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Development of Potent 3-Br-isoxazoline-Based Antimalarial and Antileishmanial Compounds

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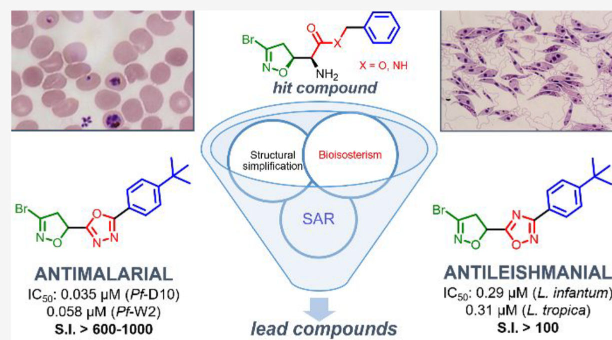
ABSTRACT: Starting from the structure of previously reported 3-Br-isoxazoline-based covalent inhibitors of *P. falciparum* glyceraldehyde 3-phosphate dehydrogenase, and with the intent to improve their metabolic stability and antimalarial activity, we designed and synthesized a series of simplified analogues that are characterized by the insertion of the oxadiazole ring as a bioisosteric replacement for the metabolically labile ester/amide function. We then further replaced the oxadiazole ring with a series of five-membered heterocycles and finally combined the most promising structural features. All the new derivatives were tested in vitro for antimalarial as well as antileishmanial activity. We identified two very promising new lead compounds, endowed with submicromolar antileishmanial activity and nanomolar antiplasmodial activity, respectively, and a very high selectivity index with respect to mammalian cells.



KEYWORDS: *malaria, leishmania, covalent inhibitor, 3-bromoisoxazoline, oxadiazole, bioisosterism*

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3-Br-isoxazoline-based derivatives endowed with antimalarial activity were previously described by our group and their biological properties were correlated to their ability to covalently and irreversibly inhibit cysteine-containing enzymes, in particular *P. falciparum* glyceraldehyde-3-phosphate dehydrogenase (*Pf*GAPDH), a key glycolytic enzyme playing a crucial role in parasite metabolism.[1] The mechanism underlying covalent inhibition is the nucleophilic attack of the catalytic cysteine residue on the Br-substituted C-3 of the isoxazoline ring.[1−3]

The most active compounds (i.e., compounds **2**–**4**, Figure 1) were all characterized by the presence of an ester or an amide function in their structure, as they were designed elaborating on the structure of the amino acid 3-Br-acivicin (compound **1**, Figure 1), whose antimalarial activity was also reported.[2]

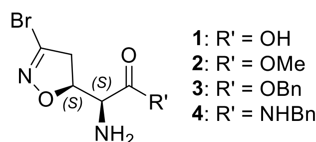

Figure 1. Structure of model compounds.

As is well-known, esters and amides may represent an Achille's heel for biologically active compounds, due to their metabolic vulnerability. Notably, in the case of compounds **2**–**4**, a metabolic conversion into the corresponding carboxylic acid derivative would produce 3-Br-acivicin, which may lead to several off-target interactions, due to the reported ability of this amino acid to inhibit enzymes of the glutamino-amido transferase family, because of its structural similarity with L-glutamine.[4] Therefore, we sought to increase the metabolic stability of these compounds through bioisosteric replacement of the ester/amide function.

Oxadiazoles are five-membered heteroaromatic rings frequently used in drug-like molecules as bioisosteric replacements for ester and amide functionalities.[5] On this ground, we first designed a new compound obtained by simply replacing the ester/amide group of compounds **3** and **4** with a 1,2,4-oxadiazole moiety, i.e., compound **5**, and then moved to the structurally simplified analogue **6a**, where the oxadiazole ring was directly linked to the 3-Br-isoxazoline ring and the amino group was eliminated (Figure 2). Compound **5** turned out to be biologically inactive, whereas compound **6a** displayed a moderate antiplasmodial activity (Table 1). From these data,

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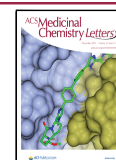

