Received: 4 December 2019 | Revised: 20 February 2020 | Accepted: 25 February 2020

DOI: 10.1002/ardp.201900363

FULL PAPER

DPhG ARCH PHARM Archiv der Pharmazie

Evaluation of the antiparasitic activities of imidazol-2-ylidene–gold(I) complexes

Waleed S. Koko[1] | Jana Jentzsch[2] | Hussein Kalie[3] | Rainer Schobert[3] | Klaus Ersfeld[2] | Ibrahim S. Al Nasr[1,4] | Tariq A. Khan[5] | Bernhard Biersack[3]

[1]College of Science and Arts in Ar Rass, Qassim University, Ar Rass, Saudi Arabia

[2]Laboratory of Molecular Parasitology, University of Bayreuth, Bayreuth, Germany

[3]Organic Chemistry Laboratory, University of Bayreuth, Bayreuth, Germany

[4]College of Science and Arts in Unaizah, Qassim University, Unaizah, Saudi Arabia

[5]College of Applied Health Sciences in Ar Rass, Qassim University, Ar Rass, Saudi Arabia

Correspondence
Bernhard Biersack, Organic Chemistry Laboratory, University of Bayreuth, Universitätsstrasse 30, 95440 Bayreuth, Germany.
Email: bernhard.biersack@yahoo.com

Funding information
Qassim University, Grant/Award Number: cosao-bs-2019-2-2-1-5619; Deutsche Forschungsgemeinschaft, Grant/Award Number: Scho 402/12-2

Abstract

A series of cationic gold(I)–carbene complexes with various 4,5-diarylimidazolylidene ligands were either newly prepared or repurposed for testing against protozoal *Leishmania major*, *Toxoplasma gondii*, and *Trypanosoma brucei* parasites. The syntheses of the new complexes **1b** and **1c** were described. Ferrocene compound **1a** showed the highest activities against *L. major* amastigotes and *T. gondii* and distinct selectivity for *T. gondii* cells when compared with the activity against nonmalignant Vero cells. The ferrocene derivatives **1a–c** are generally more active against the *L. major* amastigotes and the *T. gondii* tachyzoites than the other tested anisyl gold complexes and the approved drugs atovaquone and amphotericin B. Compounds **1a** and **1e** showed the highest selectivities for *L. major* amastigotes. Compounds **1d** and **1f** showed the highest selectivities for *L. major* promastigotes; **1f** was the most active compound against *L. major* promastigotes of this series of compounds. The 3,4,5-trimethoxyphenyl analog **1b** also exhibited a much greater selectivity for *T. b. brucei* cells when compared with its activity against human HeLa cells.

KEYWORDS

antiparasitic drugs, gold, metal-based drugs, neglected tropical diseases, *N*-heterocyclic carbene

1 | INTRODUCTION

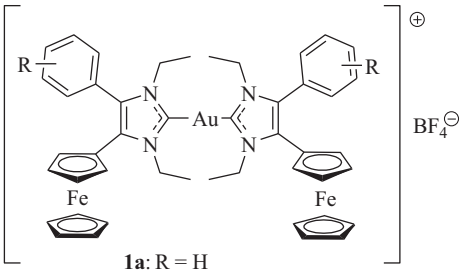
New, efficient drugs for the treatment of parasitic diseases are sought-after, and numerous efforts to identify antiparasitic drugs against neglected tropical diseases (NTDs) are already being made.[1] Both locals and travelers in tropical and subtropical countries are in danger of infection by NTDs, which will likely spread to further regions in the near future due to the ongoing climate change.[2]

Metal-based drugs have been approved for the therapy of many diseases and represent a prospering field of drug design.[3] The gold complex auranofin is a prominent example that is applied for the treatment of rheumatoid arthritis.[4] Gold complexes with antiparasitic activities have also been disclosed.[5] The X-ray structure of auranofin bound to *Leishmania infantum* trypanothione reductase revealed a dual mode of inhibition by this drug.[6] In addition, there is a continuously growing number of gold *N*-heterocyclic carbene (NHC) complexes with potent biological effects, including anticancer and antiparasitic activities.[7] Mechanistically, gold–carbene complexes can inhibit thioredoxin reductase or interact with DNA (e.g., with DNA G quadruplexes).[8–11] The high antitrypanosomal and parasite cytoskeleton-damaging activities of cationic gold(I)–NHC complexes, such as **1a**, were reported previously.[12] Complex **1a** was found to be distinctly more active against *Trypanosoma brucei* cells than against human cells, including cancer cells. These antiparasitic effects are not surprising as other

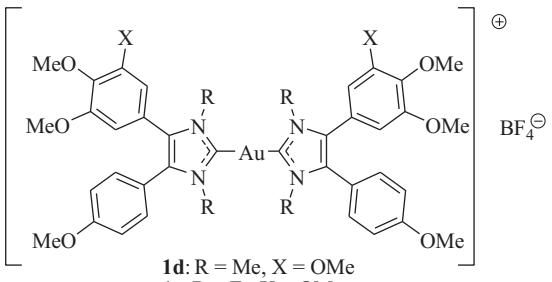
ferrocene derivatives have previously shown activities against various parasites.[13–15] In addition, imidazoles, on their own, also displayed distinct antimicrobial and antiparasitic activities.[16,17] We now evaluated the scope and structure dependence of the antiparasitic effects of a series of known and new gold(I)–NHC complexes of our lab on the protozoal parasites *Leishmania major*, *T. brucei* (both kinetoplastid parasites), and *Toxoplasma gondii* (apicomplexan parasite). Some of the known gold complexes used in this study have already shown in vivo activity against tumor xenografts with good tolerability by the laboratory animals and, thus, these complexes are suitable for repurposing against parasites.[18,19]

