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N-Cinnamoylated Aminoquinolines as Promising Antileishmanial Agents

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A series of cinnamic acid conjugates of primaquine and chloroquine were evaluated for their *in vitro* antileishmanial activities. Although primaquine derivatives had modest activity, chloroquine conjugates exhibited potent activity against both promastigotes (50% inhibitory concentration [IC_{50}] = 2.6 to 21.8 μM) and intramacrophagic amastigotes (IC_{50} = 1.2 to 9.3 μM) of *Leishmania infantum*. Both the high activity of these chloroquine analogues and their mild-to-low toxicity toward host cells make them promising leads for the discovery of new antileishmanial agents.

Leishmania parasites maintain their life cycle by alternating between promastigotes in the gut of phlebotomine insects and amastigotes inside macrophages of mammalian hosts (1, 2). These protozoa cause leishmaniasis, a complex of mammalian diseases whose clinical symptoms range from self-healing cutaneous lesions to the more severe visceralizing infection (3). Human visceral leishmaniasis (VL) results from infection with *L. donovani* and *L. infantum* and is usually fatal if left untreated, accounting for more than 50,000 deaths per year (4, 5). The management of VL relies mainly on chemotherapy with pentavalent antimonials, pentamidine, paromomycin, amphotericin B or its lipid formulations, and miltefosine (5, 6). However, the treatment of this disease is hindered by the limited efficacy, high toxicity, reduced bioavailability, high cost, and parasite resistance to the action of several of the available drugs (7). As such, it is mandatory to develop novel compounds devoid of these limitations. To respond to those needs, we have tested the activity of cinnamic acid conjugates of the 8-aminoquinoline primaquine (PQ) and the 4-aminoquinoline chloroquine (CQ) against *L. infantum*. The rationale for these tests was that (i) these conjugates have been recently reported as interesting antimalarial leads (8–10) and (ii) PQ (compound 1) and CQ (compound 3) have proven activity against several protozoans, including visceralizing *Leishmania* (11–15).

All the *N*-cinnamoylated aminoquinolines (compounds 2a to 2k and 5a to 5j) and compound 3b (CQ-C4 [see Table 2]; used as the parent compound for the synthesis of 5a to 5j) were synthesized as previously described (8, 10). Cinnamic acid (compound 4), PQ, CQ (Sigma-Aldrich, Spain), and miltefosine (Cayman Chemicals) were commercially acquired. All test compounds and the reference drugs PQ and miltefosine were dissolved in dimethyl sulfoxide (Sigma), while CQ (sodium salt; Sigma) was dissolved in phosphate-buffered saline (PBS). All solutions were stored at −20°C until use. Their antileishmanial activity was initially evaluated on the promastigote stage of *L. infantum* (MHOM/MA/67/ITMAP-263, zymodeme MON-1; laboratory reference strain [16]). Briefly, promastigotes (1×10^6/well) were cultured at 25°C in complete RPMI medium (Gibco, Life Technologies) (13) supplemented with the different compounds at concentrations between 0.63 and 80 μM. The antileishmanial activity was assessed at 72 h of culture by the resazurin methodology, as previously reported (13). All the 50% inhibitory concentration (IC_{50}) (μM) values reported herein were determined with GraphPad Prism 5.0 software (GraphPad Software Inc.) from plots of percentages of parasite growth in relation to control versus inhibitor concentrations.

We found that PQ derivatives 2b to 2d and 2j ($IC_{50} < 30$ μM) were more active than PQ (IC_{50} = 33.5 μM) and as active as miltefosine (compound 6; IC_{50} = 19.6 μM) against *L. infantum* promastigotes (Table 1). These results indicate that the conjugation of PQ with a cinnamic acid containing a substituent of *p*-Me (*p*-methyl), *p*-^{i}Pr (*p*-isopropyl), *p*-OMe, or *m*-NO_2 (compound 2b, 2c, 2d, or 2j, respectively) in its aromatic ring moderately improved the activity of PQ against the promastigote stage of *L. infantum*. The insertion of any of the other substituents (compounds 2a, 2e to 2i, and 2k) on the cinnamic acid ring did not improve the antileishmanial activity of PQ.

Regarding the 4-aminoquinolines, when tested against *L. infantum* promastigotes, CQ, CQ-C4, and most of the 5a to 5j analogues had activities that were comparable to or higher than those of PQ derivatives and miltefosine (Table 2). In particular, compounds 5a and 5c to 5g were the most effective of the series, with $IC_{50} \leq 5.5$ μM.

