compounds **3**, **4f**, **4i**, or **4m** and LPS simultaneously, did not increase nitric oxide secretion. These levels increased 3- to 4-fold in cells treated with IFN-γ and 2.7-fold in cells incubated only with compound **4m** ($p < 0.005$).

These results strongly suggest that the mode of action of the isoquinolinium salt **4m**, with its sterically demanding *i*-propyl substituent *para* to the heterobiaryl axis, may be different from that of the other methyl-substituted *N,C*-coupled isoquinolines tested and may help to limit the extent of the inflammatory response and to restore homeostasis of the host cell.

Analysis of Interactions of *N,C*-Coupled Arylisoquinolinium Salts 2, 3, 4f, and 4m with Amphotericin B against Intracellular Amastigotes In Vitro. Combination therapy has been addressed as a way to decrease (a) the cytotoxicity of individual drugs, (b) the duration of therapy, and (c) the potential development of resistance.[17] It could thus be of fundamental impact for leishmaniasis therapy. In our studies, we have explored the in vitro interaction between *N,C*-coupled arylisoquinolines and amphotericin B.

Table 5. Mean ΣFICs of the Interaction between Amphotericin B and the Arylisoquinolines **2**, **3**, **4f**, or **4m** towards Intracellular Amastigotes In Vitro

fixed relation[a]	**2**	**3**	**4f**	**4m**
1:4	0.552	0.67	2.98	<0.5
2:3	0.901	>4	>4	<0.5
3:2	0.341	>4	2.76	<0.5
4:1	2.33	3.02	>4	<0.5

[a] The different fixed relations are established as amphotericin B: compounds **2**, **3**, **4f**, and **4m**. FIC: fractional inhibitory concentrations of compounds **2**−**4f** and **4m**; ΣFICs (sum FICs), [FIC amphotericin B + FIC compounds **2**−**4f** and **4m**].

We assessed in vitro interactions using a modified fixed-ratio isobologram method and analyzed it at the EC_{50} level.[16] The fractional inhibitory concentrations (ΣFICs, sum FICs) are presented in Table 5. The results are the mean values of two independent experiments. Representative isobolograms are shown in Figure 4. Interactions were classified as synergistic with values of ΣFICs $\leq$, as antagonistic with values of ΣFICs ≥ 4, and as indifferent with values of ΣFICs between >0.5 and ≥ 4.[17]

The interaction of amphotericin B with the alkaloid ancistrocladinium B (**2**) was indifferent at all the fixed relations evaluated with values of ΣFICs between 2.33 and 0.52. Notably, the interaction of amphotericin B with structurally simplified analogues **3** and **4f** ranged from indifferent to antagonistic with ΣFICs from 0.67 to >4. Finally, the interaction of amphotericin B with compound **4m** was synergistic with ΣFIC*s* < 0.5 for all the fixed ratios evaluated, reaching values as low as 0.003 when a fixed relation (2:3) of amphotericin B to **4m** was used. This fixed relationship arises from the combination of amphotericin B (0.08 μM) with compound **4m** (0.15 μM); these concentrations lie within their corresponding EC_{50} to decrease the macrophage infection rate. Thus, the results suggest that the interaction between amphotericin B and the 4′-*i*-propyl derivative **4m** at their EC_{50} is extremely synergistic, indicating that **4m** may be a promising candidate for further investigation of its potential usefulness as an antiprotozoal drug.

Because in vitro data are based on an extended ratio and concentration range, these results strongly underline the importance of testing the possible interaction between amphotericin

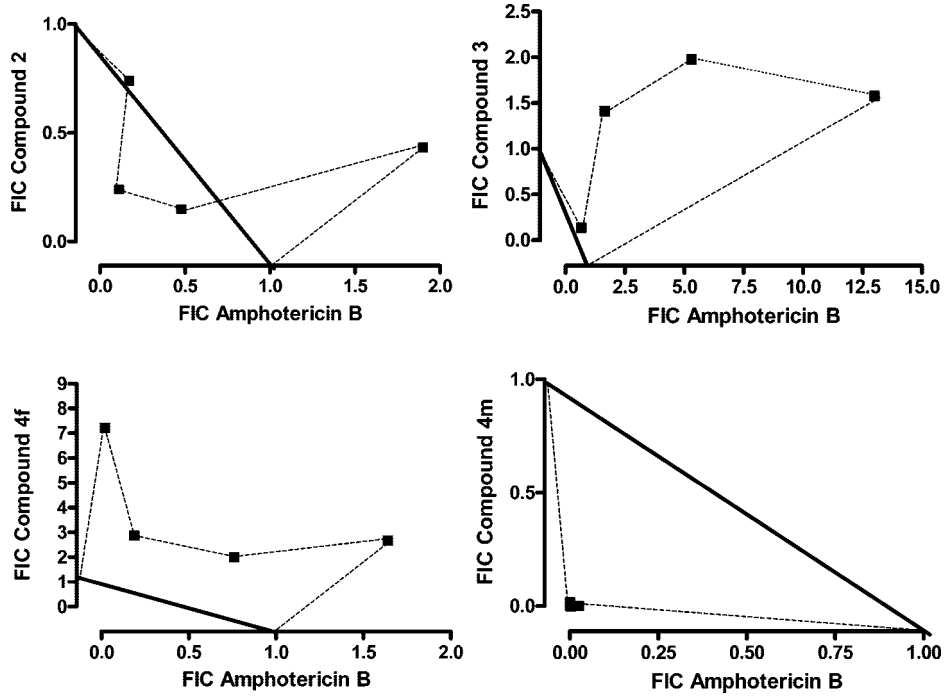


Figure 4. Representative isobolograms of in vitro interactions against intracellular amastigotes. Interactions are *g*iven at the EC_{50} levels. Numbers at the axes represent normalized FIC values of amphotericin B (*x* axes) and the partner drug **2**, **3**, **4f**, and **4m** (*y* axes).