2 | RESULTS AND DISCUSSION

The known complexes **1a** and **1d–g** were prepared according to literature procedures (Figure 1).[18,19] The new complexes **1b** and **1c** were prepared accordingly and tested to assess the influence of methoxy substituents on the activity against and the selectivity for protozoal parasites (Scheme 1). The reaction of ferrocenecarboxaldehyde with ethyl amine and TosMIC reagents **2b** and **2c**, respectively, afforded the *N*-ethyl-imidazoles **3b** and **3c** in good yields. High yield alkylation with ethyl iodide was followed by quantitative conversion of the iodides **4b** and **4c** to the BF_4 salts **5b** and **5c**. Finally, reaction of **5b** and **5c** with Ag_2O and transmetallation with 0.5 equiv.

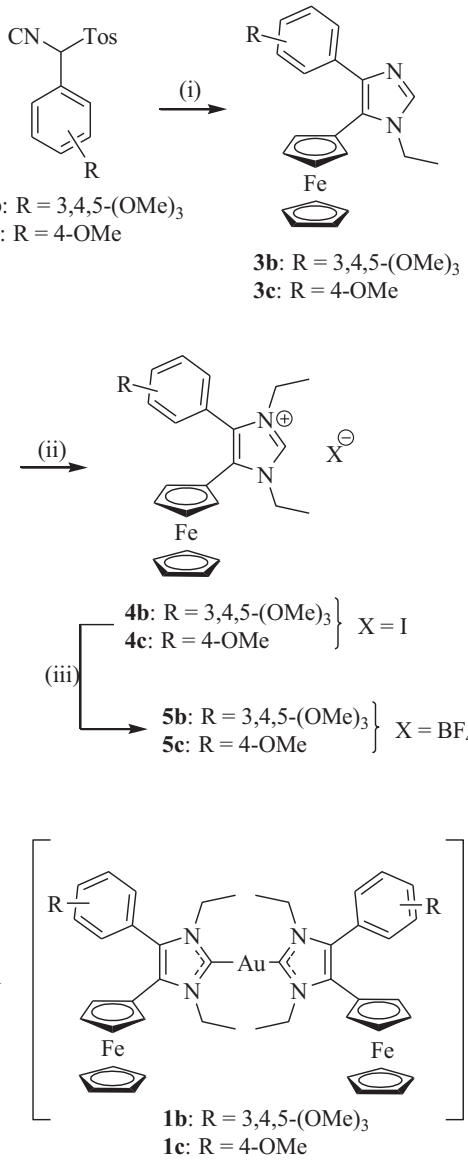


1a: R = H
1b: R = 3,4,5-(OMe)$_3$
1c: R = 4-OMe



1d: R = Me, X = OMe
1e: R = Et, X = OMe
1f: R = Me, X = Cl
1g: R = Et, X = Br

FIGURE 1 Structures of the *N*-heterocyclic carbene–gold(I) complexes **1a–g** used in this study



SCHEME 1 Synthesis of the target compounds. Reagents and conditions: (i) Ferrocenecarboxaldehyde, $EtNH_2$ (2 M in THF), AcOH, EtOH, reflux, 1 hr, then K_2CO_3, EtOH, reflux, 2 hr, 74–79%; (ii) EtI, MeCN, 85°C, 24 hr, 97–100%; (iii) $NaBF_4$, acetone, rt, 24 hr, 100%; (iv) Ag_2O, CH_2Cl_2/MeOH (1:1), rt, 5 hr, then Au(DMS)Cl, CH_2Cl_2/MeOH (1:1), rt, 24 hr, 71–79%

Au(DMS)Cl led to the target complexes **1b** and **1c** as brown solids in good yields.

The complexes **1a–g** (Figure 1) were initially tested for their activity against *T. gondii* tachyzoites (Table 1). The ferrocene derivatives **1a–c** showed distinctly higher activities against *T. gondii* (EC_{50} = 0.013–0.046 μM) than the anisyl derivatives **1d–g** (EC_{50} = 0.116–0.678 μM). Complex **1a** exhibited the highest activity of all test compounds. The ferrocenes **1a–c** also showed a reasonable selectivity for *T. gondii* cells (best for **1a**, selectivity index [SI] = 28.1) versus

TABLE 1 Inhibitory concentrations IC_{50} (in μM)[a] of the test compounds **1a–g** when applied to cells of the Vero (African green monkey kidney epithelial) cell line, effective concentrations EC_{50}[a] when applied to cells of *Toxoplasma gondii*

Compd.	EC_{50} (*T. gondii*)	IC_{50} (Vero)	SI (Vero/*T. gondii*)[b]
1a	0.013 ± 0.002	0.365 ± 0.054	28.1
1b	0.046 ± 0.008	0.662 ± 0.083	14.4
1c	0.041 ± 0.006	0.458 ± 0.070	11.2
1d	0.195 ± 0.012	0.720 ± 0.121	3.69
1e	0.678 ± 0.091	5.52 ± 1.310	8.14
1f	0.313 ± 0.007	0.573 ± 0.063	1.83
1g	0.116 ± 0.033	0.220 ± 0.046	1.90
ATO	0.07 ± 0.006	9.5 ± 1.872	136

Note: ATO (atovaquone) was applied as positive control.

