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Easy one-pot synthesis of multifunctionalized indole–pyrrole hybrids as a new class of antileishmanial agents†

Vittorio Ciccone,[‡] Aurora Diotallevi,[‡] Miriam Gómez-Benmansour, Sara Maestrini, Fabio Mantellini,[‡] Giacomo Mari,[‡] Luca Galluzzi,[‡] Simone Lucarini[‡] and Gianfranco Favi[‡]*

A chemoselective one-pot synthesis of pharmaceutically prospective indole–pyrrole hybrids by the formal [3 + 2] cycloaddition of 3-cyanoacetyl indoles (CAls) with 1,2-diaza-1,3-dienes (DDs) has been developed. The new indole–pyrrole hybrids were phenotypically screened for efficacy against *Leishmania infantum* promastigotes. The most active compounds **3c**, **3d**, and **3j** showed $IC_{50} < 20 \mu M$ and moderate cytotoxicity, lower than miltefosine. Compound **3d** was the most active with $IC_{50} = 9.6 \mu M$ and a selectivity index of 5. Consequently, **3d** could be a new lead compound for the generation of a new class of antileishmanial hybrids.

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Introduction

Leishmaniasis, one of the most dangerous and neglected tropical diseases, results in a significant burden in terms of morbidity and mortality every year worldwide. It is caused by the protozoan parasites (genus *Leishmania*), which are transmitted by the bite of phlebotomine sandflies.¹ The disease has three different forms: visceral, cutaneous (the most common) and mucocutaneous. The visceral form can be fatal if untreated.² Leishmaniasis threatens more than 350 million people in large parts of Africa, the Middle East, South America, and Asia. Today, due to global warming and migration of populations, the endemic regions of the disease have increased to non-tropical areas, such as Mediterranean Europe, where the most common forms (visceral and cutaneous) are caused by *L. infantum*.³

Currently, no efficacious vaccine against leishmaniasis is available⁴ and the commercial drugs have several limitations such as toxicity or strong side effects, induction of resistance, administration route (intravenous), and high costs.⁵ Thus, the discovery and development of new antileishmanial agents represents an important objective for the global community.^{6–9}

Indole and pyrrole are two of the most important heterocycles and are ubiquitous in natural products and several drugs. In particular, several indole-based compounds have been

proven active against leishmaniasis. For example, Singh and collaborators reported a series of *N*-(1-methyl-1*H*-indol-3-yl) methyleneamines active against *L. major*, among them, compound **6** (Fig. 1) was the most effective of this class with $IC_{50} = 0.57 \text{ mg mL}^{-1}$ ($2.3 \mu M$).¹⁰ Compound **7**, an indole-2-hydrazone, showed a better activity than compound **6** against *L. major* promastigotes ($IC_{50} = 1.9 \mu M$).¹¹ 3,3'-Diindolylmethane (DIM, Fig. 1) was reported by Roy and colleagues as potent *L. donovani* DNA topoisomerase I poison ($IC_{50} = 1.2 \mu M$).¹² Very recently, our group reported a small library of DIM derivatives, and compound **8** was the most potent against *L. infantum* promastigotes with $IC_{50} = 2.7 \mu M$. Moreover, **8** demonstrated its efficacy in the *in vitro* infection model (intracellular amastigotes), showing IC_{50} of $6.8 \mu M$.¹³

Leishmanicidal activity of pyrrole-containing compounds is also well documented in the literature. In Fig. 1, we report four examples of differently substituted pyrroles. In detail, Allocco and coworkers demonstrated the potent *in vitro* activity of trisubstituted pyrrole **9** against *L. major* promastigotes ($IC_{50} = 0.6 \mu M$) by the inhibition of casein kinase 1 of the protozoa.¹⁴ The trisubstituted pyrrole **10**, an inhibitor of *L. infantum* trypanothione reductase, was active against *L. donovani* amastigotes ($IC_{50} = 13.8 \mu M$).¹⁵ *N*-Pyridinyl-2-acyl pyrrole **11**, reported in an interesting study by Santiago *et al.*, showed activity against *L. amazonensis* and *L. donovani* promastigotes with IC_{50} of $16.9 \mu M$ and $7.8 \mu M$, respectively.¹⁶ The last example of a pyrrole-containing antileishmanial agent is compound **12** that showed $IC_{50} = 0.33 \text{ mg mL}^{-1}$ ($1.2 \mu M$) against *L. major* promastigotes.¹⁷

Among several strategies for the discovery of new hit and/or lead compounds, molecular hybridization has proven to be a good option.^{18–20} Considering the importance of pyrrole and

Department of Biomolecular Sciences, University of Urbino Carlo Bo, 61029 Urbino (PU), Italy. E-mail: simone.lucarini@uniurb.it; gianfranco.favi@uniurb.it; Tel: +39-0722303333; +39-0722303444

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‡ These authors contributed equally to this work.



