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

Waleed S. Koko1 | Jana Jentzsch2 | Hussein Kalie3 | Rainer Schobert3 | Klaus Erfeld2 | Ibrahim S. Al Nasr1,4 | Tariq A. Khan5 | Bernhard Biersack3

Abstract

A series of cationic gold(I)–carbene complexes with various 4,5-diarylimidazoylidene 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 antiparasomal 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\textsuperscript{[13–15]} In addition, imidazoles, on their own, also displayed distinct antimicrobial and antiparasitic activities\textsuperscript{[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\textsuperscript{[18,19]}

2 | RESULTS AND DISCUSSION

The known complexes 1a and 1d–g were prepared according to literature procedures (Figure 1)\textsuperscript{[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\textsubscript{4} salts 5b and 5c. Finally, reaction of 5b and 5c with Ag\textsubscript{2}O and transmetallation with 0.5 equiv. Au(DMS)Cl led to the target complexes 1b and 1c as brown solids in good yields.

**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\textsubscript{2} (2 M in THF), AcOH, EtOH, reflux, 1 hr, then K\textsubscript{2}CO\textsubscript{3}, EtOH, reflux, 2 hr, 74–79%; (ii) EtI, MeCN, 85°C, 24 hr, 97–100%; (iii) NaBF\textsubscript{4}, acetone, rt, 24 hr, 100%; (iv) Ag\textsubscript{2}O, CH\textsubscript{2}Cl\textsubscript{2}/MeOH (1:1), rt, 5 hr, then Au(DMS)Cl, CH\textsubscript{2}Cl\textsubscript{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 (EC50 = 0.013–0.046 \textmu M) than the anisyl derivatives 1d–g (EC50 = 0.116–0.678 \textmu M). 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 IC₅₀ (in µM) of the test compounds 1a-g when applied to cells of the Vero (African green monkey kidney epithelial) cell line, effective concentrations EC₅₀ when applied to cells of Toxoplasma gondii.

| Compd. | EC₅₀ (T. gondii) | IC₅₀ (Vero) | SI (Vero/T. gondii) |
|--------|------------------|------------|---------------------|
| 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.

*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.

Selectivity index (SI; IC₅₀/EC₅₀) calculated from the corresponding IC₅₀ values for the Vero cells and the EC₅₀ values against T. gondii.

TABLE 2 Effective concentrations EC₅₀ (in µM) of test compounds 1a-g when applied to promastigotes and amastigotes of Leishmania major.

| Compd. | EC₅₀ promastigotes | EC₅₀ amastigotes | SI Vero/ promastigotes | SI Vero/ amastigotes |
|--------|--------------------|-----------------|------------------------|----------------------|
| 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.

*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.

Selectivity index (SI; IC₅₀/EC₅₀) calculated from the corresponding IC₅₀ values for the Vero cells (Table 1) and the EC₅₀ values against L. major.
KOKO ET AL.

more typical of established antileishmanial drugs and drug candidates 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.

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 Trypanosoma brucei (T. b. brucei) and L. major are provided as Supporting Information.

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 Na2SO4, 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; νmax (ATR)/cm–1 3,087, 3,004, 2,958, 2,931, 2,831, 1,585, 1,511, 1,459, 1,432, 1,413, 1,199, 1,173, 1,105, 1,030, 1,001, 949, 876, 832, 744, 723, 707, 663, 644, 626; 1H NMR (300 MHz, CDCl3) δ 1.58 (3H, t, J = 7.3 Hz, CH2), 3.75 (6H, s, 2 × OCH3), 3.83 (3H, s, OCH3), 4.0–4.1 (5H, m, Ar–H), 4.2–4.3 (4H, m, Ar–H), 4.51 (2H, q, J = 7.3 Hz, CH2), 6.69 (2H, s, Ar–H), and 7.59 (1H, s, imidazole–H); 13C NMR (75.5 MHz, CDCl3) δ 17.1 (CH3), 39.8 (CH2), 56.0 (OCH3), 60.9 (OCH3), 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–CO2CH3) and 153.2 (Ar–CO2CH3); m/z (%) 447 (82) [M+], 446 (100) [M+] , 415 (7), 381 (38), 294 (13), 252 (15), 121 (23), and 56 (14).