B and the arylisoquinolinium trifluoroacetate **4m** in vivo. The mechanism of interaction that leads to the enhancement of the effects of these two agents remains to be elucidated. Amphotericin B toxicity occurs through its binding to sterols in the cell membrane, formation of aqueous pores, and the induction of programmed cell death.[32,33] Compound **4m** induces the appearance of intracellular vacuoles and its killing effect shares characteristics with programmed cell death (Ponte-Sucre et al., unpublished).

Inhibition of Human Drug-Metabolizing CYP Enzymes by *N*,*C*-Coupled Isoquinolines 3, 4f, 4i, and 4m. The most important biotransformation pathway for drugs in mammals is their oxidation by drug-metabolizing cytochrome P450 (CYP) enzymes, which are expressed mainly in the liver but also in the lung, kidney, or small intestine.[34] Among CYP enzymes involved in biotransformation reactions, the isoenzymes 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4 are responsible for the metabolism of about 90% of all known drugs.[34] To assess their relevance for drug metabolism, we tested whether the antileishmanial *N*,*C*-coupled isoquinolines **3**, **4f**, **4i**, and **4m** interfere with metabolic reactions catalyzed by the major human drug-metabolizing CYP enzymes.

Because the medical treatment of parasitic infections often requires the simultaneous application of various drugs, a low inhibitory activity of such drugs on CYP enzymes is an important prerequisite for their combined therapeutic use. In Figure 5, we present the inhibitory activity of the isoquinolines **3**, **4f**, **4i**, and **4m** and of quinidine (see Supporting Information), a strong and selective CYP2D6 inhibitor, on the applied CYP enzymes. Except for **4i**, all tested compounds are strong and selective inhibitors of CYP2D6. The inhibitory activity of **3**, **4f**, and **4m** is comparable to that of the CYP2D6 standard inhibitor quinidine (Figure 5). It is important to note that compound **4m** in particular did not inhibit the activity of the additional CYP enzymes at all, whereas compounds **3**, **4f**, and **4i**, as well as quinidine also reduced the activity of CYP2C9, CYP2C19, and CYP3A4 at a concentration of 100 μM. Thus, at the tested concentrations of 1, 10, and 100 μM, compound **4m** showed a higher selectivity toward CYP2D6 than the prototypical inhibitor quinidine (Figure 5).

Because all tested concentrations of the aryl isoquinoline reduced the activity of CYP2D6, we determined the IC_{50} values of compounds **3**, **4f**, and **4m** and of quinidine for the inhibition of CYP2D6 to compare the inhibitory activities and to evaluate the drug interaction potential of the substrates (Figure 6). Quinidine had the highest inhibitory activity for CYP2D6, with an IC_{50} value of 19.6 nM. Although compounds **3**, **4f**, and **4m** had higher IC_{50} values (0.9 μM, 56.1 nM and 109.4 nm; Figure 6), they can be considered as strong inhibitors of CYP2D6.

The inhibitory mechanism exerted by an inhibitor of drug-metabolizing CYP enzymes strongly influences the pharmacokinetics of concomitantly administered drugs metabolized via the same CYP enzymes. Herein we demonstrate that compounds **4f** and **4m** are potent and highly selective inhibitors of CYP2D6 (Figures 5 and 6). Moreover, the results shown in Figure 7, where the Lineweaver−Burk plot for the inhibition of CYP2D6 by compound **4m** is illustrated, demonstrate that, similar to quinidine (K_i 9.8 nM), compound **4m** is a strong competitive inhibitor of CYP2D6 with a K_i value of 54.7 nM. These results suggest that **4m** and the structurally closely related compounds **3** and **4f** (K_i 0.45 μM, 28.1 nM) bind with high affinities to the active site of the enzyme.

The *N*,*C*-coupled isoquinolines **3**, **4f**, and **4m** are thus strong and selective inhibitors of the drug-metabolizing CYP enzyme 2D6. This highly polymorphic isoenzyme is involved in the metabolism of 20−25% of the drugs presently in clinical use.[35] However, the currently applied medication against leishmaniasis (e.g., pentavalent antimonials, amphotericin B, or miltefosine) does not include drugs metabolized by CYP2D6. Interestingly, the isoquinolines **3**, **4f**, and **4m** are strong and highly selective competitive inhibitors of CYP2D6; therefore, adverse drug interactions due to the inhibition of metabolic pathways catalyzed by CYP1A2, 2C8/9/19, or 3A4 can be excluded. The inhibitory activity of compound **4i** on CYP2C19 (Figure 4) is