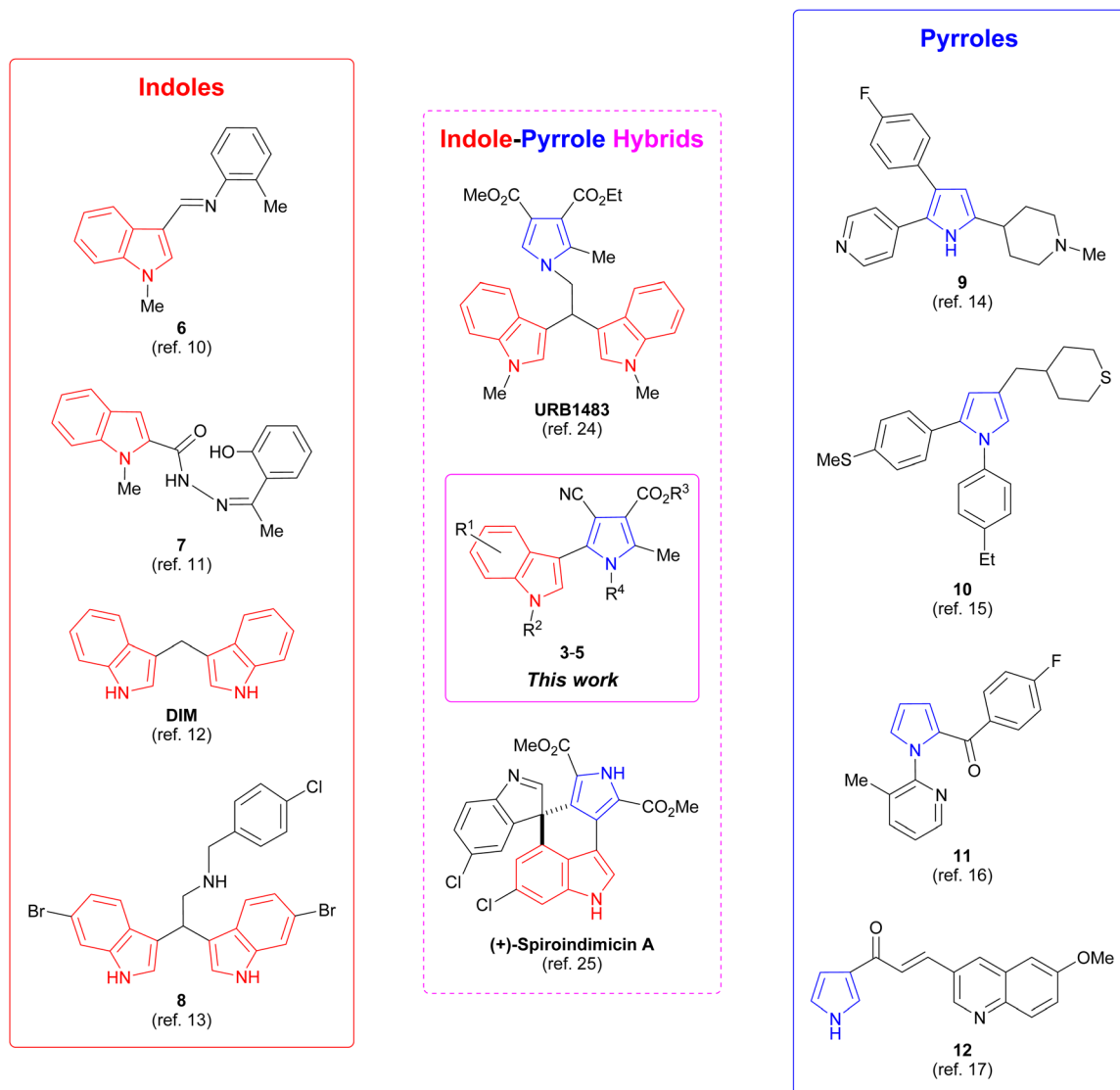


Fig. 1 Examples of indole- and pyrrole-based compounds and indole-pyrrole hybrids with antileishmanial activity.

indole scaffolds, we envisaged that hybrid entities obtained by joining these two heterocycles should have a novel greater pharmacological potency and/or reduced compound toxicity than the individual starting moieties.^{21–23} Literature already provides evidence regarding the antileishmanial activity of indole-pyrrole hybrids, but to the best of our knowledge, no information was reported about their mechanism of action.