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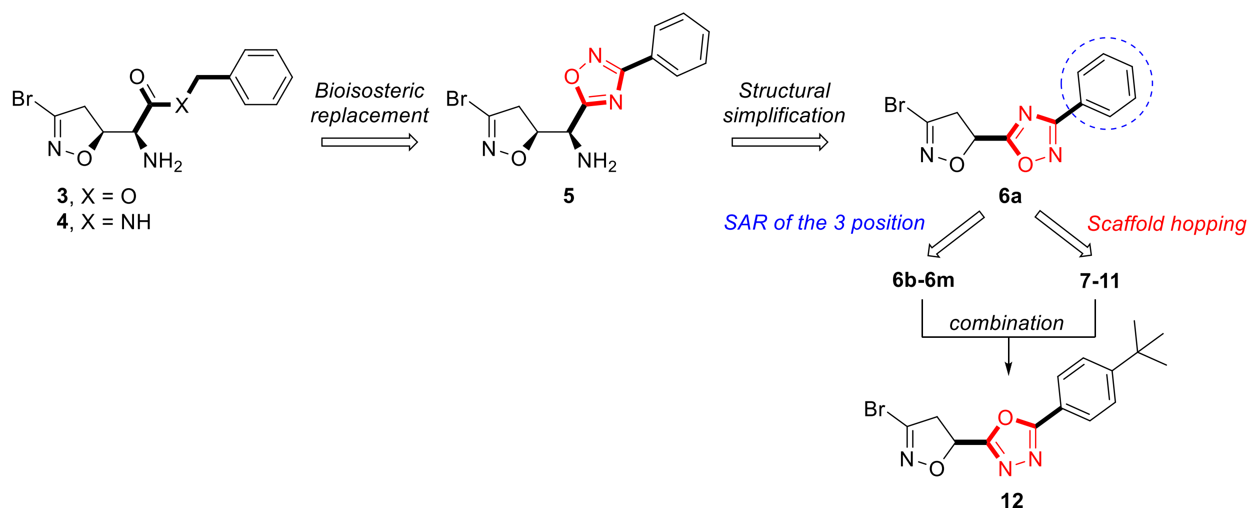

Figure 2. Med-chem strategies used for the design of new compounds starting from lead compounds **3** and **4**.

Table 1. In Vitro Activity of 3-Br-isoxazoline-Based Inhibitors against *Plasmodium falciparum* (D10 and W2 strains) and *Leishmania* spp. (*L. infantum* and *L. tropica*) Promastigotes

compd	*P. falciparum* D10 IC_{50} (μM)[a]	*P. falciparum* W2 IC_{50} (μM)[a]	*L. infantum* IC_{50} (μM)[b]	*L. tropica* IC_{50} (μM)[b]
3	0.37 ± 0.12	0.26 ± 0.05	n.d.	n.d.
4	0.36 ± 0.11	0.48 ± 0.18	n.d.	n.d.
5	inactive[c]	inactive[c]	n.d.	n.d.
6a	52.7 ± 14.4	64.8 ± 9.5	17.3 ± 7.4	43.0 ± 22.6
6b	52.7 ± 14.2	46.0 ± 3.5	26.2 ± 13.1	24.9 ± 8.26
6c	58.3 ± 8.1	48.1 ± 0.1	27.3 ± 12.3	32.6 ± 12.7
6d	inactive[c]	inactive[c]	9.0 ± 2.7	10.9 ± 2.8
6e	31.0 ± 6.9	34.0 ± 14.8	8.7 ± 4.5	6.9 ± 2.6
6f	3.1 ± 0.1	3.5 ± 0.4	0.29 ± 0.11	0.31 ± 0.03
6g	34.4 ± 5.8	42.5 ± 6.0	12.0 ± 2.0	14.7 ± 8.1
6h	29.8 ± 4.5	32.7 ± 2.8	25.6 ± 6.2	39.4 ± 11.8
6i	27.8 ± 1.1	27.2 ± 1.5	10.5 ± 4.4	5.3 ± 2.8
6j	36.9 ± 5.0	45.3 ± 4.8	inactive[c]	inactive[c]
6k	45.9 ± 7.5	40.4 ± 13.0	inactive[c]	inactive[c]
6l	45.7 ± 12.2	39.5 ± 17.0	28.8 ± 5.0	33.8 ± 24.2
6m	39.0 ± 16.3	32.5 ± 12.9	14.7 ± 6.16	25.6 ± 11.7
7	16.7 ± 8.3	15.1 ± 7.6	inactive[c]	inactive[c]
8	35.3 ± 15.9	43.0 ± 7.1	inactive[c]	inactive[c]
9	32.2 ± 14.7	39.3 ± 11.8	inactive[c]	50.7 ± 20.1
10	22.9 ± 9.6	41.3 ± 8.8	60.3 ± 6.2	inactive[c]
11	45.8 ± 3.9	39.7 ± 3.9	28.8 ± 5.0	33.8 ± 24.2
12	0.035 ± 0.014	0.058 ± 0.014	3.5 ± 1.2	7.5 ± 3.4
CQ	0.028 ± 0.004	0.406 ± 0.113	n.d.	n.d.
AMB	n.d.	n.d.	0.11 ± 0.02	0.12 ± 0.02