[a]Values are the means of at least three independent experiments ± standard deviation. They were derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 hr.

[b]Selectivity index (SI; IC_{50}/EC_{50}) calculated from the corresponding IC_{50} values for the Vero cells and the EC_{50} values against *T. gondii*.

nonmalignant Vero cells. Among the anisyl complexes **1d–g**, the *N*-ethyl 3,4,5-trimehoxyphenyl derivative **1e** is more selective than the analogous *N*-methyl derivative **1d**, indicating an influence by the *N*-alkyl group. Such an influence was not observed for the 3-halo-4,5-dimethoxyphenyl derivatives **1f** and **1g**. Compounds **1a–c** were also more active than the positive control atovaquone (ATO), which is an approved drug for the treatment of toxoplasmosis.

The activity of complexes **1a–g** against *L. major* promastigotes and amastigotes was also determined (Table 2). The ferrocenes **1a–c** were the most efficient growth inhibitors of the *L. major* amastigotes with complex **1a** showing the highest activity (EC_{50} = 0.11 μM) and a slight selectivity for the amastigotes (SI = 3.32). However, anisyl-NHC complex **1e**, while being the second least active compound against amastigotes, showed the highest selectivity for them (SI = 12.8). The anisyl-NHC complexes **1d** and **1f** were slightly more active than the ferrocenes against *L. major* promastigotes and less active against the amastigotes. For approved antileishmanial drugs, a high activity against amastigotes was observed and other drug candidates also showed higher activity against *L. major* amastigotes than against promastigotes.[20,21] When compared with the positive control amphotericin B (AmB), complexes **1a–d** showed higher activities both against the promastigotes and against the amastigotes. In addition, compound **1f** was more active than AmB against the promastigotes, and **1g** against the amastigotes. Complex **1e** showed virtually the same activity as AmB against amastigotes and considerable selectivity.

Compounds **1a** and **1e** were already described by our groups as antitrypanosomal compounds.[12] Hence, the new ferrocenes **1b** and **1c**, which are close analogs of **1a**, were selected and also tested for their trypanocidal activity against bloodstream-form *T. b. brucei* parasites by the Alamar Blue (AB) assay. The obtained results were compared with those previously observed for **1a** and **1e** (Table 3).[12] In particular, the new complex **1b** exhibited high activity against *T. b. brucei* (IC_{50} = 5 nM) and a high selectivity for *T. b. brucei* cells versus human HeLa cervix carcinoma cells. The selectivity of **1b** (SI = 168) for *T. b. brucei* exceeded even those of **1a** (SI = 148) and the 4-anisyl-5-(3,4,5-trimethoxyphenyl)-imidazol-2-ylidene complex **1e**. Among the new ferrocenes, the 3,4,5-trimethoxyphenyl group of **1b** proved to be more conducive to overall activity against and selectivity for the parasite than the anisyl group of **1c**.

TABLE 2 Effective concentrations EC_{50} (in μM) of test compounds **1a–g** when applied to promastigotes and amastigotes of *Leishmania major*[a]

Compd.	EC_{50} promastigotes	EC_{50} amastigotes	SI Vero/ promastigotes[b]	SI Vero/ amastigotes[b]
1a	0.37 ± 0.042	0.11 ± 0.008	1.0	3.32
1b	0.42 ± 0.035	0.22 ± 0.065	1.57	3.01
1c	0.45 ± 0.061	0.19 ± 0.057	1.02	2.41
1d	0.33 ± 0.017	0.38 ± 0.038	2.16	1.89
1e	3.11 ± 0.983	0.43 ± 0.097	1.78	12.8
1f	0.31 ± 0.072	0.46 ± 0.086	1.86	1.25
1g	1.34 ± 0.349	0.26 ± 0.074	0.16	0.85
AmB	0.83 ± 0.164	0.47 ± 0.089	9.6	16.4

Note: AmB (amphotericin B) was applied as positive control.

[a]Values are the means of at least three independent experiments ± standard deviation. They were derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 hr.

[b]Selectivity index (SI; IC_{50}/EC_{50}) calculated from the corresponding IC_{50} values for the Vero cells (Table 1) and the EC_{50} values against *L. major*.

TABLE 3 Inhibitory concentrations IC_{50} (in μM) of test compounds **1a–c** and **1e** when applied to *Trypanosoma brucei brucei* cells and human HeLa cells[a]

Compd.	IC_{50} (*T. b. brucei*)	IC_{50} (HeLa)	SI (HeLa/*T. b. brucei*)[b]
1a	0.00093[c]	0.138[c]	148[c]
1b	0.005 ± 0.001	0.840 ± 0.170	168
1c	0.028 ± 0.005	0.277 ± 0.035	9.89
1e	0.003[c]	0.231[c]	77[c]
Pentamidine	0.000042[c]	1.47[c]	35,000[c]

[a]Values are the means of at least three independent experiments ± standard deviation. They were derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 hr.
[b]Selectivity index (SI) calculated from the corresponding IC_{50} values for the HeLa cells and the IC_{50} values for *T. b. brucei*.
[c]Value is taken from Reference [12].