In detail, an example is URB1483 (Fig. 1), described in our previous work,²⁴ which was selected from a phenotypic screening of anazole-bisindole chemical library and showed $IC_{50} = 3.7 \mu M$ against *L. infantum* with no quantifiable cytotoxicity in mammalian cells. Moreover, URB1483 reduced the infection index of both human and canine macrophages with an effect comparable to the clinically used drug pentamidine. In addition, we also hypothesized an inhibition of the topoisomerase IB of the parasite for the lead URB1483 by molecular modelling studies, but the compound resulted inactive against the isolated enzyme.²⁴ Another interesting example of a hybrid

compound is the natural alkaloid (+)-spiroindimicin A, elegantly synthesized by the group of Zhang. (+)-Spiroindimicin A is a cyclic molecule containing a direct bond between the indole and pyrrole scaffolds that showed a very good activity against *L. amazonensis* promastigotes ($IC_{50} = 1.3 \mu M$); however, the authors did not report any information about its mechanism of action.²⁵

Although considerable efforts have been made to obtain indole-pyrrole conjugates,^{26–30} only a few have been dedicated to exploring multifunctionalized C3–C2' indole linked pyrrole hybrids.^{31–33} In view of our continued interest in fabricating heterocycles as antileishmanial compounds,²⁴ a novel series of densely functionalized indole-pyrrole hybrids was designed (Fig. 1), prepared and assessed for their *in vitro* anti-parasitic activity. Our strategy involves the construction of the pyrrole ring of bi-heterocyclic hybrids (3–5) by a formal [3 + 2] cycloaddition reaction of 3-cyanoacetyl indoles (1) with 1,2-diaza-1,3-dienes (2).

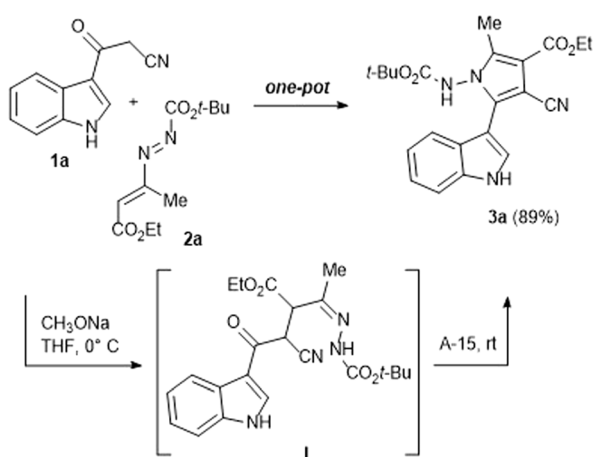


Result and discussion

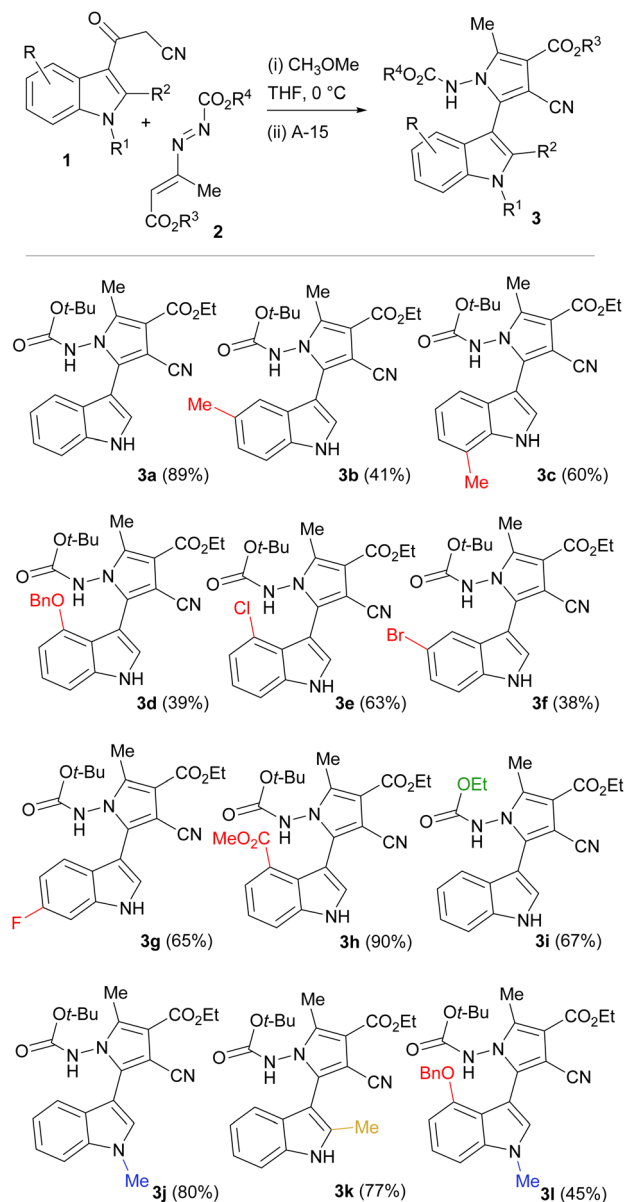
Based on our previous work,^{34,35} we initiated the study selecting 3-cyanoacetyl indole (**1a**) and 1,2-diaza-1,3-diene (**2a**) as model substrates to check the reaction progress. We registered a rapid and efficient formation of 1,4-adduct intermediate (**I**) as the main component from a mixture of **1a** (0.4 mmol) and **2a** (0.4 mmol) in THF (6 mL) at 0 °C upon addition of 20 mol% of sodium methoxide (0.08 mmol) (10 min, TLC monitoring) (Scheme 1). Although the pyrrole ring formation was previously obtained by Cu-assisted azacyclization,³⁵ our orienting experiments revealed that none of the copper salts tested [$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, CuCl_2 , CuBr_2 , $\text{Cu}(\text{OAc})_2$, and $\text{Cu}(\text{TfO})_2$] led to the desired biheterocycle **3a**. In order to establish suitable reaction conditions for the direct assembly of indole–pyrrole hybrids under green conditions, we turned our attention to Amberlyst 15(H) (A-15), a commercially available heterogeneous sulfonic acid catalyst.³⁶ A-15 stands out among commonly used Brønsted acids because it is inexpensive, non-toxic, easily handleable, recoverable and recyclable. Thus, after the initial base promoted (MeONa) formation of the adduct **I**, A-15 (0.6 mmol) was subsequently added to the reaction mixture. To our great satisfaction, the *in situ* transformation of the intermediate proceeded smoothly, affording the desired product **3a** with excellent isolated yield (12 h, 89%).