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) Ferrocencarboxaldehyde (90 mg, 0.42 mmol) was dissolved in EtOH and EtNH2 (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 K2CO3 (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 Na2SO4, 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; νmax (ATR)/cm–1 3,087, 3,004, 2,958, 2,931, 2,831, 1,585, 1,511, 1,459, 1,432, 1,413, 1,199, 1,173, 1,105, 1,030, 1,001, 949, 876, 832, 744, 723, 707, 663, 644, 626; 1H NMR (300 MHz, CDCl3) δ 1.58 (3H, t, J = 7.3 Hz, CH2), 3.75 (6H, s, 2 × OCH3), 3.83 (3H, s, OCH3), 4.0–4.1 (5H, m, Ar–H), 4.2–4.3 (4H, m, Ar–H), 4.51 (2H, q, J = 7.3 Hz, CH2), 6.69 (2H, s, Ar–H), and 7.59 (1H, s, imidazole–H); 13C NMR (75.5 MHz, CDCl3) δ 17.1 (CH3), 39.8 (CH2), 56.0 (OCH3), 60.9 (OCH3), 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–CO2CH3) and 153.2 (Ar–CO2CH3); 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) Ferrocencarboxaldehyde (90 mg, 0.42 mmol) was dissolved in EtOH and EtNH2 (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 K2CO3 (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 Na2SO4, 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; νmax (ATR)/cm–1 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; 1H NMR (300 MHz, CDCl3) δ 1.54 (3H, t, J = 7.3 Hz, CH3), 3.80 (3H, s, OCH3), 4.0–4.1 (5H, m, FC–H), 4.2–4.3 (4H, m, FC–H), 4.45 (2H, q, J = 7.3 Hz, CH2), 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); 13C NMR (75.5 MHz, CDCl3) δ 17.0 (CH3), 40.0 (CH2), 60.4 (OCH3), 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–CO2CH3); 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: \( \nu_{\text{max}} \) (ATR/cm) 3313, 3037, 2975, 2937, 2835, 1580, 1566, 1510, 1488, 1463, 1444, 1429, 1412, 1390, 1353, 1343, 1294, 1238, 1196, 1159, 1122, 1087, 1060, 1032, 1021, 1001, 962, 918, 887, 840, 826, 816, 800, 777, 727, 674, 663, 623, and 600; \(^1^H\) NMR (300 MHz, CDCl\(_3\)) \( \delta \) 1.49 (3H, t, \( J = 7.3 \) Hz, CH\(_3\)), 1.76 (3H, t, \( J = 7.3 \) Hz, CH\(_3\)), 3.83 (6H, \( s \) \( 2 \times \text{OCH}_3\)), 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\(_3\)), 4.3–4.4 (2H, m, Fc–H), 4.72 (2H, q, \( J = 7.3 \) Hz, CH\(_3\)), 6.53 (2H, s, Ar–H), and 10.30 (1H, s, imidazolium–H); \(^1^C\) NMR (75.5 MHz, CDCl\(_3\)) \( \delta \) 15.6, 61.0 (OCH\(_3\)), 65.1, 68.4, 69.1, 69.2, 69.5, 70.0, 70.4, 80.3 (Fc–COCH\(_3\)).

Bis-[1,3-diethyl-4-ferrocenyl-5-(3,4,5-trimethoxyphenyl)imidazolium-2-ylidine]gold(I) (1b)

Compound 5b (157 mg, 0.28 mmol) was dissolved in CH\(_2\)Cl\(_2\)/MeOH (1:1, 30 ml) and Ag\(_2\)O (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 and dried in vacuum. Yield: 156 mg (0.31 mmol, 100%); brown oil: \( \nu_{\text{max}} \) (ATR/cm) 3417, 2976, 2935, 2836, 1616, 1599, 1562, 1519, 1487, 1456, 1411, 1386, 1343, 1292, 1249, 1175, 1106, 1022, 963, 919, 883, 838, 768, 724, 639, and 615; \(^1^H\) NMR (300 MHz, CDCl\(_3\)) \( \delta \) 1.39 (3H, t, \( J = 7.3 \) Hz, CH\(_3\)), 1.71 (3H, q, \( J = 7.3 \) Hz, CH\(_3\)), 3.84 (3H, s, OCH\(_3\)), 3.9–4.0 (5H, m, Fc–H), 4.65 (2H, q, \( J = 7.3 \) Hz, CH\(_3\)), 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); \(^1^C\) NMR (75.5 MHz, CDCl\(_3\)) \( \delta \) 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, 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), 178 (100); \( \nu \) max(ATR)/cm 3,417, 2,976, 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, 1022, 963, 919, 883, 838, 768, 724, 639, and 615.
temperature for 24 hr. The suspension was filtered, the filtrate was concentrated in vacuum and the residue was redissolved in CH$_2$Cl$_2$, filtered over MgSO$_4$/celite. The filtrate was concentrated and the remainder was recrystallized from CH$_2$Cl$_2$/n-hexane and dried in vacuum. Yield: 127 mg (0.114 mmol, 71%); brown solid of mp 190–193°C. $\gamma_{\text{max}}$(ATR)/cm$^{-1}$ 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$_3$) $\delta$ 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$_3$) $\delta$ 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$_{16}$H$_{22}$AuBF$_4$F$_2$Na$_2$O$_2$: 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 $3 \times 10^6$ parasite/ml. The virulence of $L$. major parasites was maintained by passing in female BALB/c mice by injecting hind footpads with $1 \times 10^6$ stationary-phase promastigotes. After 8 weeks, $L$. major amastigotes were isolated from mice. Isolated amastigotes were transformed to promastigotes 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 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 $5 \times 10^5$ 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 ($5 \times 10^3$ 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% CO2 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 %):

\[
\text{Inhibition(\%)} = \left( \frac{I_{\text{Control}} - I_{\text{Experimental}}}{I_{\text{Control}}} \right) \times 100.
\]

where \(I_{\text{Control}}\) 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 EC50 (effective concentration at 50%) values. Obtained EC50 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% CO2 atmosphere.[23]