[a]pLDH method; data are the mean of three different experiments ± SD; D10: CQ-susceptible *Pf* strain; W2: CQ-resistant *Pf* strain; n.d.: not determined. [b]MTT assay; data are the mean of three different experiments ± SD; n.d.: not determined. [c]No activity observed at the highest concentration tested (60 μM).

we initiated an investigation of the SARs around this new scaffold, with the aim of increasing its antiparasitic activity. At first, we studied the effect of different functionalization on the aromatic ring appended at the 3 position, and then we replaced the oxadiazole ring with several different five-membered heterocyclic scaffolds. Finally, on the basis of the results of the phenotypic assays and the SAR analysis, we combined the best structural features all in one (Figure 2).

Considering that GAPDH, the putative target of this series of compounds, is not only a key enzyme in *P. falciparum* metabolism but plays also a pivotal role in the glycolytic pathway of kinetoplastida protozoan parasites,[6] we decided to broaden our biological investigation, evaluating their activity both against *P. falciparum* and two species of *Leishmania*, i.e., *L. tropica* and *L. infantum*.

The synthesis of the 1,2,4-oxadiazole derivatives **6** started from the appropriate nitriles (**14a–m**), which were commercially available or, in the case of nitriles **14i** and **14k**, were synthesized from their corresponding aldehydes (**13i**, **13k**) using a one-pot procedure that involves the transformation of the aldehyde group into an aldoxime, acetylation, and loss of acetic acid to afford the nitrile[7] (Scheme 1).