3 | CONCLUSIONS

The evaluation of a series of NHC gold(I) complexes against pathogenic parasites such as *T. gondii*, *T. b. brucei* and *L. major* led to promising results. Both high activities and considerable selectivities were observed. The ferrocene derivatives **1a** and **1b**, in particular, were highly active against all tested parasites. The anisyl-NHC derivatives **1d** and **1f** exhibited remarkable activities against *L. major* promastigotes, which is worthy of note as most of the other tested complexes were more active against *L. major* amastigotes, which is also more typical of established antileishmanial drugs and drug candidates currently in the pipeline. More research into the mechanisms of action and their structure–activity dependencies is necessary to pinpoint the reason for these peculiar differences. According to present knowledge, investigational applications of some of the tested gold complexes for the treatment of cutaneous leishmaniasis (i.e., *L. major* infection) appear promising, as do their combinations with approved antiparasitic drugs such as pentamidine or miltefosine to reduce the necessary doses and possible side-effects.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All starting compounds were purchased from Aldrich. The known complexes **1a** and **1d–g** and the TosMIC reagents **2b** and **2c** were prepared according to literature procedures.[18,19,22] The analytical data of these compounds were in agreement with the published data. The following instruments were applied for this study: melting points (uncorrected), Gallenkamp; infrared (IR) spectra, Perkin–Elmer Spectrum One FT-IR spectrophotometer with ATR-sampling unit; nuclear magnetic resonance spectra, Bruker Avance 300 spectrometer; chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as internal standard; mass spectra, Varian MAT 311A (EI), UPLC/Orbitrap (ESI); microanalyses, Perkin–Elmer 2400 CHN elemental analyzer.

The compound codes together with the nuclear magnetic resonance (NMR) spectra of the new compounds **1b** and **1c** are provided as Supporting Information.

*1-Ethyl-5-ferrocenyl-4-(3,4,5-trimethoxyphenyl)-imidazole (**3b**)*

Ferrocenecarboxaldehyde (90 mg, 0.42 mmol) was dissolved in EtOH and $EtNH_2$ (2 M in THF, 1.05 ml, 2.10 mmol) and AcOH (150 μl, 2.63 mmol) were added. The reaction mixture was stirred under reflux for 1 hr. Compound **2b** (159 mg, 0.44 mmol) and K_2CO_3 (500 mg, 3.62 mmol) were added, and the reaction mixture was stirred under reflux for 2 hr. The solvent was evaporated, and the residue was suspended in ethyl acetate, washed with water, dried over Na_2SO_4, filtered, and the filtrate was concentrated in vacuum. The residue was purified by column chromatography (silica gel 60, ethyl acetate/methanol 9:1). Yield: 138 mg (0.31 mmol, 74%); brown oil; ν_{max}(ATR)/cm 3,087, 3,004, 2,958, 2,931, 2,831, 1,585, 1,511, 1,459, 1,432, 1,413, 1,390, 1,354, 1,344, 1,286, 1,235, 1,198, 1,185, 1,124, 1,062, 1,033, 1,010, 959, 885, 842, 818, 770, 743, 732, 697, 664, 651, 641, and 626; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (3H, t, J = 7.3 Hz, CH_3), 3.75 (6H, s, 2 × OCH_3), 3.83 (3H, s, OCH_3), 4.0–4.1 (5H, m, Fc–H), 4.2–4.3 (4H, m, Fc–H), 4.51 (2H, q, J = 7.3 Hz, CH_2), 6.69 (2H, s, Ar–H), and 7.59 (1H, s, imidazole–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.1 (CH_3), 39.8 (CH_2), 56.0 (OCH_3), 60.9 (OCH_3), 66.6, 68.2, 69.2, 75.4 (Fc–C), 105.8, 113.2, 124.1, 130.4, 131.0, 136.1, 136.6, 136.9, 140.1 (Ar–C or imidazole–C), 152.7 (Ar–$COCH_3$), and 153.2 (Ar–$COCH_3$); *m/z* (%) 447 (82) [M^+], 446 (100) [M^+], 415 (7), 381 (38), 294 (13), 252 (15), 121 (23), and 56 (14).

*1-Ethyl-4-anisyl-5-ferrocenylimidazole (**3c**)*

Ferrocenecarboxaldehyde (90 mg, 0.42 mmol) was dissolved in EtOH and $EtNH_2$ (2 M in THF, 1.05 ml, 2.10 mmol) and AcOH (150 μl, 2.63 mmol) were added. The reaction mixture was stirred under reflux for 1 hr. Compound **2c** (133 mg, 0.44 mmol) and K_2CO_3 (500 mg, 3.62 mmol) were added and the reaction mixture was stirred under reflux for 2 hr. The solvent was evaporated and the residue was suspended in ethyl acetate, washed with water, dried over Na_2SO_4, filtered, and the filtrate was concentrated in vacuum. The residue was purified by column chromatography (silica gel 60, ethyl acetate/methanol 9:1). Yield: 128 mg (0.33 mmol, 79%); brown oil; ν_{max}(ATR)/cm 3,093, 2,973, 2,935, 2,835, 1,613, 1,577, 1,562, 1,516, 1,456, 1,412, 1,378, 1,350, 1,290, 1,242, 1,199, 1,173, 1,105, 1,030, 1,001, 949, 876, 832, 744, 723, 707, 663, 635, and 600; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.54 (3H, t, J = 7.3 Hz, CH_3), 3.80 (3H, s, OCH_3), 4.0–4.1 (5H, m, Fc–H), 4.2–4.3 (4H, m, Fc–H), 4.45 (2H, q, J = 7.3 Hz, CH_2), 6.84 (2H, d, J = 8.9 Hz, Ar–H), 7.39 (2H, d, J = 8.9 Hz, Ar–H), and 7.58 (1H, s, imidazole–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.0 (CH_3), 40.0 (CH_2), 60.4 (OCH_3), 68.1, 68.9, 69.1, 75.7 (Fc–C), 113.3, 113.8, 123.6, 127.6, 128.4, 128.8, 130.0, 132.1, 136.1, 136.3, 140.0 (Ar–C or imidazole–C), 158.5 (Ar–$COCH_3$); *m/z* (%) 386 (100) [M^+], 321 (47), 308 (47), 264 (22), 193 (26), 121 (31), and 56 (21).