After establishing the reaction conditions for the rapid assembly of **3a**, the reaction with indole derivatives having diverse substituents was carried out (Scheme 2).

For example, substrates with methyl and halogen groups (Cl, Br, and F) at the 4-, 5-, 6-, and 7-position of the indole unit were positioned to give the corresponding product. Furthermore, 3-cyanoacetyl-4-benzyloxy indole reacted with DD **2a** to provide the indole–pyrrole hybrid **3d**, albeit in modest yield. On the other hand, the use of methyl indole-4-carboxylate furnished the relative hybrid compound **3h** in excellent yield. While the use of a DD partner with ethyl ester protection at the *N*-terminus (**2b**) provided the desired compound **3i** in lower yield than those from **2a**, the introduction of a methyl group at the N1- and C2-position of the indole ring was well tolerated (**3j** and **3k**).



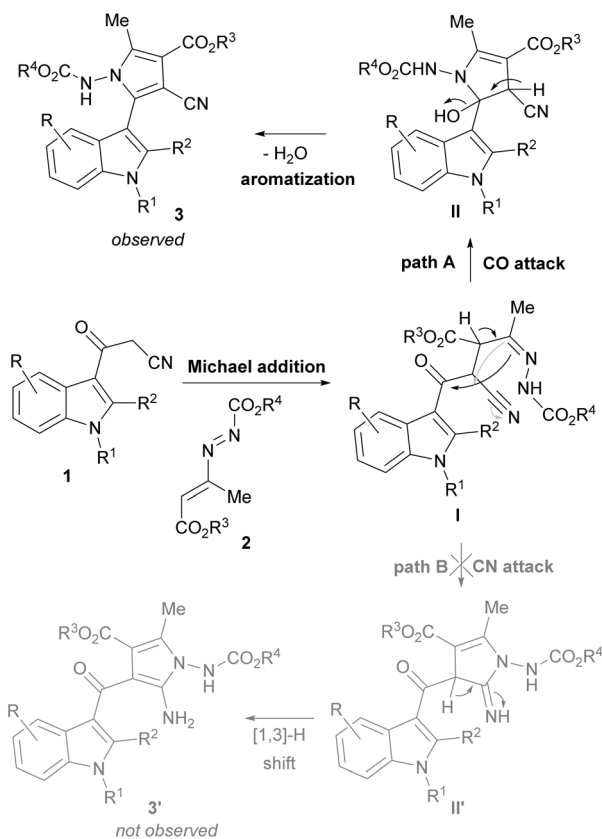
Scheme 1 One-pot two-step synthesis of indole–pyrrole hybrid **3a**.



Scheme 2 Substrate scope of highly functionalized indole–pyrrole hybrids **3**. Reagents and conditions: (i) **1** (0.4 mmol), **2** (0.4 mmol), MeONa (0.08 mmol), THF, 0 °C; (ii) A-15 (1.5 eq.), r.t.