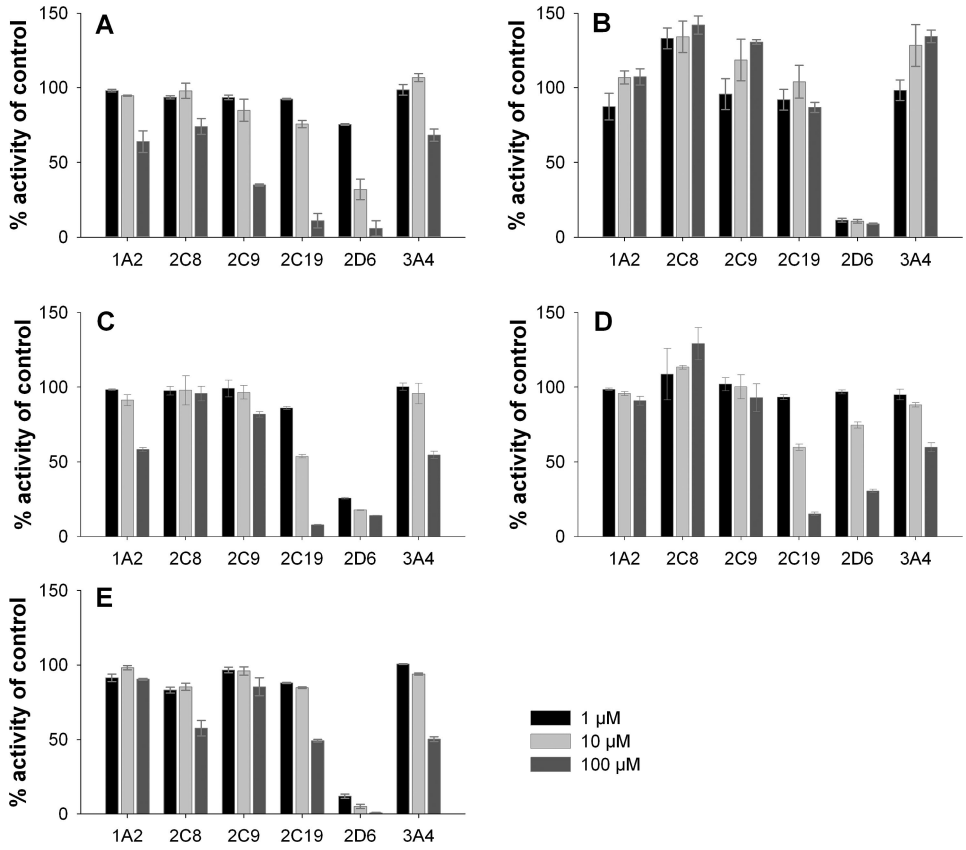


Figure 5. Inhibition of CYP1A2, 2C8/9/19, 2D6, and 3A4 by 1, 10, and 100 μM of compound **3** (A), **4m** (B), **4f** (C), or **4i** (D), or quinidine (E). The results represent mean values of triplicate determinations (±standard deviation).

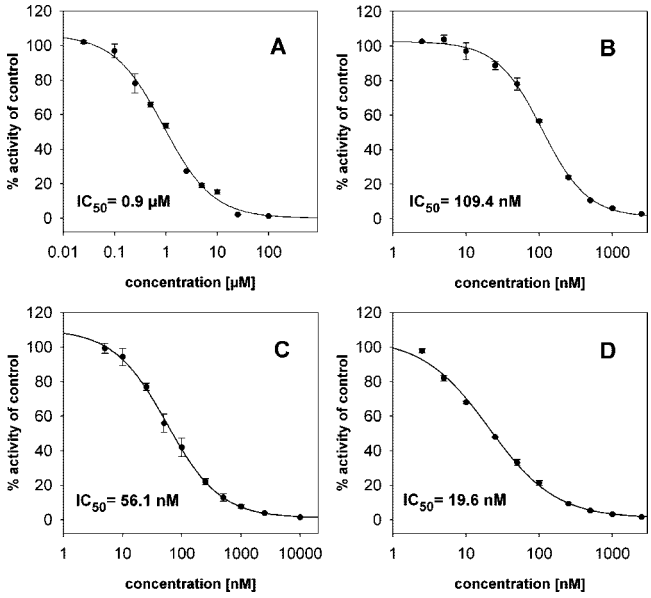


Figure 6. Inhibition curves and IC_{50} values for the impairment of CYP2D6 by compound **3** (A), **4m** (B), and **4f** (C) and of quinidine (D). The results represent mean values of triplicate determinations (± standard deviation).

too low to cause clinically relevant drug interactions with drugs preferentially metabolized by this isoenzyme. This means that compounds **3**, **4i**, **4f**, or **4m** could be safely prescribed together with antileishmanial drugs in a combination therapy approach, once the therapeutic index of these aryl isoquinolines is further enhanced. The strong inhibitory activity of compounds **4f** and **4m** on CYP2D6 (K_i values 28.1 nM and 54.7 nM) is remarkable and the competitive inhibition of CYP2D6 points to a direct interaction of these substrates with the catalytic center of the enzyme. This high-affinity binding might be due to a coordination of the ferriprotoporphyrin IX (heme) of CYP2D6 as demonstrated in the complexation of ferriprotoporphyrin IX by antimalarial drugs like chloroquine and the antiplasmodial *C*,*C*-linked naphthylisoquinoline alkaloid dioncophylline C.[36]

Conclusions

In summary, the presented study on the SAR of *N*,*C*-coupled arylisoquinolines showed that, in addition to the decisive impact of the biarylic structure for the activity, the aryl portion of these compounds also plays an important role for both activity and toxicity of this new class of antileishmanial agents. These findings now provide the basis for a more specific QSAR-guided design, selection, and synthesis of novel antileishmanial *N*,*C*-linked synthetic arylisoquinolines with an improved toxicity−activity ratio.

Furthermore, the results strongly suggest that the significant decrease in macrophage infection rate induced by *N*,*C*-coupled arylisoquinolinium salts is based on a direct effect of the compounds on the intracellular parasites. However, the effect of the 4′-*i*-propyl derivative **4m** might involve both a direct leishmanicidal effect and the modulation of the cytokine activity and nitric oxide production by host macrophages.

Finally, because compound **4m** acts synergistically with amphotericin B in vitro, it is a promising candidate for further investigations on its potential usefulness as an antiprotozoal drug, although the toxicity of this newly synthesized structurally simplified *N*,*C*-coupled arylisoquinolinium salt against the host cells is still high. Additionally, because antileishmanial drugs are not metabolized by the CYP2D6 isoenzyme, the risk of

adverse drug reactions with conventional antileishmanial drugs and of drug interactions caused by inhibition of other drug-metabolizing enzymes is excluded. In vivo experiments on the combination of amphotericin B with **4m** will be fundamental to confirm the present data and to evaluate further the potency of the arylisoquinolinium salt **4m** in combination therapy.