*1,3-Diethyl-4-ferrocenyl-5-(3,4,5-trimethoxyphenyl)-imidazolium iodide (**4b**)*

Compound **3b** (130 mg, 0.29 mmol) was dissolved in MeCN (15 ml) and iodoethane (1.0 ml, 12.4 mmol) was added. The reaction mixture was stirred at 85°C for 24 hr. The solvent was evaporated and the residue was dried in vacuum. Yield: 170 mg (0.28 mmol, 97%); brown oil; ν_{max}(ATR)/cm 3,133, 3,037, 2,975, 2,937, 2,835, 1,580, 1,566, 1,510, 1,488, 1,463, 1,444, 1,429, 1,412, 1,390, 1,353, 1,343, 1,294, 1,238, 1,196, 1,159, 1,122, 1,087, 1,060, 1,032, 1,021, 1,001, 962, 918, 887, 840, 826, 816, 800, 777, 727, 674, 663, 623, and 600; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (3H, t, J = 7.3 Hz, CH_3), 1.76 (3H, t, J = 7.3 Hz, CH_3), 3.83 (6H, s, 2 × OCH_3), 3.92 (3H, s, OCH_3), 4.0–4.1 (5H, m, Fc–H), 4.1–4.2 (2H, m, Fc–H), 4.20 (2H, q, J = 7.3 Hz, CH_2), 4.3–4.4 (2H, m, Fc–H), 4.72 (2H, q, J = 7.3 Hz, CH_2), 6.53 (2H, s, Ar–H), and 10.30 (1H, s, imidazolium–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 15.9 (CH_3), 16.5 (CH_3), 43.4 (CH_2), 56.6 (OCH_3), 61.1 (OCH_3), 67.3, 68.7, 69.3, 69.7, 69.8, 70.0, 70.4, 80.3 (Fc–C), 106.3, 108.3, 120.9, 129.7, 130.1, 130.2, 135.7, 138.0, 140.0 (Ar–C or imidazolium–C), 154.0 (Ar–$COCH_3$), and 154.4 (Ar–$COCH_3$); *m/z* (%) 474 (2) [M^+], 445 (18), 142 (42), 127 (21), and 66 (100).

*1,3-Diethyl-4-anisyl-5-ferrocenylimidazolium iodide (**4c**)*

Compound **3c** (127 mg, 0.33 mmol) was dissolved in MeCN (15 ml) and iodoethane (1.0 ml, 12.4 mmol) was added. The reaction mixture was stirred at 85°C for 24 hr. The solvent was evaporated and the residue was dried in vacuum. Yield: 179 mg (0.33 mmol, 100%); brown oil; ν_{max}(ATR)/cm 3,439, 2,977, 2,934, 2,837, 1,616, 1,599, 1,562, 1,519, 1,487, 1,455, 1,411, 1,386, 1,343, 1,292, 1,249, 1,175, 1,106, 1,022, 1,006, 963, 919, 883, 838, 768, 724, 639, 626, and 616; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40 (3H, t, J = 7.3 Hz, CH_3), 1.71 (3H, t, J = 7.3 Hz, CH_3), 3.84 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 3.9–4.0 (5H, m, Fc–H), 4.0–4.1 (4H, m, Fc–H), 4.2–4.3 (2H, m, CH_2), 4.66 (2H, q, J = 7.3 Hz, CH_2), 7.01 (2H, d, J = 8.9 Hz, Ar–H), 7.23 (2H, d, J = 8.9 Hz, Ar–H), and 10.23 (1H, s, imidazolium–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 15.6 (CH_3), 16.3 (CH_3), 43.1 (CH_2), 43.4 (CH_2), 55.4 (OCH_3), 67.3, 68.7, 69.2, 69.5, 69.6, 70.1, 80.3 (Fc–C), 106.5, 114.6, 115.1, 117.6, 130.0, 130.1, 131.6, 131.8, 132.4, 135.4, 137.9, 147.0 (Ar–C or imidazolium–C), and 161.2 (Ar–$COCH_3$); *m/z* (%) 415 (53) [M^+], 401 (60), 387 (100), 373 (55), 348 (35), 309 (27), 186 (72), 142 (37), 121 (34), and 66 (78).