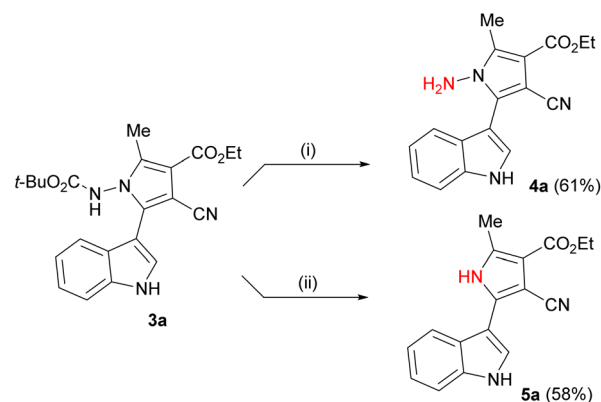
Moderate result in terms of yield was finally obtained by introducing both benzyloxy and methyl groups at C4 and N1 of the indole moiety, respectively (**3l**).

According to the experimental results and previous studies, a plausible mechanism is proposed (Scheme 3). Initially, a base-catalyzed Michael addition between **1** and **2** gives the adduct intermediate **I**, which undergoes an intramolecular pyrrole formation to furnish 2,3-dihydro-1*H*-pyrrol-2-ol intermediate **II**. Finally, this latter undergoes aromatization to give compound **3**. Although an intramolecular nucleophilic attack of hydrazonic nitrogen at the cyano group *via* a *path B* mechanism to produce product **3'** from **I** could also be possible, we did not observe this occurrence. The compound **3** was obtained with exclusive chemoselectivity, suggesting that the reaction proceeds *via*

Scheme 3 Plausible mechanism for the formation of **3**.

a carbonyl function attack through a *path A* mechanism, a result which is supported by the previous literature data.^{31,32}

In addition, the presence of an alkoxy carbonyl amino residue at the pyrrole *N*-atom makes these biheterocycles excellent precursors for further synthetic manipulations such as *N*-Boc deprotection and *N*-*N* bond cleavage (Scheme 4).

Scheme 4 Synthetic transformations of **3a**. (i) TFA (5 eq.), CH₂Cl₂, r.t.; (ii) (1) BrCH₂CO₂Et (1.5 eq.), Cs₂CO₃ (2.5 eq.), CH₃CN, 50 °C; (2) Cs₂CO₃ (2.5 eq.), CH₃CN, 80 °C.

Accordingly, compound **3a** was subjected to removal of Boc protecting group by treatment with TFA,³⁷ to give 3-(1*H*-pyrrol-1-amine-2-yl)-1*H*-indole derivative **4a**. For the *N*-*N* bond cleavage, the conversion to **5a** was successfully obtained employing the Magnus conditions.³⁸

The *in vitro* anti-parasitic activity of all fourteen new indole-pyrrole hybrids was first evaluated on *L. infantum* MHOM/TN/80/IPT1 promastigotes at a single dose of 20 μM for 72 h. Only compounds **3j**, **3c** and **3d** showed an anti-leishmanial activity >40% at this concentration. In particular, the non-substituted indole **3a** did not show any inhibition of *L. infantum* promastigotes. Methylation of the indole ring in position 1 (compound **3j**) and 7 (compound **3c**) gave active compounds with inhibition of 47.6% and 44.1%, respectively (entries 2 and 5, Table 1). On the other hand, 2- and 5-methyl indole derivatives **3k** and **3b** did not show any activity. Halogenated derivatives **3e**–**g** also showed no inhibition of the promastigote's viability. Position 6 was also explored by electron withdrawing (–COOMe, compound **3h**) or donating (–OBn, compound **3d**)

Table 1 Summary of indole-pyrrole hybrid activity on *L. infantum* promastigotes and THP-1 cells and corresponding selectivity indexes. IC₅₀ and CC₅₀ values are reported as mean and 95% CI, from duplicate experiments. Miltefosine (MILT) was used a positive control

	Cmp	Inhibition of <i>L. infantum</i> ^a at 20 μM (%)	<i>L. infantum</i> ^a IC ₅₀ (μM) (95% CI)	THP-1 CC ₅₀ (μM) (95% CI)	SI ^b
1	3a	–31.6			
2	3j	47.6	16.7 (15.1–18.8)	61.2 (58.6–63.5)	3.7
3	3k	–48.9			
4	3b	–4.1			
5	3c	44.1	20.7 (18.9–23.9)	65.2 (53.0–77.4)	3.2
6	3g	–29.8			
7	3e	–15.6			
8	3f	–75.7			
9	3h	–13.9			
10	3d	63.0	9.6 (8.6–10.7)	43.1 (36.2–51.3)	4.5
11	3l	35.6			
12	3i	–91.5			
13	4a	–49.8			
14	5a	–46.6			
15	MILT	97.8	3.7 (3.3–4.1)	36.6 (32.8–40.6)	9.9

