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Metal Anticancer Compounds

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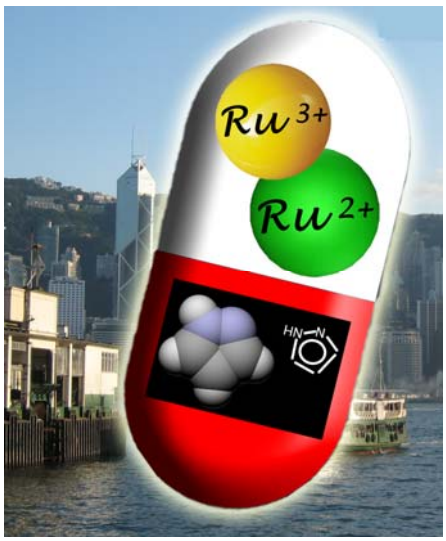

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Synthesis and biological studies of some gold(I) complexes containing functionalised alkynes

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The propargyl ethers 7-chloro-(4-propargyloxy)quinoline, 1-propargyloxynaphthalene and 2-propargyloxybenzophenone react with [AuCl(PPh$_3$)] in the presence of KOH to give the gold(I) alkynyl complexes [Au(C≡COCH$_2$Ar)(PPh$_3$)] in good yields. The compounds were fully characterised by spectroscopic methods and were subsequently examined for their biological activity against four tumour cell lines as well as their activity against *Plasmodium falciparum*, the parasite responsible for malaria. The compounds show antiproliferative effects in human cancer cells with IC$_{50}$ values ranging from 0.4–12 μM.

Introduction

Cancer and malaria are both diseases responsible for a significant number of deaths world-wide thus creating a huge economic and social burden.[1-3] Given that within the next century the number of people affected by these diseases will increase,[4] the development of a cure is an important field of chemical and biomedical research. Cisplatinum, *cis*-[PtCl$_2$(NH$_3$)$_2$], has been in clinical use for the treatment of various cancers for more than 30 years. However, despite the success of this compound, it has significant drawbacks including high toxicity (resulting in severe side-effects for the patient) as well as poor solubility and development of cellular resistance.[5] In the case of malaria, the most active and also most used drug Chloroquine is today almost completely ineffective, due to cell resistance. Research groups all over the world have therefore developed new approaches in the design of novel metal-based anti-cancer and anti-malaria drugs.[6-10] The use of gold containing medicines for the treatment of various ailments has a long history; today there are several anti-arthritis drugs containing gold(I) thiolate complexes in clinical use. Recently, the anti-tumour activity of gold(I) and gold(III) complexes have also been recognised.[9,11] Navarro *et al.* reported the anti-malaria activity of some cationic gold(I) phosphine complexes containing chloroquine.[12,13] These complexes showed a significant increase of anti-malaria activity against *Plasmodium* parasites compared to that of Chloroquine itself. Given our interest in metal complexes with biological activity,[14-17] we wished to prepare some gold complexes containing derivatives of known anti-malaria drugs, in which the gold atom is bound tightly to the ligand through a strong Au-C bond. The preparation and biological studies of these complexes is presented herein.

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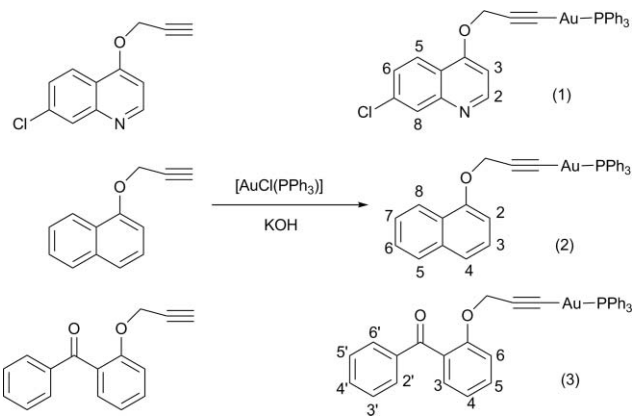
Results and discussion

The propargyl ethers 7-chloro-(4-propargyloxy)quinoline, 1-propargyloxynaphthalene and 2-propargyloxybenzophenone react with [AuCl(PPh$_3$)] in the presence of KOH to afford the colourless, air- and moisture-stable gold(I) alkynyl complexes [Au(C≡COCH$_2$Ar)(PPh$_3$)] (**1-3**) in good yields (Scheme 1).



Scheme 1

Complexes **1-3** were fully characterised by spectroscopic techniques including 1D ^{1}H, ^{13}C and ^{31}P NMR spectroscopy as well as 2D (^{1}H-^{13}C HSQC and HMBC) NMR techniques. Unfortunately, we were unable to grow crystals suitable for X-ray diffraction, since solutions of the complexes decomposed and deposited metallic gold after a few days. The spectral data is nevertheless fully consistent with the proposed structures. The ^{31}P{^{1}H} NMR spectra of complexes **1-3** show singlet resonances with chemical shifts typically observed for Ph$_3$PAu complexes. Deprotonation of the propargyl ethers is evident from the ^{1}H NMR spectra of **1-3**: the triplet resonance of the acetylenic proton has disappeared and the OCH$_2$ signal has collapsed to a singlet. Further evidence of deprotonation is seen in the IR spectra of the complexes, in which the C≡C-H stretching frequency has disappeared. The most interesting feature of the ^{13}C NMR spectra of complexes **1-3** is the

Table 1 *In vitro* anti-malaria activity of complexes **1-3** and chloroquine in two strains of *Plasmodium falciparum*