Conjugate 5a was the most interesting compound of this series, with an IC_{50} against *L. infantum* promastigotes of 3.1 μM, which is 6-fold lower than that of its parent 3b compound (IC_{50} = 18 μM). In order to understand how the conjugation with cinnamic acid improved the activity of 3b, we evaluated the activity of cinnamic acid alone (compound 4) but found no significant activity against this form of the parasite ($IC_{50} > 80$ μM). Interestingly, the

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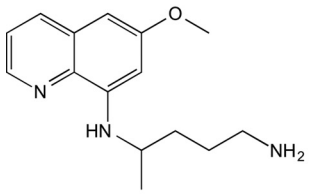
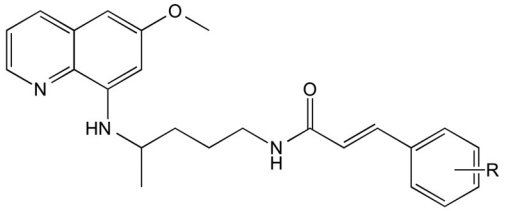
TABLE 1 Antiparasitic activities of primaquine, its cinnamic acid derivatives, and the reference drug miltefosine, with estimated ClogP values



1



2a-k

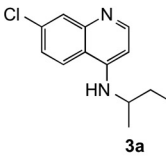
Compound[a]	*R*[b]	IC_{50} (μM) for *L. infantum* promastigotes at 72 h (mean ± SD)	ClogP value[c]
1		33.5 ± 8.7	2.28
2a	H	39.0 ± 4.5	4.30
2b	*p*-Me	18.3 ± 1.3	4.68
2c	*p*-^{i}Pr	16.0 ± 3.0	5.46
2d	*p*-OMe	22.3 ± 1.8	4.23
2e	*m*-F	52.9 ± 17.4	4.46
2f	*p*-F	35.7 ± 11.2	4.47
2g	*p*-Cl	35.1 ± 6.3	5.04
2h	*p*-Br	30.1 ± 9.0	5.04
2i	*o*-NO_2	35.4 ± 5.2	4.13
2j	*m*-NO_2	24.7 ± 2.9	4.14
2k	*p*-NO_2	>160	4.15
6		19.6 ± 4.3	4.39

[a] 1, primaquine; 2a to 2k, cinnamic acid primaquine derivatives; 6, the reference drug miltefosine.
[b] *R*, cinnamic acid substituent. Me, methyl; ^{i}Pr, isopropyl.
[c] ClogP values were calculated using ALOGPS 2.1 (http://www.vcclab.org/lab/alogps/).

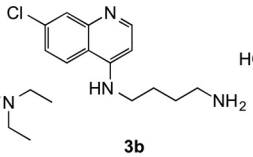
equimolar mixture of 3b and 4 also did not have higher activity than 3b alone (IC_{50} = 18 μM). Hence, activity displayed by the conjugates is not merely due to the sum of the intrinsic activities of the respective building blocks but is due to the action of new features generated by their covalent linkage.

It should be noted that none of conjugates 5a to 5j ($IC_{50} \geq 2.6$ μM) was more active than CQ (IC_{50} = 1.3 μM) against *L. infantum* promastigotes. This could signify that the positively charged tertiary amine in the aliphatic chain of CQ is important for activity against the promastigote stage of *L. infantum*. In fact, the mild

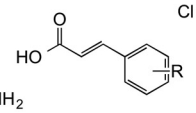
TABLE 2 Antiparasitic activities and macrophage toxicities of chloroquine, CQ-C4, cinnamic acid, cinnamic acid analogues of chloroquine, and the reference drug miltefosine, with calculated selectivity indices and estimated ClogP values[a]