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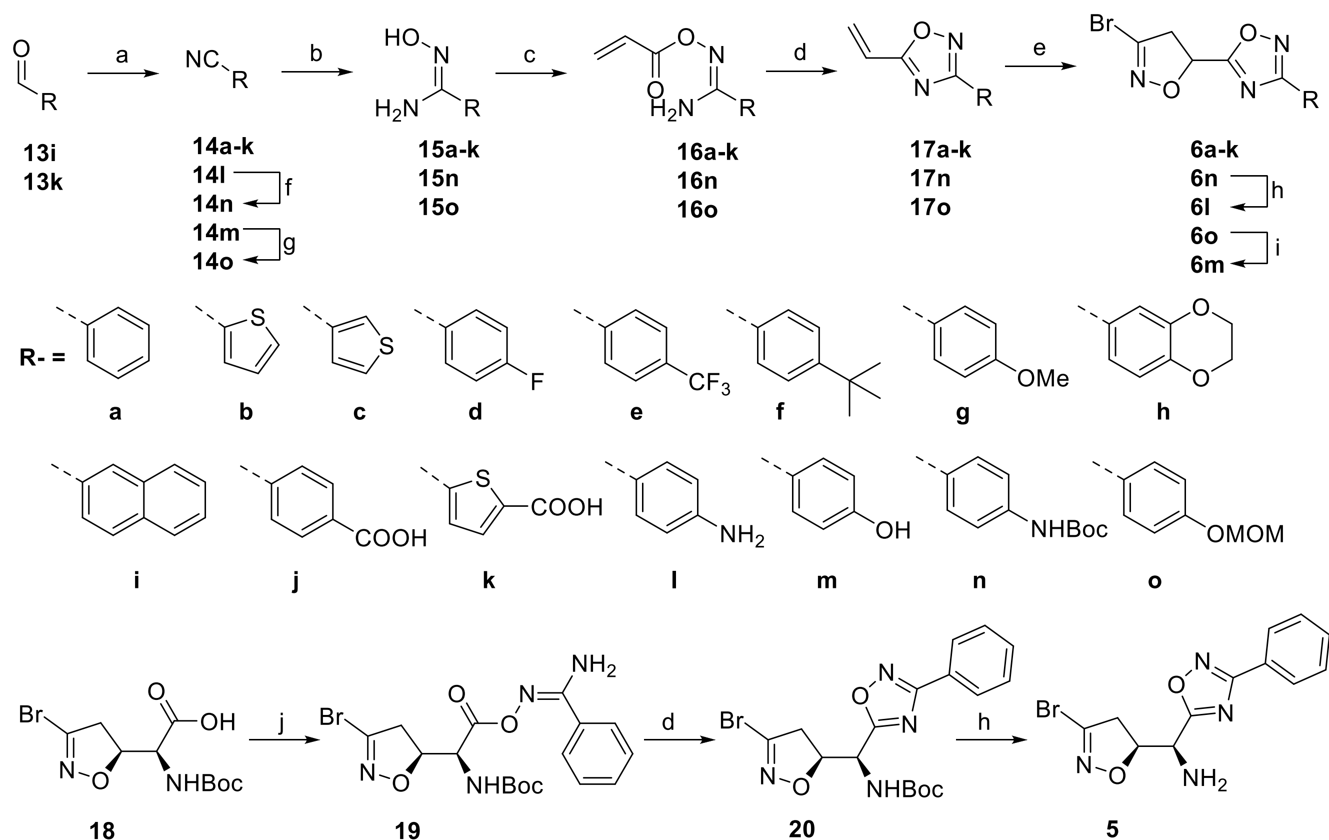
Scheme 1. Synthesis of Compounds 5 and 6a−m[a]



[a]Reagents and conditions: (a) NH_2OH*HCl, pyridine, Ac_2O, reflux, 3 h; (b) NH_2OH*HCl, Na_2CO_3, EtOH/H_2O (10:1 v/v), reflux, 4−24 h; (c) acryloyl chloride, dry THF, 1−3 h; (d) 1 M TBAF in dry THF, 30−60 min; (e) DBF, $NaHCO_3$, EtOAc, overnight; (f) Boc_2O, I_2, overnight; (g) MOMCl, DIPEA, dry DCM, 2 h; (h) 30% TFA/DCM, 4 h; (i) $BiCl_3$, CH_3CN, H_2O, 50 °C; (j) benzamidoxime, PyBOP, DIPEA, dry DCM, overnight.

Before the following step, nitrile **14l** had to be protected as *N*-Boc (**14n**) using di-*tert*-butyldicarbonate (Boc_2O) in the presence of a catalytic amount of molecular iodine under solvent-free conditions,[8] whereas nitrile **14m** was converted into the corresponding *O*-MOM protected derivative (**14o**) by reacting it with chloromethyl methyl ether (MOMCl) in the presence of diisopropylethylamine (DIPEA) (Scheme 1). Nitriles **14** were converted into amidoximes **15**, by refluxing them with hydroxylamine hydrochloride and sodium carbonate in EtOH/water. The so obtained amidoximes were chemoselectively *O*-acylated with acryloyl chloride obtaining *O*-acrylamidoximes **16**. The following cyclization reaction represents the key step of this synthetic route. A systematic analysis of the cyclization conditions was reported in the literature by Baykov and co-workers,[9] who highlighted the use of the superbase system KOH/DMSO as the more efficient method for a wide range of heat-labile intermediates. However, in a recent publication,[10] the same research group used this method to prepare a number of variously decorated vinyl-1,2,4-oxadiazoles and obtained compound **17a** in only an 11% yield, explaining this outcome with the generation of a base-initiated anionic polymerization subproduct due to the Michael acceptor behavior. Therefore, we decided to explore different reaction conditions, using compound **16a** as a model substrate and four different bases (DBU, Py, TBAF,[11] K_2CO_3) at three different temperatures (rt, 60 °C, 100 °C) in dry THF. In our hands, the best results in terms of yield and purity were achieved using a catalytic amount of TBAF (0.2 equiv) at room temperature, affording oxadiazole **17a** in only 1 h and 97% yield. Accordingly, all the desired 3-substituted-5-vinyl-1,2,4-oxadiazoles **17** were prepared following this optimized procedure.