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% CO2, 95% humidity, 37°C). AB reagent (10 µl of 500 mM 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 IC50 values were determined with the Quest Graph™ IC50 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 x 104 cells per well per 200 µl) for 24 hr in 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 IC50 values (concentration that caused a 50% reduction in viable cells). Obtained IC50 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

REFERENCES
[1] P. M. Cheuka, G. Mayoka, P. Mutai, K. Chibale, Molecules 2017, 22, 58.
[2] A. K. Mitra, A. R. Mason, Trop. Med. Infect. Dis. 2017, 2, 36.
[3] C. S. Allardyce, P. J. Dyson, Dalton Trans. 2016, 45, 3201.
[4] C. F. Shaw III, Chem. Rev. 1999, 99, 2589.
[5] M. Navarro, Coord. Chem. Rev. 2009, 253, 1619.
[6] A. Ilari, P. Baiocco, L. Messori, A. Fiorillo, A. Boffi, M. Gramiccia, T. di Muccio, G. Coletti, Amino Acids 2012, 42, 403.
[7] M. Mora, M. C. Gimeno, R. Visbal, Chem. Soc. Rev. 2019, 48, 447.
[8] E. Schuh, C. Pflüger, A. Citta, A. Folda, M. P. Rigobello, A. Bindoli, A. Casini, F. Mohr, J. Med. Chem. 2012, 55, 5518.
[9] C. Schmidt, B. Karge, R. Misgeld, A. Prokop, M. Brönstrup, J. Ott, Med. Chem. Commun. 2017, 8, 1681.
[10] D. Wragg, A. de Almeida, R. Bonsignore, F. E. Kühn, S. Leoni, A. Casini, Angew. Chem. Int. Ed. 2018, 57, 14524.
[11] C. Schmidt, L. Albrecht, S. Balasupramaniam, R. Misgeld, B. Karge, M. Brönstrup, A. Prokop, K. Baumann, S. Reichl, J. Ott, Metalloccics 2019, 11, 533.
[12] I. Winter, J. Lockhauserbäumer, G. Lallinger-Kube, R. Schobert, K. Ersfeld, B. Biersack, Mol. Biochem. Parasitol. 2017, 214, 112.
[13] A. Baramee, A. Coppin, M. Mortualaire, L. Pelinski, S. Tomavo, J. Brocard, Bioorg. Med. Chem. 2006, 14, 1294.
[14] F. Dubar, J. Khalife, J. Brocard, D. Dive, C. Biot, Molecules 2008, 13, 2900.
[15] A. M. A. Velásquez, A. I. Francisco, A. A. N. Kohatsu, F. A. da Silva, D. F. Rodrigues, R. G. da Silva Teixeira, B. G. Chiari, M. G. de Almeida, V. L. Isac, M. D. Vargas, R. M. Cicarelli, Bioorg. Med. Chem. Lett. 2014, 24, 1707.
[16] G. Sujatha, P. Ramanathan, T. Pazhanisamy, V. Anusuya, DJ. J. Environ. Chem. Fuel 2016, 1, 60.
[17] A. M. Jarrad, A. Debnath, Y. Miyamoto, K. A. Hansford, R. Pelengon, M. S. Butler, T. Bains, T. Karoli, M. A. T. Blaskovich, L. Eckmann, M. A. Cooper, Eur. J. Med. Chem. 2016, 120, 353.
[18] J. K. Muenzner, B. Biersack, H. Kalie, I. C. Andronache, L. Kaps, D. Schuppam, F. Sasse, R. Schobert, ChemMedChem 2014, 9, 1195.
[19] J. K. Muenzner, B. Biersack, A. Albrecht, T. Rehm, U. Lacher, W. Milius, A. Casini, J. Zhang, I. Ott, V. Brabec, O. Stuchlikova, I. C. Andronache, L. Kaps, D. Schuppam, R. Schobert, Chem. Eur. J. 2016, 22, 18953.
[20] K. Griewank, C. Gazeau, A. Eichhorn, E. von Stebut, Antimicrob. Agents Chemother. 2010, 54, 652.
Additional supporting information may be found online in the Supporting Information section.

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