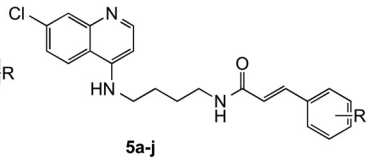
3a



3b



4



5a-j

Compound	R[b]	IC_{50} (μM) for *L. infantum* promastigotes at 72 h (mean ± SD)	IC_{50} (μM) for *L. infantum* intramacrophagic amastigotes at 72 h (mean ± SD)	IC_{50} (μg/ml) for *L. infantum* intramacrophagic amastigotes at 72 h (mean ± SD)	CC_{50} (μM) for mouse bone marrow-derived macrophages at 72 h (mean ± SD)	CC_{50} (μg/ml) for mouse bone marrow-derived macrophages at 72 h (mean ± SD)	SI	ClogP value[c]
3a		1.3 ± 0.2	23.0 ± 12.1	11.9 ± 6.2	71.3 ± 2.7	36.8 ± 1.4	3.1	4.42
3b		18.0 ± 2.9	43.9 ± 5.8	11.0 ± 1.4	74.5 ± 1.8	18.6 ± 0.4	1.7	2.47
4	H	>80.0	47.1 ± 16.1	7.0 ± 2.4	>80.0	>11.9	>1.7	2.00
3b + 4	H	18.0 ± 5.0	65.5 ± 34.5		34.1 ± 16.9			
5a	H	3.1 ± 1.8	1.2 ± 0.8	0.5 ± 0.3	25.4 ± 6.7	9.6 ± 2.5	21.2	4.49
5b	*p*-Me	21.8 ± 15.4						4.86
5c	*p*-^{i}Pr	2.6 ± 0.4	4.0 ± 0.9	1.7 ± 0.4	13.5 ± 6.2	5.7 ± 2.6	3.4	5.65
5d	*p*-OMe	3.9 ± 2.0	9.3 ± 1.9	3.8 ± 0.8	61.9 ± 26.5	25.4 ± 10.9	6.7	4.41
5e	*m*-F	3.3 ± 1.4	4.2 ± 2.9	1.7 ± 1.2	17.6 ± 0.6	7.0 ± 0.2	4.2	4.66
5f	*p*-F	5.5 ± 2.1	5.7 ± 2.7	2.3 ± 1.1	22.2 ± 5.9	8.8 ± 2.3	3.9	4.67
5g	*p*-Cl	3.1 ± 0.9	6.6 ± 2.3	2.7 ± 1.0	7.7 ± 2.1	3.2 ± 0.9	1.2	5.09
5h	*p*-Br	10.8 ± 1.6						5.23
5i	*o*-NO_2	7.3 ± 0.4						4.37
5j	*m*-NO_2	21.0 ± 7.2						4.38
6		19.6 ± 4.3	4.1 ± 3.3	1.7 ± 1.3	25.5 ± 4.5	10.4 ± 1.8	6.2	4.39

[a] 3a, chloroquine; 3b, CQ-C4; 4, cinnamic acid; 5a to 5j, cinnamic acid analogues of chloroquine; 6, the reference drug miltefosine; SI, calculated selectivity indices.
[b] *R*, cinnamic acid substituent. Me, methyl; ^{i}Pr, isopropyl.
[c] ClogP was calculated using ALOGPS 2.1 (http://www.vcclab.org/lab/alogps/).

basicity conferred to CQ by its tertiary amine has been considered a key factor for CQ's activity against the malaria agent *Plasmodium* (17). However, we have recently shown that the same cinnamic acid CQ conjugates described in this paper display nanomolar activities against blood-stage malaria parasites (8, 9), suggesting that the tertiary amine is not as important for CQ's antimalarial activity as previously supposed. What we suggest, instead, is that the *N*-cinnamoylated conjugates exert their antimalarial activity by a mechanism(s) of action (MOA) distinct from that of CQ. In fact, (i) the conjugates' ability to block hemozoin formation, the MOA traditionally attributed to CQ (17), is not fully correlated with their antimalarial activities (8, 9), and (ii) unlike CQ, the conjugates are also significantly active against liver-stage plasmodia (9). Alternatively, the decreased antipromastigote activity of cinnamoyl conjugates may be related to stereoelectronic features of the cinnamoyl group compared to those of the *N*,*N*-diethyl moiety in CQ, eventually affecting the compound's binding to its target in the *L. infantum* promastigote.

Finally, no clear correlation between lipophilicity and activity could be established among CQ analogues 5a to 5j or PQ derivatives 2a to 2k (IC_{50} and ClogP values in Tables 1 and 2).

Given that CQ and its analogues 5a and 5c to 5g displayed potent activity against promastigotes, we then explored their potential against *L. infantum* intramacrophagic amastigotes. For that purpose, differentiated macrophages (4×10^5/well), derived from the bone marrow of BALB/c mice, were infected with stationary promastigotes of *L. infantum* (4×10^6/well), as previously described (13). Compounds were added after 24 h of infection at concentrations between 2.5 and 40 μM. Cells were fixed and stained with Hemacolor (Merck, Germany) 72 h later. Representative pictures were obtained with an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging software CellB̂, Olympus). A minimum of 1,600 macrophages were counted per experimental condition using the Cell Note software (18), and the "parasite index" was calculated as the number of amastigotes per 100 macrophages. The IC_{50} (μM) values were determined with GraphPad software from plots of percentages of parasite growth reduction in relation to control versus inhibitor concentration.