*1,3-Diethyl-4-ferrocenyl-5-(3,4,5-trimethoxyphenyl)-imidazolium tetrafluoroborate (**5b**)*

Compound **4b** (170 mg, 0.28 mmol) was dissolved in acetone (15 ml) and $NaBF_4$ (47 mg, 0.43 mmol) was added. The reaction mixture was stirred at room temperature for 24 hr. The solution was filtered over $MgSO_4$, the filtrate was concentrated and dried in vacuum. Yield: 157 mg (0.28 mmol, 100%); brown oil; ν_{max}(ATR)/cm 2,987, 2,928, 2,828, 1,583, 1,568, 1,511, 1,489, 1,458, 1,428, 1,412, 1,393, 1,351, 1,296, 1,240, 1,197, 1,164, 1,124, 1,107, 1,032, 1,010, 921, 889, 858, 825, 778, 727, 675, 642, and 627; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (3H, t, J = 7.3 Hz, CH_3), 1.74 (3H, t, J = 7.3 Hz, CH_3), 3.81 (6H, s, 2 × OCH_3), 3.89 (3H, s, OCH_3), 4.0–4.1 (5H, m, Fc–H), 4.1–4.2 (4H, m, Fc–H), 4.3–4.4 (2H, m, CH_2), 4.70 (2H, q, J = 7.3 Hz, CH_2), 6.54 (2H, s, Ar–H), and 10.18 (1H, s, imidazolium–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 15.8 (CH_3), 16.4 (CH_3), 43.4 (CH_2), 56.6 (OCH_3), 61.0 (OCH_3), 67.3, 68.6, 69.2, 69.6, 70.0, 70.3 (Fc–H), 106.4, 108.3, 120.9, 130.0, 130.1, 135.4, 137.8, 139.8 (Ar–C or imidazolium–C), 153.8 (Ar–$COCH_3$), and 154.1 (Ar–$COCH_3$).

*1,3-Diethyl-4-anisyl-5-ferrocenylimidazolium tetrafluoroborate (**5c**)*

Compound **4c** (166 mg, 0.31 mmol) was dissolved in acetone (15 ml) and $NaBF_4$ (51 mg, 0.47 mmol) was added. The reaction mixture was stirred at room temperature for 24 hr. The solution was filtered over $MgSO_4$, the filtrate was concentrated and dried in vacuum. Yield: 156 mg (0.31 mmol, 100%); brown oil; ν_{max}(ATR)/cm 3,417, 2,976, 2,935, 2,836, 1,616, 1,599, 1,562, 1,519, 1,487, 1,456, 1,411, 1,386, 1,343, 1,292, 1,249, 1,175, 1,106, 1,022, 1,006, 963, 919, 883, 838, 768, 724, 639, and 615; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 (3H, t, J = 7.3 Hz, CH_3), 1.71 (3H, t, J = 7.3 Hz, CH_3), 3.84 (3H, s, OCH_3), 3.9–4.0 (5H, m, Fc–H), 4.0–4.1 (4H, m, Fc–H), 4.2–4.3 (2H, m, CH_2), 4.65 (2H, q, J = 7.3 Hz, CH_2), 7.01 (2H, d, J = 8.9 Hz, Ar–H), 7.23 (2H, d, J = 8.9 Hz, Ar–H), and 10.19 (1H, s, imidazolium–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 15.6 (CH_3), 16.3 (CH_3), 43.0 (CH_3), 43.4 (CH_3), 55.5 (OCH_3), 67.3, 68.7, 69.2, 69.3, 69.5, 69.6, 70.1, 80.4 (Fc–H), 106.5, 114.6, 115.1, 117.6, 130.0, 130.1, 131.6, 131.8, 132.0. 132.4, 135.3, 137.9 (Ar–C or imidazolium–C), and 161.3 (Ar–$COCH_3$).

*Bis-[1,3-diethyl-4-ferrocenyl-5-(3,4,5-trimethoxyphenyl)-imidazol-2-ylidene]gold(I) (**1b**)*

Compound **5b** (157 mg, 0.28 mmol) was dissolved in CH_2Cl_2/MeOH (1:1, 30 ml) and Ag_2O (108 mg, 0.47 mmol) was added. The reaction mixture was stirred at room temperature for 5 hr. Au(DMS)Cl (41 mg, 0.14 mmol) was added and the reaction mixture was stirred at room temperature for 24 hr. The suspension was filtered, the filtrate was concentrated in vacuum and the residue was redissolved in CH_2Cl_2, filtered over $MgSO_4$/celite. The filtrate was concentrated and the remainder was recrystallized from CH_2Cl_2/*n*-hexane and dried in vacuum. Yield: 136 mg (0.11 mmol, 79%); brown solid of mp 176–178°C; ν_{max}(ATR)/cm 2,933, 1,580, 1,509, 1,460, 1,411, 1,346, 1,327, 1,287, 1,236, 1,185, 1,165, 1,124, 1,107, 1,047, 1,030, 1,002, 916, 886, 822, 779, 727, and 670; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.3–1.5 (6H, m, 2 × CH_3), 1.6–1.8 (6H, m, 2 × CH_3), 3.83 (12H, s, 4 × OCH_3), 3.9–4.0 (6H, m, 2 × OCH_3), 4.0–4.3 (22H, m, Fc–H, 2 × CH_2), 4.6–4.7 (4H, m, 2 × CH_2), and 6.51 (4H, s, Ar–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.7 (CH_3), 18.2 (CH_3), 44.1 (CH_2), 44.3 (CH_2), 56.5 (OCH_3), 61.0 (OCH_3), 65.1, 68.4, 69.1, 69.2, 69.5, 72.4 (Fc–H), 108.1, 108.4, 123.6, 128.7, 129.2, 130.5 (Ar–C or imidazolium–C), 149.9 (Ar–$COCH_3$), 153.6 (Ar–$COCH_3$), and 182.8 (Au–C); *m/z* (ESI, %) 1,145.0 (80) [M^+] and 475.2 (100). Anal calcd. $C_{52}H_{60}AuBF_4Fe_2N_4O_6$: C, 50.67; H, 4.91; N, 4.55; Found, C, 50.79; H, 4.99; N, 4.60%.