Experimental Section

General Information. All used solvents were distilled before use. Commercially available material was used without further purification. Reactions with $NaBH_4$ were carried out in predried glassware under argon. Melting points were determined on a Reichert−Jung Thermovar hot plate and uncorrected NMR spectra (^{1}H NMR: 400 MHz; ^{13}C NMR: 101 MHz) were recorded on a Bruker AMX, using the solvents as the internal ^{1}H and ^{13}C standards with coupling constants (J) in Hertz (Hz). HRESIMS was measured on a Bruker Daltonik micrOTOF-focus. The combustion analyses were performed on a Leco CHNS 932 analyzer. Thin-layer chromatography was carried out using silica gel 60 F254 or C-18 F254s aluminum foil. Detection of the compounds was achieved by fluorescence quenching at 254 nm or fluorescence at 356 nm. Gel chromatography was performed using Sephadex LH-20. Analytical HPLC was performed on a Jasco System (DG-1580, LG-1580, PU-1580, CO-1560, AS-1555, MD-1510) using a Chromolith Performance RP-18e (100 mm × 4.6 mm) with a MeCN (A)/H_2O (B) solvent mixture complemented by 0.05% TFA: 0 min 5% A, 5 mL/min; 5 min 50% A, 5 mL/min; 9 min 100% A, 5 mL/min. Preparative HPLC was carried out on a Jasco System (PU-2087 Plus, MD-2010 Plus) with a Waters 600 Controller and a Waters 996 Photodiode Array Detector. As a column a Chromolith SemiPrep-RP-18e (100 mm × 100 mm) was used with a MeCN (A)/H_2O(B) solvent mixture with 0.05% TFA: 0 min 5% A, 10 mL/min; 5 min 50% A, 10 mL/min; 7 min 100% A, 10 mL/min.

***N*-(4′-Methylphenyl)-6,8-dimethoxy-1,3-dimethylisoquinolinium Tetrafluoroborate (4f).** A mixture of 288 mg (0.95 mmol) of 6,8-dimethoxy-1,3-dimethyl-2-benzopyrylium tetrafluoroborate (**5**) and 100 mg (0.95 mmol) of 4-tolylamine in 10 mL glacial acetic acid was stirred at room temperature for 8 h. The yellow solid was filtered and washed with diethyl ether. The mother liquor was diluted with methanol and purified by chromatography on Sephadex-LH20 material with methanol as the eluent. The crude products were combined and recrystallized from a mixture of methanol, diethyl ether, and *n*-hexane to afford 364 mg (0.92 mmol, 97%) of the isoquinolinium tetrafluoroborate **4f** as a white solid, mp >250 °C. ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H), 2.50 (s, 3H), 2.92 (s, 3H), 4.00 (s, 3H), 4.05 (s, 3H), 6.91 (d, J = 2.1 Hz, 1H), 7.03 (d, J = 2.1 Hz, 1H), 7.23 (d, J = 8.3 Hz, 2H), 7.47 (d, J = 8.1 Hz, 2H), 7.3 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 22.05, 22.82, 24.05, 57.35, 57.46, 100.1, 103.2, 116.1, 123.8, 126.8, 132.4, 137.6, 142.2, 143.4, 145.1, 159.5, 162.1, 168.2. Anal. ($C_{20}H_{22}NO_2BF_4$) C, H, N.

***N*-(2′,6′-Dimethylphenyl)-6,8-dimethoxy-1,3-dimethylisoquinolinium Tetrafluoroborate (4i).** Yield = 78%; brown needles, mp 198 °C. ^{1}H NMR ($CDCl_3$) δ 1.82 (s, 6H), 2.19 (s, 3H), 2.75 (s, 3H), 3.97 (s, 3H), 4.10 (s, 3H), 6.75 (d, J = 2.3 Hz, 1H), 7.29 (d, J = 2.3 Hz, 1H), 7.33 (d, J = 8.1 Hz, 2H), 7.42 (t, J = 7.1 Hz, 1H), 8.38 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 17.94, 21.39, 21.83, 57.44, 57.79, 100.8, 103.6, 115.9, 125.4, 131.1, 131.9, 133.7, 138.4, 143.5, 143.8, 157.9, 161.2, 161.5, 161.9, 168.7. HRESIMS calcd for $C_{21}H_{24}NO_2^+$ $(M)^+$, 322.18016; found, 322.18011.

***N*-(4′-*i*-Propylphenyl)-6,8-dimethoxy-1,3-dimethylisoquinolinium Trifluoroacetate (4m).** A mixture of 288 mg (0.94 mmol) of 6,8-dimethoxy-1,3-dimethyl-2-benzopyrylium tetrafluoroborate (**5**) and 128 mg (0.95 mmol) of 4-*i*-propylphenyl amine in 10 mL glacial acetic acid was stirred at room temperature for 8 h. The solvent was evaporated; the brown oil was dissolved in 2 mL MeOH and purified by chromatography on Sephadex-LH20 material with methanol as the eluent. The crude product was submitted to preparative HPLC. The obtained fractions were combined and the solvent evaporated to yield 376 mg (0.84 mmol, 88%) of compound **4m** as a yellow solid, mp 217 °C. ^{1}H NMR ($CDCl_3$) δ = 1.34 (d, J = 7.0 Hz, 6H), 2.30 (s, 3H), 2.89 (s, 3H), 3.07 (q, J = 6.8 Hz, 1H), 3.97 (s, 3H), 4.06 (s, 3H), 6.94 (d, J = 2.1 Hz, 1H), 7.08 (d, J = 2.9 Hz, 1H), 7.25 (d, J = 8.3 Hz, 2H), 7.53 (d, J = 8.3 Hz, 2H), 8.03 (s, 1H). ^{13}C NMR ($CDCl_3$): δ = 22.82, 24.09, 24.47, 34.67, 57.34, 57.46, 100.1, 103.3, 116.0, 120.6, 123.8, 126.8, 129.9, 137.6, 143.2, 145.1, 153.1, 159.4, 161.1, 162.0, 168.3; HRESIMS calcd for $C_{22}H_{26}NO_2^+$ $(M)^+$, 336.19581; found, 336.19621.