All tested CQ analogues (compounds 5a and 5c to 5g; $IC_{50} \leq 9.3$ μM) were more active than CQ ($IC_{50} = 23.0$ μM) against intramacrophagic amastigotes of *L. infantum* in the order 5a(H) → 5c(*p*-^{i}Pr) = 5e(*m*-F) → 5f(*p*-F) = 5g(*p*-Cl) → 5d(*p*-OMe), as shown in Table 2. Moreover, they presented activity that was higher than or comparable to that of miltefosine ($IC_{50} = 4.1$ μM). No clear correlation was found between the activity of CQ and its conjugates against intramacrophagic amastigotes (Table 2) and their lipophilicity (ClogP values) or electronic effects of substituents on the aromatic ring of the cinnamoyl moiety. Still, different explanations might account for the efficacy of CQ conjugates being higher than that of the parent CQ against amastigotes: CQ might be more promptly metabolized by the macrophage into a less effective compound, in this way explaining its reduced activity toward amastigotes compared to promastigotes (Table 2). Alternatively, amastigotes might have increased capacity to efflux CQ, but not its cinnamic acid analogues, through, e.g., the previously characterized ABCG6 transporter of *L. infantum* (19). Lastly, the cytotoxic properties of the conjugates, compared to that of CQ, may also account for different activities against intramacrophagic parasites, as explained below.

We also evaluated the cytotoxicity of the most interesting compounds, by the resazurin methodology (13). Macrophages (8×10^4/well) were treated with CQ and compounds 5a and 5c to 5g for 72 h at 37°C, in a concentration range of 0.63 to 100 μM. The concentrations that caused a 50% loss of host cell viability (CC_{50} [in μM]) (Table 2) were determined with GraphPad software from plots of percentages of viability in relation to the control concentration relative to the inhibitor concentration. All cinnamic acid analogues were more cytotoxic than the parent CQ compound ($CC_{50} = 71.3$ μM), which may also justify their increased activity against intramacrophagic amastigotes. However, with the exception of compound 5g ($CC_{50} = 7.7$ μM), no CQ analogue was more toxic to macrophages ($CC_{50} \geq 13.5$ μM) than the reference drug miltefosine ($CC_{50} = 25.5$ μM). The presence of *p*-Cl, unlike that of the other substituents, in the aromatic ring of the cinnamic acid favors both antileishmanial activity and toxicity.

Interestingly, compound 5a, which had the best activity against promastigotes, also exhibited high activity against *L. infantum* intracellular amastigotes ($IC_{50} = 0.5$ μg/ml) while maintaining a relatively low toxicity to the host cells ($CC_{50} = 9.6$ μg/ml, comparable to that of miltefosine) (Table 2). Actually, this compound exhibits a selectivity index (SI = 21.2) that is more than 3-fold higher than that of the reference drug miltefosine (SI = 6.2). This means that compound 5a conforms to the World Health Organization's criteria for hit identification against *Leishmania* intramacrophagic amastigotes, since it displays an $IC_{50} < 1$ to 2 μg/ml and a selectivity index (SI) > 20 (20). Similarly to what happened in promastigote cultures, cinnamic acid alone (compound 4) or equimolar mixtures of compounds 4 and 3b had no significant activity against *L. infantum* amastigotes, indicating that new molecular features generated by the covalent linkage of the two pharmacophores were responsible for the antileishmania activity.

Overall, by conjugating the heteroaromatic core of CQ to substituted cinnamic acids, we have found five compounds with potent activity against *L. infantum* and moderate toxicity toward mammalian host cells. Therefore, they represent good candidates for further development as antileishmanial agents.

Keeping in mind that the tested compounds were recently reported as antimalarial leads, this work reinforces the notion that common and/or related chemical scaffolds have the potential to be active in multiple parasites, given the phylogenetic relatedness of the target organisms. Studies on the possible mechanism(s) underlying the antileishmanial activity displayed by the *N*-cinnamoylated aminoquinolines will follow. As a recent work has suggested that CQ may act against visceralizing *Leishmania* (11, 12, 19) by promoting the alkalinization of the parasite's acidocalcisomes (21), a role of the antileishmanial lead 5a as such a promoter will be investigated. Hopefully, this and subsequent work will contribute to the development of new effective antiparasitic agents and to the unveiling of their mode(s) of antiprotozoal action.

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