*Bis-[1,3-diethyl-4-anisyl-5-ferrocenylimidazol-2-ylidene]gold(I) (**1c**)*

Compound **5c** (156 mg, 0.31 mmol) was dissolved in CH_2Cl_2/MeOH (1:1, 20 ml) and Ag_2O (120 mg, 0.52 mmol) was added. The reaction mixture was stirred at room temperature for 5 hr. Au(DMS)Cl (45 mg, 0.16 mmol) was added and the reaction mixture was stirred at room

temperature for 24 hr. The suspension was filtered, the filtrate was concentrated in vacuum and the residue was redissolved in CH_2Cl_2, filtered over $MgSO_4$/celite. The filtrate was concentrated and the remainder was recrystallized from CH_2Cl_2/*n*-hexane and dried in vacuum. Yield: 127 mg (0.114 mmol, 71%); brown solid of mp 190–193°C; ν_{max}(ATR)/cm 2,960, 2,933, 2,872, 2,841, 1,621, 1,599, 1,573, 1,517, 1,462, 1,414, 1,380, 1,346, 1,306, 1,290, 1,250, 1,177, 1,106, 1,046, 1,027, 970, 913, 885, 837, 815, 790, 773, 727, and 645; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.3–1.4 (6H, m, 2 × CH_3), 1.6–1.7 (6H, m, 2 × CH_3), 3.88 (6H, s, 2 × OCH_3), 4.0 –4.1 (10H, m, Fc–H), 4.1–4.2 (8H, m, Fc–H), 4.2–4.3 (4H, m, 2 × CH_2), 4.69 (4H, q, J = 7.3 Hz, 2 × CH_2), 7.03 (4H, d, J = 8.8 Hz, Ar–H), and 7.2–7.3 (4H, m, Ar–H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.3 (CH_3), 18.2 (CH_3), 44.1 (CH_2), 55.4 (OCH_3), 67.0, 68.4, 69.1, 69.2, 69.4, 69.7, 72.5, 81.5 (Fc–C), 109.0, 114.4, 114.7, 114.9, 119.4, 120.3, 128.8, 130.0, 130.4, 131.5, 131.8, 132.0. 132.4, 134.6 (Ar–C or imidazolium–C, 160.7 (Ar–$COCH_3$), and 182.3 (Au–C); *m/z* (ESI, %) 1,025.0 (100) [M^+], 946.9 (45), and 506.6 (25). Anal calcd. $C_{48}H_{52}AuBF_4Fe_2N_4O_2$: C, 51.83; H, 4.71; N, 5.04; Found, C, 51.95; H, 4.80; N, 5.11%.

4.2 | Biological assays

4.2.1 | *Leishmania major* cell isolation, culture conditions, and assays

Promastigotes of *L. major* were isolated from a Saudi male patient in February 2016 and maintained at 26°C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and antibiotics in a tissue culture flask with weekly transfers. Promastigotes were cryopreserved in liquid nitrogen at concentrations of 3×10^6 parasite/ml. The virulence of *L. major* parasites was maintained by passing in female BALB/c mice by injecting hind footpads with 1×10^6 stationary-phase promastigotes. After 8 weeks, *L. major* amastigotes were isolated from mice. Isolated amastigotes were transformed to promastigote forms by culturing at 26°C in Schneider's medium supplemented with 10% FBS and antibiotics. For infection, amastigote-derived promastigotes with less than five in vitro passages were used. Male and female BALB/c mice were obtained from Pharmaceutical College, King Saud University, Kingdom of Saudi Arabia, and maintained in specific pathogen-free facilities.

To evaluate the activity of test compounds against *L. major* promastigotes, promastigotes from logarithmic-phase cultured in phenol red-free RPMI-1640 medium (Invitrogen) with 10% FBS were suspended on 96-wells plates to yield 10^6 cells/ml (200 µl/well) after hemocytometer counting. Compounds were added to obtain the final concentrations (50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 µg/ml). Negative control wells containing cultures with dimethyl sulfoxide (DMSO; 1%) and without compound and positive control wells containing cultures with decreasing concentration of AmB (reference compound, 50, 25, 12.5, 6.25, 3.13, 1.65, 0.75 µg/ml) were used. Plates were incubated at 26°C for 72 hr to evaluate the antiproliferative effect. The number of viable promastigotes were assessed by colorimetric method using the tetrazolium salt colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT). It measures the reduction of the MTT component into an insoluble formazan product. This colored product was solubilized by adding a detergent solution to lyse the cells. The samples were analyzed using an enzyme-linked immunosorbent assay reader at 570 nm. Obtained EC_{50} values resulted from three independent experiments.[23]

To evaluate the activity of test compounds against amastigotes in macrophages, peritoneal macrophages from female BALB/c mice (6–8 weeks of age) were collected by aspiration, then 5×10^4 cells per well were seeded on 96-wells plates in phenol red-free Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 10% FBS for 4 hr at 37°C in 5% CO_2 atmosphere to promote cell adhesion. The medium was discarded and washed with phosphate-buffered saline (PBS). 200 µl containing *L. major* promastigotes solution (at a ratio of 10 promastigotes to 1 macrophage in RPMI-1640 medium with 10% FBS) was added per well. Plates were incubated for 24 hr at 37°C in a humidified 5% CO_2 atmosphere to allow infection and amastigote differentiation. Then, the infected macrophages were washed three times with PBS to remove the free promastigotes and overlaid with fresh phenol red-free RPMI-1640 medium containing compounds at final concentrations (50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 µg/ml) were added and cells were incubated at 37°C in humidified 5% CO_2 atmosphere for 72 hr. Negative control containing cultures with DMSO (1%) and without compounds and positive control wells containing cultures with decreasing concentration of AmB (reference compound, 50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 µg/ml) were used. The percentage of infected macrophages were evaluated microscopically after removing medium, washing, fixation, and Giemsa staining. Obtained EC_{50} values resulted from three independent experiments (for the EC_{50} calculation see Section 4.2.2.).[23]