^a *L. infantum* MHOM/TN/80/IPT1 promastigotes. ^b Selectivity index = CC₅₀/IC₅₀.



substituents. Although, **3h** was not active, **3d** resulted the most active compound of the series with an inhibition of 63% of the protozoa viability. *N*-Methylation of **3d** gave a less active compound with an inhibition of 35.6% of the promastigotes (compound **3l**, entry 11), contrary to what was shown by compound **3a**. Switching from Boc carbamate **3a** to ethyl carbamate **3i** showed no improvement in terms of activity (entry 12). Cleavage of *N*-Boc protecting group (compound **4a**) or N–N bond (compound **5a**) on the pyrrole ring did not give any active compounds (entries 13–14).

The most promising compounds **3j**, **3c**, and **3d** were tested on *L. infantum* MHOM/TN/80/IPT1 promastigotes for 72 h with scalar dilution 1 : 2 and 2 : 3 (from 20 to 0.31 μM) to determine IC_{50} . While **3j** and **3c** showed IC_{50} of 16.7 μM and 20.7 μM , compound **3d** displayed $\text{IC}_{50} < 10 \mu\text{M}$ (9.6 μM , entry 10). Later, the exact cytotoxicity (CC_{50}) of these three most active compounds was evaluated on THP-1 cells as described in the methods. All three compounds showed moderate cytotoxicity ($43 \mu\text{M} < \text{CC}_{50} < 65 \mu\text{M}$). The most active compound **3d** displayed a cytotoxicity comparable to miltefosine (entries 10 and 15, Table 1).

Conclusions

Over the last 150 years of research, about 91% of small-molecule drugs were discovered through phenotypic screening. The data shows the importance of this type of research which could possibly counteract the actual productivity crisis of target-based drug discovery.³⁹ Along this line, a novel series of densely functionalized indole–pyrrole bi-heterocycles has been prepared and phenotypically screened against *L. infantum* promastigotes. The most active compounds **3d**, **3j**, and **3c** showed IC_{50} of 9.6, 16.7 and 20.7 μM , respectively. All these three compounds displayed moderate toxicity on human macrophage-like THP-1 cells comparable or lower than the reference drug miltefosine. Although the most potent compound **3d** showed a moderate selectivity index ($\text{SI} \approx 5$), it may represent a lead compound of a new class of multi-functionalized indole–pyrrole hybrid active against leishmaniasis. Even if at the present we do not have information about the biological target(s) of this class of molecules, this work does represent a further successful example of molecular hybridization strategy and consequent phenotypic screening for the discovery of new antiparasitic agents.

Experimental

Chemistry

General remarks. All the commercially available reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA), BLDpharm (Shanghai, China), Fluorochem (Haddington, UK), or TCI (Tokyo, Japan) and they were used without further purification. In particular, indoles (**1a–k**), cyanoacetic acid (**A**) and acetic anhydride (**B**) were commercial materials; 3-cyanoacetyl indoles (CAIs) **1a–k** and 1,2-diaza-1,3-dienes (DDs) **2a,b** were prepared according to literature procedures (see ESI†). Chromatographic purification of compounds was carried

out on silica gel (60–200 μm). TLC analysis was performed on Merck silica gel plates (silica gel 60 F254); compounds were visualized by exposure to UV light and by dipping the plates in 1% $\text{Ce}(\text{SO}_4) \cdot 4\text{H}_2\text{O}$, 2.5% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 10% sulphuric acid followed by heating on a hot plate. All ^1H NMR and ^{13}C NMR spectra were recorded at 400 and 101 MHz, respectively, using $\text{DMSO}-d_6$ or CDCl_3 as solvent on a Bruker Ultrashield 400 spectrometer (Bruker, Billerica, MA, USA) and analyzed using TopSpin 1.3 (2013) software package. Chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent and are sorted in descending order within each group. The following abbreviations are used to describe peak patterns where appropriate: s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, sept = septet, m = multiplet and br = broad signal. All coupling constants (J value) are given in Hertz [Hz]. High-resolution mass spectra were performed by slow direct infusion ($5 \mu\text{L min}^{-1}$) of $\approx 0.1 \mu\text{g mL}^{-1}$ solution (acetonitrile/0.1% aqueous formic acid 1 : 1) of new compounds, using Orbitrap Exploris 240 mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an ESI source; only molecular ions $[\text{M} + \text{H}]^+$ are given. Melting points were determined by Buchi (Gallen, Switzerland) B-540 in open capillary tubes and are uncorrected.