Parasites. The cloned virulent *L. major* isolate MHOM/IL/81/FE/BNI was maintained by passage in BALB/c mice (6−8 weeks old). Promastigotes were grown in blood agar cultures at 26 °C, 5% CO_2, 95% humidity. Prior to their use, promastigotes were washed twice with phosphate-buffered saline and suspended at 1 × 10^8 cells/mL in Click RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES buffer pH 7.2, 100 μg/mL penicillin, 160 μg/mL gentamicin, 7.5% $NaHCO_3$, and 5 × 10^{-5} M 2-mercaptoethanol (complete medium).

Cells and Cell Lines. The macrophage cell line J774.1 was maintained in complete medium. Prior to their use, cells were detached from the flasks with a rubber policeman, washed twice with phosphate-buffered saline, and suspended at 2 × 10^6 cells/mL in complete medium. Peritoneal macrophages were obtained from BALB/c mice by a previously described protocol.[14] Dendritic cells were generated from bone marrow progenitors of BALB/c mice as described.[14] The mouse embryo fibroblast cell line NIH 3T3 was grown to 80−90% confluence using a previously described protocol.[14] For the experimental procedures, cells were detached from the flasks with a rubber policeman, washed with phosphate-buffered saline, and suspended in Dulbecco's modified Eagle medium (DMEM) at 2 × 10^6 cells/mL.

Analysis of In Vitro Antiproliferative Activity. The analysis of the in vitro antiproliferative activity of the compounds was done using Alamar Blue.[14,19] Amphotericin B was used as a reference compound and positive control. For in vitro studies, the compounds were dissolved in dimethyl sulfoxide and further diluted in medium. The final concentration of dimethyl sulfoxide in the medium never exceeded 1% (v/v) and had no effect on the proliferation of extracellular or intracellular parasites. For each experiment, every drug concentration was assayed in duplicate wells. Optical density values at 48 h were used to calculate the concentration that inhibits 50% cell proliferation (IC_{50}) via linear interpolation.[37] The index included in Table 1 was calculated as the ratio of the IC_{50} value for macrophages to the IC_{50} for *L. major*.

Analysis of Macrophage Infection Rate. The activity of compounds on the macrophage infection rate was analyzed as described previously.[14] Amphotericin B was used as a reference compound and positive control. Intracellular parasites were quantified by staining with acridine orange and ethidium bromide and analyzed by fluorescence microscopy at 495 nm as described previously.[38] Data on the infection rate are expressed as mean values ± standard errors of the mean of at least three experiments in which 300 macrophages were analyzed for each drug concentration. The program Graph-pad was used to fit the data to nonlinear regression and to determine the concentration that decreases the infection rate to 50% (the 50% effective concentration [EC_{50}]).

Analysis of Cytokine and Nitric Oxide Production. The analysis of the effect of compounds on the cytokine production by infected macrophages was done following a protocol described previously.[39] The potent macrophage activators IFN-γ (100 U/mL) or LPS (15 μg/mL) were used as positive controls for the production of cytokines. A concentration of 3 μM of compounds **3**, **4f**, **4i**, and **4m**, i.e., close to the IC_{50} against *L. major* promastigotes, was used for this assay. After 24 h, the culture supernatants were collected and the levels of IL-1β, IL-6, IL-10, IL-12, TGF-β, and TNF-α were quantified by the sandwich enzyme-linked immunosorbent assay. The developing color in the wells was read at a test wavelength of 405 nm and a reference wavelength of 490 nm using a Multiskan Ascent ELISA reader. Data were calculated as ng/mL. Cytokines secreted by noninfected, untreated macrophages were normalized to 100% for further analysis of the results. The detection thresholds were 30.72 pg/mL for IL-1β, 39.00 pg/mL for IL-4, 27.24 pg/mL for IL-6, 78.00 pg/mL for IL-10, 72.00 pg/mL

for IL-12, 222.00 pg/mL for IFN-γ, 118.00 pg/mL for TGF-β, and 40.00 pg/mL: for TNF-α.

The analysis of the effect of compounds on the nitric oxide production by infected macrophages was done following a protocol described in literature.[39] The nitrite concentrations were determined using sodium nitrite as a standard. Data are expressed as micromolar concentrations of nitrite. Nitrite concentrations reflect the nitric oxide levels released by macrophages.

Analysis of Plasma Protein Binding of Compounds 4f, 4i, 4k, 4m, 4n, and 4x. The binding of the compounds to albumin was determined by means of a continuous ultrafiltration method. In this procedure, an ultrafiltration cell with an ultrafiltration membrane (molecular weight cutoff of 10 kDa) was connected to a Bischoff DAD 3 L EU. Initially a buffered (3×10^{-2} M, pH 7.4 sodium-phosphate) solution of the substances (10 mg/L) was pumped continuously through the ultrafiltration cell. During this procedure the absorbance of the ultrafiltrate is measured at 264 nm and plotted versus time. The system is then rinsed with phosphate buffer and 1 mL of bovine serum albumin 40 mg/mL solution is injected into the system. The ultrafiltration membrane retains the protein molecules in the cell. The buffered solution of each compound is pumped again through the ultrafiltration cell, and the absorbance of the ultrafiltrate is measured again. An interaction between the compound and the proteins present in the ultrafiltration cell leads to a shift of this second curve to the right compared to the curve obtained initially. The extent of the shift to the right of the curve corresponds to the protein avidity of the compound. The data is evaluated with a special software written by Prof. Nickel and Dr. Reyer (Bonn).