4.2.2 | *Toxoplasma gondii* cell line, culture conditions, and assay

Serial passages of the cell line Vero (ATCC® CCL81™) were used for the cultivation of *T. gondii* tachyzoites of the RH strain (a gift from Dr. Saeed El-Ashram, State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing, China). Vero cells were cultured by using a complete RPMI-1640 medium with heat-inactivated 10% FBS in a humidified 5% CO_2 atmosphere at 37°C. For the cultivation of the Vero cells, 96-well plates (5×10^3 cells per well in 200 µl RPMI-1640 medium) were used and then the cells were incubated at 37°C and 5% CO_2 for 1 day, followed by removal of medium and washing the cells with PBS. Then, RPMI-1640 medium with 2% FBS containing tachyzoites (RH strain) of *T. gondii* at a ratio of 5 (parasite) to 1 (Vero cells) was added. After incubation at 37°C and 5% CO_2 for 5 hr, cells were washed with PBS and then treated as described below.

Negative control (control): Wells containing cultures with DMSO (1%) without test compound.

Experimental: Medium + compounds (dissolved in DMSO) (50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg/ml).

Positive control (reference drug): Medium + ATO (dissolved in DMSO; 50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg/ml).

After incubation at 37°C and 5% CO_2 for 72 hr, the cells were stained with 1% toluidine blue after washing with PBS and fixation in 10% formalin. The cells were examined under an inverted photomicroscope to determine the infection index (number of cells infected from 200 cells tested) of *T. gondii*. The following equation was used for the calculation of the observed inhibition (in %):

$$\text{Inhibition}(\%) = (I_{\text{Control}} - I_{\text{Experimental}})/(I_{\text{Control}}) \times 100,$$

where I_{Control} refers to the infection index of untreated cells and $I_{\text{Experimental}}$ refers to the infection index of cells treated with test compounds.

Then effects of test compounds on parasite growth were expressed as EC_{50} (effective concentration at 50%) values. Obtained EC_{50} values resulted from three independent experiments.[24]

4.2.3 | *Trypanosoma* cell line and culture conditions

Cultivation of the *T. b. brucei* bloodstream-form cell strain Lister 427 was carried out in HMI-9 medium, pH 7.5, supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.[25]

4.2.4 | Alamar Blue (AB) assay

Viable cells after treatment with drug candidates were identified via the AB assay.[26–29] Pink resorufin is formed in intact cells from the irreversible reaction of the blue dye resazurin and NADH. *T. b. brucei* cells (8,000/well) were seeded on 96-well microplates, test compounds (dissolved in DMSO) were added and the cells were incubated for 72 hr (5% CO_2, 95% humidity, 37°C). AB reagent (10 μl of 500 μM resazurin sodium salt in PBS) was added and the cells were incubated for an additional 4 hr at 37°C. Fluorescence (extinction at 544 nm, emission at 590 nm) was determined on an Omega Fluostar (BMG Labtech) fluorescence plate reader. The IC_{50} values were determined with the Quest Graph™ IC_{50} Calculator (AAT Bioquest Inc.).

4.2.5 | In vitro cytotoxicity assay

MTT assay was carried out for cytotoxicity evaluation of compounds. Briefly, Vero cells were cultured in 96-well plates (5×10^3 cells per well per 200 μl) for 24 hr in RPMI-1640 medium with 10% FBS and 5% CO_2 at 37°C. Cells were washed with PBS and treated with test compounds for 72 hr at varying concentrations (50, 25, 12.5, 6.25, 3.13, 1.65, and 0.75 μg/ml) in medium with 10% FBS. As a negative control, cells were treated with the medium in 2% FBS. Thereafter, the supernatant was removed and 100 ml RPMI-1640 medium containing 10 ml MTT (5 mg/ml) was added and incubated for 4 hr. After that, the supernatant was removed and 200 ml DMSO was added to dissolve the formazan. FLUOstar OPTIMA spectrophotometer was applied for colorimetric analysis (λ = 540 nm). Cytotoxic effects were expressed by IC_{50} values (concentration that caused a 50% reduction in viable cells). Obtained IC_{50} values resulted from three independent experiments.[30,31]

ACKNOWLEDGMENTS

R.S. thanks the Deutsche Forschungsgemeinschaft for financial support (grant Scho 402/12-2). We are grateful to Qassim University and the Deanship of Scientific Research for material support of this study (number cosao-bs-2019-2-2-1-5619) during the academic year 1440 AH/2019 AD.

ORCID

Bernhard Biersack http://orcid.org/0000-0001-7305-346X

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How to cite this article: Koko WS, Jentzsch J, Kalie H, et al. Evaluation of the antiparasitic activities of imidazol-2-ylidene–gold(I) complexes. *Arch Pharm.* 2020;353:e1900363. https://doi.org/10.1002/ardp.201900363