The vinyl oxadiazoles **17a−k**, **17n**, and **17o** were then submitted to 1,3-dipolar cycloaddition reaction with bromonitrile oxide, generated in situ from its stable precursor dibromoformaldoxime (DBF), in the presence of solid $NaHCO_3$ as heterogeneous base and ethyl acetate as a solvent, according to a well-established literature procedure for the synthesis of 3-bromo-isoxazoline derivatives.[12]

The cycloaddition reaction afforded the desired 3-Br-isoxazoline compounds **6a−k**, **6n**, and **6o** (Scheme 1).

At this stage, **6n** was deprotected with a 30% solution of TFA in DCM to give the amine derivative **6l**, whereas *O*-MOM derivative **6o** was converted into the corresponding phenol **6m** with $BiCl_3$ as a Lewis acid catalyst.[13]

The same optimized procedure for the 1,2,4-oxadiazole synthesis was used to prepare compound **5**. In this case, the oxadiazole ring formation was performed after the 1,3-dipolar cycloaddition step, using the already available synthetic intermediate **18**, previously described by us,[2] which was reacted with benzamidoxime, using benzotriazol-1-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and DIPEA as coupling agents to give the *N*-acyloxybenzimidamide derivative **19**. Cyclization with catalytic TBAF in THF

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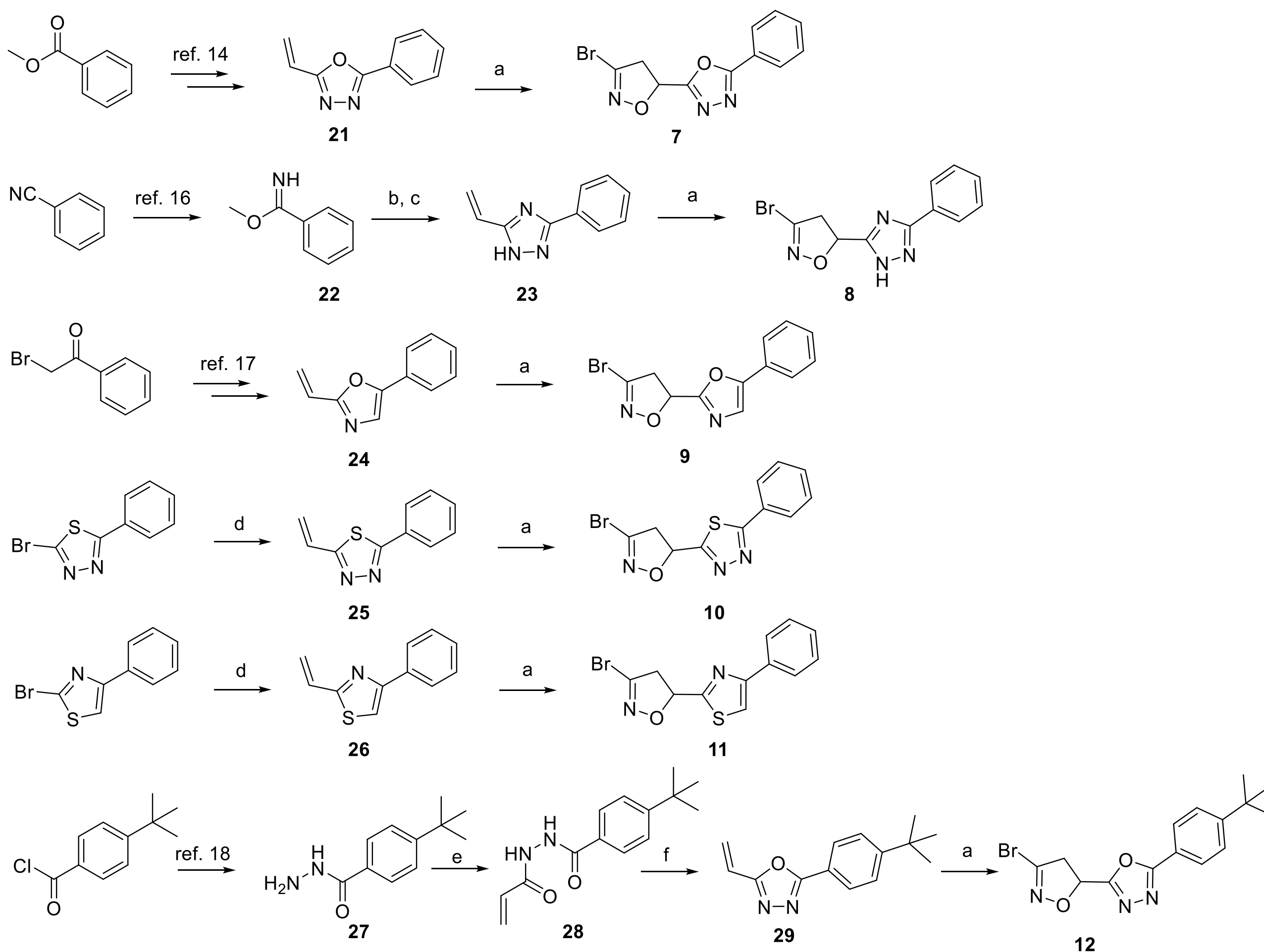
Scheme 2. Synthesis of Compounds 7−12[a]