Analysis of the Interaction of Compounds 2, 3, 4f, and 4m with Amphotericin B against Intracellular Amastigotes in Peritoneal Macrophages In Vitro. In vitro drug interactions were assessed using a modified fixed-ratio isobologram method.[15] The predetermined EC_{50} value of each drug was used to choose the top concentrations of the individual drugs in the assay. In this way, we ensured that the EC_{50} fell near the midpoint of a six-point 2-fold dilution series. Top concentrations were 0.2 μM for amphotericin B in a 72 h assay and 0.150, 0.08, 0.4, and 0.25 μM for each compound. The top concentrations were used to prepare solutions with fixed ratios (5:0, 4:1, 3:2, 2:3, 1:4, 0:5 of amphotericin B and the corresponding compound). These solutions were five times further diluted in a 2-fold dilution series. Peritoneal macrophages were treated in the same way as in the macrophage infection rate assay and the interaction assay mixtures were incubated at 37 °C, 5% CO_2, and 95% air humidity for further 48 h. Drug activity was determined from the percentage of infected cells in drug-treated cultures in relation to nontreated cultures after acridine orange and ethidium bromide staining. From the known concentration of amphotericin B in the fixed-ratio solutions, EC_{50} values were calculated by sigmoidal analysis using the program Graph-pad. For each drug combination, an EC_{50} was obtained from the fixed-ratio solutions at ratios 5:0 and 0:5. Solutions at ratios 4:1, 3:2, 2:3, and 1:4 yielded the EC_{50} of each of the drug combinations.[17]

Inhibitory Activity of Compounds 3, 4f, 4i, and 4m on Human Drug-Metabolizing CYP Enzymes. Details of the herein described methods have been published previously.[15] Stock solutions of CYP substrates were prepared in DMSO (4−50 mM). The *N,C*-coupled isoquinolines **3**, **4f**, **4i**, and **4m** were also dissolved in DMSO (stock solution 25 mM), and the final assay concentration ranges were 0.01−100 μM.

The test compounds (**3**, **4f**, and **4m**) as well as quinidine were incubated in a 100 mM Na/K-phosphate buffer containing 3 mM $MgCl_2$ (pH 7.4) with a commercially available mixture of CYP1A2/2C8/9/19/2D6 and 3A4 (0.1 mg/mL; Supermix, NatuTec, Frankfurt, Germany), NADPH (1 mM), and a substrate cocktail consisting of tacrine (CYP1A2), paclitaxel (CYP2C8), tolbutamide (CYP2C9), imipramine (CYP2C19), dextromethorphan (CYP2D6), and midazolam (CYP3A4) at 37 °C for 30 min. Control incubations (100% activity) were performed with pure DMSO. The reaction was stopped by addition of 150 μL ice-cold MeOH containing the internal standard reserpine. Finally, samples were centrifuged at 12000*g* for 6 min and the clear supernatants were used for liquid chromatography/mass spectrometry (LC/ESI-MS) analysis. The determination of the IC_{50} values was determined by incubating compounds **3**, **4f**, or **4m** or quinidine with 5 pmol/mL of CYP2D6 from baculovirus-infected insect cells (NatuTec) and the corresponding substrate dextromethorphan as described above. By incubation of various concentrations of dextromethorphan (0, 2.5, 5, 10, and 20 μM) with CYP2D6 (5 pmol/mL) and compound **4m** (0, 50, 100, 250 nM), a Lineweaver−Burk plot was constructed to determine the inhibition mechanism. All incubations were performed in triplicate.

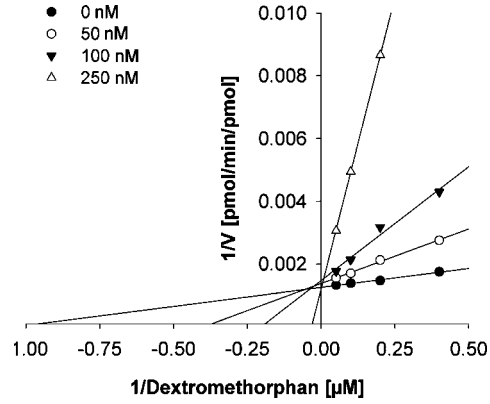


Figure 7. Lineweaver−Burk plot for the inhibition of CYP2D6 by compound **4m** (0, 50, 100, 250 nM). The results represent mean values of triplicate determinations.

The metabolites 1-hydroxytacrine, 6α-hydroxypaclitaxel, 4-hydroxytolbutamide, desipramine, dextrorphan, 1′-hydroxymidazolam, and reserpine as the internal standard were analyzed with LC/ESI-MS after automated online extraction on an Agilent (Palo Alto, CA) LC/MSD system. The inhibitory activity was calculated by dividing the metabolite to internal standard peak area ratios by the peak area ratios obtained with control incubations and was expressed as "% activity of control". IC_{50} values were calculated by nonlinear regression analysis using SigmaPlot version 10.0 (SPSS Inc., Chicago, IL). The K_i values of **3**, **4f**, and **4m**, and of quinidine for the inhibition of CYP2D6 were calculated using the Cheng−Prusoff equation $K_i = IC_{50}/(1 + [S]/K_m)$.[40]

Statistical Analysis. Data on the antiproliferative activity of the compounds (from at least two experiments) were analyzed with Thermo Electron Ascent Software and Microsoft Excel. Differences in IC_{50} and EC_{50} values of treated or untreated macrophages were tested for statistical significance by the unpaired Student's *t* test using the Graph-pad program. FIC values and sum FICs (ΣFICs[FIC amphotericin B + FIC compounds **2**, **3**, **4f**, and **4m**]) were calculated as follows: FIC amphotericin B = EC_{50} of drug in combination/EC_{50} of drug alone. The same was applied to each compound as the partner drug. FICs and ΣFICs were calculated for all fixed-ratio solutions and FICs were used to construct isobolograms. Mean ΣFICs were used to classify the nature of the interaction.[18]

Acknowledgment. The authors gratefully acknowledge the financial support from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 630), the Fonds der Chemischen Industrie (fellowship to T. Gulder and supplies), and the Hochschul- und Wissenschaftsprogramm of the University of Würzburg (fellowship to T. Gulder). We thank Christina de Witt, Christine Hambrecht, and Melanie Pavlov for technical assistance, and Dr. Marcela Fajardo-Moser and Dr. Katharina Remer for fruitful discussions.