General procedure for the one-pot two-step synthesis of indole–pyrrole hybrids 3a–l. To a stirred solution of the 3-cyanoacetyl indole **1** (0.4 mmol) in THF (2 mL) in the presence of a catalytic amount of sodium methoxide (0.08 mmol) was added dropwise a solution of azoalkene **2** (0.4 mmol) in THF (2 mL) at 0 $^\circ\text{C}$. The mixture was magnetically stirred at 0 $^\circ\text{C}$ until consumption of the starting material (TLC check). Once the Michael addition was completed (the formation of two isomers of adduct intermediate as major components was revealed by TLC), Amberlyst 15(H) (1.5 equiv.) was added, and the reaction was stirred for an additional 12 hours. After completion of the reaction (monitored by TLC), the resin was filtered off, washed with THF and the solvent was removed under reduced pressure. The crude residue was purified by silica gel column chromatography using cyclohexane and EtOAc as an eluent to obtain the corresponding product **3**. As an example, we report below the characterization of compound **3a**. The characterization of the compounds **3b–l** is reported in the ESI.†

Ethyl 1-((tert-butoxycarbonyl)amino)-4-cyano-5-(1H-indol-3-yl)-2-methyl-1H-pyrrole-3-carboxylate (3a). Compound **3a** was isolated by column chromatography (ethyl acetate/cyclohexane 30 : 70) in 89% yield (145.4 mg); white solid; mp: 188–190 $^\circ\text{C}$; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.73 (s, 1H), 10.56 (s, 1H), 7.53 (d, $J = 2.8$ Hz, 1H), 7.50 (d, $J = 8.0$ Hz, 2H), 7.20 (t, $J = 7.6$ Hz, 1H), 7.10 (t, $J = 7.6$ Hz, 1H), 4.30 (q, $J = 7.2$ Hz, 2H), 2.41 (s, 3H), 1.36 (s, 9H), 1.33 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 162.8, 154.7, 139.0, 138.4, 136.3, 127.2, 126.0, 122.4, 120.3, 119.9, 116.0, 112.5, 110.2, 101.8, 90.4, 81.9, 60.5, 28.2, 14.5, 10.9; HRMS (ESI-Orbitrap, m/z): calcd for $\text{C}_{22}\text{H}_{25}\text{N}_4\text{O}_4$ $[\text{M} + \text{H}]^+$ 409.1870; found 409.1882.

Ethyl 1-amino-4-cyano-5-(1H-indol-3-yl)-2-methyl-1H-pyrrole-3-carboxylate (4a). Compound **4a** was prepared according to the literature procedure.³⁷ To a solution of **3a** (81.7 mg, 0.2 mmol) in dichloromethane (2 mL) was added TFA (96.0 mg,

1.0 mmol) dropwise at 0 °C. The mixture was stirred at r.t. until TLC showed complete consumption of starting material. Water was added and the mixture was extracted with AcOEt (2 × 10 mL). The combined organic layer was washed with brine, separated, dried over Na₂SO₄ and filtered. After the solvent was removed under reduced pressure, the residue was purified by silica gel column chromatography (ethyl acetate/cyclohexane 40 : 60) to afford **4a** as white solid (37.6 mg, 61%). Mp: 209–211 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.65 (s, 1H), 7.75 (d, *J* = 2.3 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.49 (d, *J* = 8.0 Hz, 2H), 7.19 (t, *J* = 7.5 Hz, 1H), 7.11 (d, *J* = 7.5 Hz, 2H), 4.26 (q, *J* = 7.2 Hz, 2H), 1.31 (t, *J* = 7.2 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.2, 139.1, 138.0, 136.3, 127.7, 126.2, 122.1, 120.5, 120.0, 116.9, 112.3, 109.9, 102.9, 88.9, 60.1, 14.6, 11.6. HRMS (ESI-Orbitrap, *m/z*): calcd for C₁₇H₁₇N₄O₂ [M + H]⁺ 309.1346; found 309.1341.