[a]Reagents and conditions: (a) DBF, $NaHCO_3$, EtOAc, overnight; (b) acryloyl chloride, K_2CO_3, Et_2O, overnight; (c) N_2H_4*H_2O, MeOH, 2 h; (d) potassium trifluorovinylborate, $Pd(PPh_3)_4$, 2 M aq. Na_2CO_3, EtOH, toluene, reflux, 2 h; (e) acrylic acid, EDC*HCl, DCM, overnight; (f) hexachloroethane, PPh_3, DIPEA, MeCN, 2 h.

at room temperature afforded the 1,2,4-oxadiazole **20**. Finally, treatment with a 30% solution of TFA in DCM afforded the final compound **5**, which was obtained as a free amine after basic aqueous work up (Scheme 1).

The second aim of the present SAR investigation was the replacement of the 1,2,4-oxadiazole ring with different heterocyclic scaffolds, including 1,3,4-oxadiazole, 1,2,4-triazole, oxazole, 1,3,4-thiadiazole, and thiazole, keeping the rest of the molecule unmodified, as in model compound **6a**.

Compounds **7−11** were prepared through 1,3-dipolar cycloaddition of bromonitrile oxide to alkenes **21**, **23**, and **24−26**, containing the different heterocyclic scaffolds, using the procedure already described above (Scheme 2).

The desired alkenes were prepared as follows. Compound **21** was prepared starting from commercially available methyl benzoate, following the synthetic route reported by Milinkevich and co-workers.[14] The 1,2,4-triazole **23** was prepared employing the synthetic route developed by Azzouni et al.[15] with some minor modifications: methylbenzimidate **22**, in turn obtained from benzonitrile according to a published procedure,[16] was treated with acryloyl chloride in the presence of solid K_2CO_3 to give the intermediate methyl *N*-acryloylbenzimidate, which was then cyclized with hydrazine to afford **23**. Oxazole **24** was prepared as reported in the literature, starting from commercially available 2-Br-acetophenone.[17] Alkenes **25** and **26** were obtained through a Suzuki cross-coupling by reacting the commercially available brominated precursors with potassium vinyltrifluoroborate and Pd-tetrakis. (Scheme 2).