Supporting Information Available: Chemical structures, experimental section, purity data, and HPLC analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Ponte-Sucre, A. Physiological consequences of drug resistance in *Leishmania* and their relevance for chemotherapy. *Kinetop. Biol. Dis. 2* **2003**, *14*, http://www.kinetoplastids.com/home/.

(2) Ouellette, M.; Drummelsmith, J.; Papadopoulou, B. Leishmaniasis: drugs in the clinic, resistance and new developments. *Drug Resist. Updates* **2004**, *7*, 257–266.

(3) Croft, S. L.; Sundar, S.; Fairlamb, A. H. Drug resistance in leishmaniasis. *Clin. Microbiol. Rev.* **2006**, *19*, 111–126.

(4) Bringmann, G., Günther, C., Ochse, M., Schupp, O., Tasler, S. Biaryls in Nature: A Multi-facetted Class of Stereochemically, Biosynthetically, and Pharmacologically Intriguing Secondary Metabolites. In *Progress in the Chemistry of Organic Natural Products*, Herz, W., Falk, H., Kirby, G. W., Moore, R. E., Eds.; Springer-Verlag: New York, 2001; Vol. 82, pp 1−293.

(5) François, G.; Bringmann, G.; Dochez, C.; Schneider, C.; Timperman, G.; Aké Assi, L. Activities of extracts and naphthylisoquinoline alkaloids from *Triphyophyllum peltatum*, *Ancistrocladus abbreviatus*, and *Ancistrocladus barteri* against *Plasmodium berghei* (Anka strain) in vitro. *J. Ethnopharmacol* **1995**, *46*, 115–120.

(6) François, G.; Timperman, G.; Holenz, J.; Aké Assi, L.; Geuder, T.; Maes, L.; Dubois, J.; Hanocq, M.; Bringmann, G. Naphthylisoquinoline alkaloids exhibit strong growth-inhibiting activities against *Plasmodium falciparum* and *P. berghei* in vitro − structure−activity relationships of dioncophylline C. *Ann. Trop. Med. Parasitol.* **1996**, *90*, 115–123.

(7) François, G.; Timperman, G.; Eling, W.; Aké Assi, L.; Holenz, J.; Bringmann, G. Naphthylisoquinoline alkaloids against malaria: evaluation of the curative potentials of dioncophylline C and dioncopeltine A against *Plasmodium berghei* in vivo. *Antimicrob. Agents Chemother.* **1997**, *41*, 2533–2539.

(8) Bringmann, G.; Hörr, V.; Holzgrabe, U.; Stich, A. Antitrypanosomal naphthylisoquinoline alkaloids and related compounds. *Pharmazie* **2003**, *58*, 343–346.

(9) Bringmann, G.; Hamm, A.; Günther, C.; Michel, M.; Brun, R.; Mudogo, V. Ancistroealaines A and B, two new bioactive naphthylisoquinolines, and related naphthoic acids from *Ancistrocladus ealaensis*. *J. Nat. Prod.* **2000**, *63*, 1465–1470.

(10) Bringmann, G.; Messer, K.; Brun, R.; Mudogo, V. Ancistrocongolines A−D, new naphthylisoquinoline alkaloids from *Ancistrocladus congolensis*. *J. Nat. Prod* **2002**, *65*, 1096–1101.

(11) Bringmann, G.; Dreyer, M.; Faber, J. H.; Dalsgaard, P. W.; Stærk, D.; Jaroszewski, J.; Ndangalasi, H.; Mbago, F.; Brun, R.; Reichert, M.; Maksimenka, K.; Christensen, S. B. Ancistrotanzanine A, the first 5,3′-coupled naphthylisoquinoline alkaloid, and two further, 5,8′-linked related compounds from the newly described species *Ancistrocladus tanzaniensis*. *J. Nat. Prod* **2003**, *66*, 1159–11165.

(12) Yang, L.-K.; Glover, R. P.; Yonganathan, K.; Sarnaik, J. P.; Godbole, A. J.; Soejarto, D. D.; Buss, A. D.; Butler, M. S. Ancisheynine, a novel naphthylisoquinolinium alkaloid from *Ancistrocladus heyneanus*. *Tetrahedron Lett.* **2003**, *44*, 5827–5829.

(13) Bringmann, G.; Kajahn, I.; Pedersen, S. E. H.; Reichert, M.; Faber, J. H.; Gulder, T.; Brun, R.; Christensen, S. B.; Ponte-Sucre, A.; Moll, H.; Heubl, G.; Mudogo, V. Ancistrocladinium A and B, the first *N*,*C*-coupled naphthyldihydroisoquinoline alkaloids from a Congolese *Ancistrocladus* species. *J. Org. Chem.* **2006**, *71*, 9348–9356.

(14) Ponte-Sucre, A.; Faber, J. H.; Gulder, T.; Kajahn, I.; Pedersen, S. E. H.; Schultheis, M.; Bringmann, G.; Moll, H. Activity of naphthylisoquinoline alkaloids and synthetic analogs against *Leishmania major*. *Antimicrob. Agents Chemother.* **2007**, *51*, 188–194.

(15) Fivelman, Q. L.; Adagu, I. S.; Warhurst, D. C. Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **2004**, *48*, 4097–4102.

(16) Bringmann, G.; Jansen, J. R. Einfache Synthesen nützlicher Diketo-Bausteine für biomimetische Isochinolin- und Naphthalin-Synthesen. *Liebigs Ann. Chem.* **1985**, 2116–2125.