Ethyl 4-cyano-5-(1*H*-indol-3-yl)-2-methyl-1*H*-pyrrole-3-carboxylate (5a). Compound **5a** was prepared according to a modified version of the Magnus method.³⁸ To a solution of **3a** (81.7 mg, 0.2 mmol) in acetonitrile (5 mL), ethyl bromoacetate (0.033 mL, 0.3 mmol) and Cs₂CO₃ (162.9 mg, 0.5 mmol) were added. The mixture was stirred at 50 °C (oil bath) until the disappearance of the starting material (0.5 h, TLC check). The solvent was removed under vacuum, water (5 mL) was added, and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layer was dried over Na₂SO₄ and filtered. After the solvent was removed under reduced pressure, the residue was dissolved in acetonitrile (5 mL) and Cs₂CO₃ (162.9 mg, 0.5 mmol) was added. The mixture was stirred at 80 °C until TLC showed complete consumption of intermediate. The solvent was removed under vacuum, water (5 mL) was added, and the mixture was extracted with ethyl acetate (3 × 10 mL). The collected organic phase was washed with brine, dried over Na₂SO₄ and filtered. After the solvent was removed under vacuum, the residue was purified by column chromatography (ethyl acetate/cyclohexane 30 : 70) to afford compound **5a** as a white solid (34.1 mg, 58% yield). Mp: 231–233 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.11 (s, 1H), 11.65 (s, 1H), 7.75–7.71 (m, 2H), 7.50 (d, *J* = 8.0 Hz, 1H), 7.21 (t, *J* = 8.0 Hz, 1H), 7.14 (t, *J* = 8.0 Hz, 1H), 4.25 (q, *J* = 7.2 Hz, 2H), 2.51 (s, 3H), 1.31 (t, *J* = 7.2 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.3, 137.9, 136.5, 136.0, 125.9, 125.0, 122.5, 120.2, 120.1, 117.4, 112.5, 112.0, 105.0, 89.1, 60.0, 14.7, 13.1. HRMS (ESI-Orbitrap, *m/z*): calcd for C₁₇H₁₆N₃O₂ [M + H]⁺ 294.1237; found 294.1252.

In vitro studies

Parasite and cell cultures. The WHO international reference strain *L. infantum* MHOM/TN/80/IPT1 (ATCC® 50134™) was routinely cultured in Evans's Modified Tobie Medium (EMTM) at 26–28 °C. To test the indole–pyrrole hybrid compounds, the parasites were maintained in RPMI-PY medium as described previously.¹³ The human monocytic cell line THP-1 (ECACC 88081201) was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg mL^{−1} streptomycin, 100 U L^{−1} penicillin, and maintained in a humidified incubator at 37 °C and 5% CO₂. All

cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

***L. infantum* viability assay.** The activity of indole–pyrrole hybrid compounds was first evaluated on late log/stationary phase *L. infantum* promastigotes resuspended in complete RPMI-PY medium in 96-well plates with a density of 2.5 × 10⁶ parasites per mL (100 µL per well). The anti-parasitic activity was initially investigated at a single dose of 20 µM of each compound for 72 h at 26 °C. Those compounds showing anti-parasitic activity >40% were further tested with scalar dilutions 1 : 2 or 2 : 3 (from 20 to 0.31 µM) on promastigotes to determine IC₅₀. As positive control, the anti-leishmanial drug miltefosine (Sigma-Aldrich, Milan, Italy) was included. All conditions were carried out in duplicate. The promastigotes viability was established using the CellTiter 96H aqueous non-radioactive cell proliferation assay (Promega, Madison, Wisconsin, USA), as previously reported.²⁴ The IC₅₀ values were determined using the nonlinear regression curves in GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA). The equation used for data fitting was $Y = 100 / (1 + 10^{((\log IC_{50} - X) \times \text{hillslope}))}$ (hillslope not constrained), where *X* is equal to the log of concentration and *Y* is the normalized response.

Evaluation of cytotoxicity on THP-1 cells. The cytotoxicity of indole–pyrrole hybrid compounds was evaluated on THP-1 cells seeded at a density of 5 × 10⁶ cells per mL, 100 µL per well in a 96-well plate and treated for 48 h with 20 ng mL^{−1} phorbol myristic acid (PMA) to induce differentiation into macrophage-like cells. After cell adhesion, selected compounds were tested to determine CC₅₀ with scalar dilutions 1 : 2 (from 100 to 3.12 µM). Not-treated cells (negative control) and the anti-leishmanial drug miltefosine were included in each experiment. Each condition was carried out in duplicate. The CellTiter 96H Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was performed to evaluate the selected compounds cytotoxicity, as described above. The selectivity index, calculated as the ratio between cytotoxicity in THP-1 and activity against *L. infantum* promastigotes, was included for each compound.

Statistical analysis. The evaluation of IC₅₀ in promastigotes and CC₅₀ in mammalian cells following indole–pyrrole hybrid compounds treatment was performed by nonlinear regression analysis and expressed as means and 95% confidence interval. All statistical tests were performed using GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, CA, USA). 277 A *p*-value ≤ 0.05 was considered significant.

Author contributions

Conceptualization: SL and GF; data curation: AD, FM and GM; funding acquisition: FM, LG, SL and GF; investigation: VC, AD, MGB and SM; methodology: AD, FM and GM; project administration: FM, LG, SL and GF; supervision: LG, SL and GF; writing – original draft: SL and GF; writing – review & editing: VC, AD, MGB, FM, GM, SL and GF.

Conflicts of interest

There are no conflicts to declare.



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