Finally, after having submitted both series of compounds **6a−m** and **7−11** to biological evaluation, as discussed in detail below, we combined the structural features of the more active derivative of each series, i.e., **6f** and **7** (Table 1) in the same derivative, thus designing compound **12** (Figure 2), which was synthesized as reported in Scheme 2. 4-*tert*-Butylbenzohydrazide **27**, prepared according to a literature procedure,[18] was acylated with acrylic acid in the presence of EDC*HCl to afford intermediate **28**, which was then cyclodehydrated with hexachloroethane/PPh_3 and DIPEA, to give **29**, following the same procedure used to prepare compound **21**.[14] Alkene **29** was finally submitted to the 1,3-dipolar cycloaddition step to produce **12**.

The phenotypic assays were conducted on D10 (chloroquine-sensitive) and W2 (chloroquine-resistant) strains of *P. falciparum*, using the parasite lactate dehydrogenase (pLDH) method and chloroquine (CQ) as control,[19,20] and on *L. tropica* and *L. infantum* promastigotes, using the MTT (3-(4,5-

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dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method and amphotericin-B (AMB) as a control.[21,22]

The activity of the tested compounds is reported in Table 1. The antiplasmodial activity was compared to that previously reported for our model compounds **3** and **4**.[1]

When the ester or amide function of **3** and **4** was bioisosterically replaced with the 1,2,4-oxadiazole group (**5**), quite surprisingly, the antiplasmodial activity was completely lost. Conversely, the activity was restored in simplified derivatives, where the amino group was eliminated and the oxadiazole ring was directly linked to the 3-Br-isoxazoline moiety, although this first series of compounds, i.e., **6a**−**m**, displayed only a modest antiplasmodial activity. The IC_{50}s were in the midmicromolar range, with the only exception of compound **6f**, which exhibited an IC_{50} of 3.1 μM against D10 strain and 3.5 μM against W2 strain.

As a general trend, all compounds were 2−10 times more potent against *Leishmania* parasites, compared to *P. falciparum* strains. Compound **6f** confirmed to be the most active compound, reaching a submicromolar activity against promastigotes of both *Leishmania* species (IC_{50} = 0.29 μM against *L. infantum* and IC_{50} = 0.31 μM against *L. tropica*).

In the second set of derivatives **7**−**11**, we replaced the 1,2,4-oxadiazole ring with different pentatomic heterocyclic scaffolds, while keeping the rest of the molecule constant as in compound **6a**. However, all the modifications generally led to a modest or negligible increase in the biological activity. The most active derivative of this second set was the 1,3,4-oxadiazole derivative **7**, displaying a 3−4 times increase in the antiplasmodial activity if compared to its analogue **6a** (IC_{50} 16.7 μM vs 52.7 μM on D10 strain and 15.1 vs 64.8 μM on W2 strain), although it was inactive against *Leishmania* parasites.

As a final effort, we combined the structural features of **6f**, the best performing derivative of the first set, and those of compound **7**, the best of the second set. This structural modification resulted in compound **12**, which showed a surprisingly potent antiplasmodial activity. Indeed, this compound displayed an IC_{50} against D10 strain (CQ-sensible *P. falciparum*) of 0.035 μM, comparable to the one of the reference compound chloroquine (IC_{50} = 0.028 μM). Moreover, contrarily to CQ, compound **12** was highly active (IC_{50} = 0.058 μM) also against the CQ-resistant *P. falciparum* strain (W2). Interestingly, it also proved to be moderately active against stage IV−V gametocytes from the 3D7 *P. falciparum* strain, although with a significantly lower potency (IC_{50} 10.4 ± 4.8 μM).

The activity of **12** against the promastigotes of two *Leishmania* species was in the low micromolar range (IC_{50} = 3.5 μM against *L. infantum* and IC_{50} = 7.5 μM against *L. tropica*).