(17) Seifert, K.; Croft, S. L. In vitro and in vivo interactions between miltefosine and other antileishmanial drugs. *Antimicrob. Agents Chemother.* **2006**, *50*, 73–79.

(18) Unger, M.; Frank, A. Simultaneous determination of the inhibitory potency of herbal extracts on the activity of six major cytochrome P450 enzymes using liquid chromatography/mass spectrometry and automated online extraction. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2273–2281.

(19) Mikus, J.; Steverding, D. A simple colorimetric method to screen drug cytotoxicity against Leishmania using the dye Alamar Blue. *Parasitol. Int.* **2000**, *48*, 265–259.

(20) Escobar, P.; Matu, S.; Marques, C.; Croft, S. L. Sensitivities of *Leishmania* species to hexadecylphosphocoline (miltefosine), ET-18-OCH_3 (edelfosine), and amphotericin B. *Acta Trop.* **2002**, *81*, 151–157.

(21) Heinze, A.; Holzgrabe, U. Determination of the extent of protein binding of antibiotics by means of an automated continuous ultrafiltration method. *Int. J. Pharm.* **2006**, *10*, 8–112.

(22) Brunton, L. L., Lazo, J. S., Parker, K. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 11th ed.; McGraw-Hill: New York, 2005; pp 1851 and 1876.

(23) Matte, C.; Maion, G.; Mourad, W.; Olivier, M. *Leishmania donovani*-induced macrophages cyclooxygenase-2 and prostaglandin E2 synthesis. *Parasite Immunol.* **2001**, *23*, 177–184.

(24) Gantt, K. R.; Schultz-Cherry, S.; Rodriguez, N.; Jeronimo, S. M.; Nascimento, E. T.; Goldman, T. L.; Recker, T. J.; Miller, M. A.; Wilson, M. E. Activation of TGF-β by *Leishmania chagasi*: importance for parasite survival in macrophages. *J. Immunol.* **2003**, *170*, 2613–2620.

(25) Olivier, M.; Gregory, D. J.; Forget, G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin. Microbiol. Rev.* **2005**, *8*, 293–305.

(26) Moll, H.; Scharner, A.; Kämpgen, E. Increased interleukin 4 (IL-4) receptor expression and IL-4-induced decrease in IL-12 production by Langerhans cells infected with *Leishmania major*. *Infect. Immun.* **2002**, *70*, 1627–1630.

(27) Berberich, C.; Ramírez-Pineda, J. R.; Hambrecht, C.; Alber, G.; Skeiky, Y. A. W.; Moll, H. Dendritic cell (DC)-based protection against an intracellular pathogen is dependent upon DC-derived IL-12 and can be induced by molecularly defined antigens. *J. Immunol.* **2003**, *170*, 3171–3179.

(28) Omer, F. M.; Kurtzhals, J. A.; Riley, E. M. Maintaining the immunological balance in parasitic infections: a role for TGF-β. *Parasitol. Today* **2000**, *16*, 358–359.

(29) Murphy, M. L.; Wille, U.; Villegas, E. N.; Hunter, C. A.; Farrell, J. P. IL-10 mediates susceptibility to *Leishmania donovani* infection. *Eur. J. Immunol.* **2001**, *31*, 2848–2856.

(30) Awasthi, A.; Mathur, R. K.; Saha, B. Immune response to *Leishmania* infection. *Indian J. Med. Res* **2004**, *119*, 238–258.

(31) Padigel, U. M.; Farrell, J. P. Control of infection with *Leishmania major* in susceptible BALB/c mice lacking the common gamma-chain for FcR is associated with reduced production of IL-10 and TGF-β by parasitized cells. *J. Immunol.* **2005**, *174*, 6340–6345.

(32) Ramos, H.; Valdivieso, E.; Gamargo, M.; Dagger, F.; Cohen, B. E. Amphotericin B kills unicellular leishmanias by forming aqueous pores permeable to small cations and anions. *J. Membr. Biol.* **1996**, *152*, 65–75.

(33) Lee, N.; Bertholet, S.; Debrabant, A.; Müller, J.; Duncan, R.; Nakhasi, H. L. Programmed cell death in the unicellular protozoan parasite *Leishmania*. *Cell. Death Differ.* **2002**, *9*, 53–64.

(34) Lewis, D. F. Human cytochromes P450 associated with the phase 1 metabolism of drugs and other xenobiotics: a compilation of substrates and inhibitors of the CYP1, CYP2, and CYP3 families. *Curr. Med. Chem.* **2003**, *10*, 955–972.

(35) Eichelbaum, M.; Ingelman-Sundberg, M.; Evans, W. E. Pharmacogenomics and individualized drug therapy. *Annu. Rev. Med.* **2006**, *57*, 119–137.

(36) Schwedhelm, K. F.; Horstmann, M.; Faber, J. H.; Reichert, Y.; Bringmann, G.; Faber, C. The novel antimalarial compound dioncophylline C forms a complex with heme in solution. *Chem. Med. Chem.* **2007**, *2*, 541–548.

(37) Huber, W.; Koella, J. C. A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. *Acta Trop.* **1993**, *55*, 257–261.

(38) Ponte-Sucre, A.; Campos, Y.; Fernández, M.; Moll, H.; Mendoza-León, A. Growth and survival of *Leishmania* sp. are impaired by ion channel blockers. *Exp. Parasitol.* **1998**, *88*, 11–16.

(39) Ponte-Sucre, A.; Vicik, R.; Schultheis, M.; Schirmeister, T.; Moll, H. Aziridine-2,3-dicarboxylates: peptidomimetic cysteine protease inhibitors with antileishmanial activity. *Antimicrob. Agents Chemother.* **2006**, *50*, 2439–2447.

(40) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

JM801084U