The two more interesting compounds identified in the present study, **6f** and **12**, were then tested against the BMDM murine macrophage cell line to assess the selectivity indices for parasites versus mammalian cells (Table 2). Compound **6f** displayed a selectivity index of 11−12 for *P. falciparum vs* murine macrophages and >100 for both *Leishmania* spp. vs murine macrophages.

The latter observation is crucial for the treatment of *Leishmania* amastigotes, which normally reside and grow inside macrophages, while sparing normal cells.

Compound **12** exhibits a S.I. > 1000 for D10 and >600 for W2 *P. falciparum* strains vs murine macrophages and only >5−10 for *Leishmania* spp. *vs* murine macrophages. Comparable results in terms of selectivity indices were obtained testing compound **12** against human endothelial HMEC cells (data not shown).

Table 2. Cytotoxicity of 6f and 12 against Murine BMDM Cells and Selectivity Indices for Parasites vs Murine Cells

compd	BMDM (IC_{50} μM)	S.I.[a] *P. falciparum* D10/BMDM	S.I. *P. falciparum* W2/BMDM	S.I. *L. infantum*/BMDM	S.I. *L. tropica*/BMDM
6f	37.3 ± 1.5	12	11	129	120
12	40.0 ± 1.1	1143	690	11	5

[a]Selectivity index (S.I.) was calculated based on the IC_{50}s against the parasites reported in Table 1.

Thus, in the present work, starting from the structure of previously reported compounds and using some classical medicinal chemistry strategies such as bioisosterism, structural simplification, and SAR studies, we discovered two very promising antiparasitic agents with potent antiproliferative activity against two different protozoan parasites, *P. falciparum* and two species of *Leishmania*. Compared to the initial model compounds **3** and **4**, the new derivatives **6f** and **12** have completely lost the structural resemblance with the amino acid 3-Br-acivicin, and thus their activity cannot be in anyway led back to their ability to mimic L-glutamine. In view of a future administration in vivo, the toxicity issues deriving from a possible metabolic conversion into 3-Br-acivicin and related metabolites are definitively ruled out. Moreover, the lipophilicity is significantly improved, suggesting better membrane permeability properties compared to the initial compounds; in fact, the calculated Log $D_{7.4}$ values are 1.41 for compound **3**, 0.76 for compound **4**, 3.64 for compound **6f**, and 3.35 for compound **12**.[23] These new compounds can be prepared through simple chemical steps starting from readily available commercial synthons.

The putative target, on the basis of previous studies on analogue derivatives, is GAPDH, although a target-identification approach will be pursued in the near future to confirm the engagement of GAPDH and identify other possible targets that may covalently bind to the 3-Br-isoxazoline nucleus of **6f** and **12**.

Interestingly, on passing from the 1,2,4 oxadiazole of compound **6f** to the 1,3,4 oxadiazole of **12**, we observed a decrease in the antileishmanial activity and a pronounced increase of the antiplasmodial activity. This may underline subtle differences in the binding pocket of GAPDH in the two different parasites or the contribution of a different target.

Compound **6f** has a low micromolar potency as antiplasmodial agent and a submicromolar antileishmanial activity, whereas **12** exhibits low micromolar activity against *Leishmania* spp. and nanomolar potency against *P. falciparum*. Considering also the very good selectivity of **6f** for *Leishmania vs* murine cells and the even higher selectivity of **12** for *P. falciparum vs* murine cells, we can conclude that we have identified two very promising lead compounds that deserve further biological investigations as potential antileishmanial (**6f**) and antimalarial (**12**) compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00354.

Material and methods used in this work, including reagents, synthetic procedures, compound characterization and biological assays (PDF)

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Notes

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ABBREVIATIONS

AMP, amphotericin-B; BMDM, bone marrow-derived macrophages; Boc, *tert*-butyloxycarbonyl; CQ, chloroquine; DBF, dibromoformaldoxime; DBU, 1,8-diazabicyclo [5.4.0] undec-7-ene; DIPEA, *N,N*-diisopropylethylamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMEC, human microvascular endothelial cells; MOM, methoxymethyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pLDH, parasite lactate dehydrogenase; Py, pyridine; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; SAR, structure–activity relationship; TBAF, tetra-*n*-butylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran

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