	IC_{50} [μM]	
Compound	3D7	K1
1	14.8	14.8
2	7.2	19.5
3	14.5	23.6
Chloroquine	0.01	0.3

location of the resonances from the C≡CAu unit. The signal of the carbon atom attached directly to gold appears as a doublet at *ca.* 131 ppm with coupling constants of approximately 142 and 129 Hz for complexes **1** and **3**, respectively. The corresponding signal of complex **2** could not be observed. These values are very similar to those reported by Schmidbaur *et al.* for [Au(C≡CH){P(p-tol)$_3$}].[18] Similarly, in all three complexes the carbon signal of the acetylenic carbon not attached to gold could be observed at *ca.* 100 ppm. In this case, the P-C coupling constants ranged from 0 to 26 Hz. Similar observations of such variations in P-C coupling constants have been observed in gold alkynyl complexes by us and others previously.[18,19]

The *in vitro* anti-malaria activity of the three complexes was examined in two different strains of *plasmodium falciparum* cultures. The results (Table 1) show that these complexes have low activity against the malaria parasite strains 3D7 (chloroquine sensitive) and K1 (chloroquine resistant). IC_{50} values for growth inhibition of the blood stages of *P. falciparum* were between 7.2 and 23 μM. A collateral resistance with chloroquine was not observed. The IC_{50} values of the complexes were several orders of magnitude higher than that observed for chloroquine itself (0.01 and 0.3 μM for the 3D7 and K1 strain, respectively).

These results were unexpected given that Navarro *et al.*[12,13] observed that cationic chloroquine gold derivatives show higher (or at least similar) anti-malaria activity than free chloroquine. It may, however, simply be that the weak nitrogen-gold bond in their complex is broken under physiological conditions, allowing release of free chloroquine. The AuPPh$_3$ moiety might thus simply help to bring the molecule to the site of action within the cell. In our system, the gold-carbon bond is expected to be considerably stronger than a gold-nitrogen bond, which means that hydrolysis of the complexes under physiological conditions is unlikely. The very low anti-malaria activity of these complexes might then be explained by the fact that the molecule can simply not reach the site of action. Given these disappointing results, we wished to investigate if complexes **1-3** showed any other *in vitro* bioactivity. These compounds were therefore screened in four human tumour cell lines. To the best of our knowledge, these results are amongst the first such studies carried out on mononuclear gold(I) compounds. The results, shown in Fig. 1 and Table 2, were much more encouraging, yielding IC_{50} values in the high nanomolar (cell line CH1) to low micromolar range (all other cell lines).

Out of the three complexes, compound **3** shows the highest activity in all four cell lines tested, but differences in the cytotoxic potencies of these compounds are not very pronounced and mainly within the ranges of variation. In particular, complex **3** gives IC_{50} values of similar magnitude to those of cisplatin in the broadly chemosensitive ovarian cancer cell line CH1 and in the colon cancer cell line SW480. On the other hand, the resistance mechanisms of SK-OV-3, an ovarian cancer cell line with intrinsic resistance to classic platinum drugs probably related to its distinct genomic aberrations (tetraploidy, double minutes),[20] seem to affect sensitivity to compounds **1-3**, too. Comparison of the results for complexes **1** and **2** shows that the presence of a chloride substituent at the 7-position and nitrogen at position 4 of the naphthyl ring seems to have no significant effect on their cytotoxicity. In general, the similarity of the cytotoxic potencies seems to suggest that the observed activity is governed to a higher degree by the (triphenylphosphine)gold(I)propargyl moiety common to all three compounds rather than by the variable part of the structure. The mechanism of action of anti-tumour active gold complexes is to date not well understood. Mechanistic considerations have shifted in recent years from DNA to mitochondrial targets[21] and in particular thioredoxin reductase,[22] a redox enzyme with a selenocysteine residue suitable as a binding site for gold. However, it remains unclear whether the effects reported for gold(I) phosphine complexes can be generalized to our gold compounds.

Table 2 *In vitro* cytotoxicity of complexes **1-3** and cisplatin in four human cancer cell lines

	IC_{50} [μM][a]			
Compound	CH1	SK-OV-3	HeLa	SW480
1	1.5 ± 0.3	10 ± 4	4.6 ± 0.2	9.3 ± 1.0
2	0.6 ± 0.2	12 ± 6	6.0 ± 2.3	10 ± 2
3	0.4 ± 0.1	7.4 ± 0.9	4.1 ± 0.6	4.5 ± 0.7
cist-[PtCl$_2$(NH$_3$)$_2$]	0.16 ± 0.03	1.9 ± 0.3	0.37 ± 0.06	3.5 ± 0.3

[a] 50% inhibitory concentration in the assay (96 h exposure). Values are means ± standard deviations of at least three independent experiments.

At the moment, the major achievement of this study is to have demonstrated for the first time that mononuclear organometallic gold(I) complexes can indeed show antiproliferative activity in human cancer cells *in vitro* in a reasonable range of concentrations. Further studies of this well known class of compounds are therefore clearly warranted and ongoing in our laboratories.

Experimental

General

^{1}H, ^{13}C and ^{31}P{^{1}H} NMR spectra were recorded on a 400 MHz Bruker ARX spectrometer. Chemical shifts are quoted relative to external SiMe$_4$ (^{1}H, ^{13}C) and 85% H$_3$PO$_4$ (^{31}P). The splitting of proton resonances in the reported ^{1}H NMR spectra are defined as s = singlet, d = doublet, t = triplet and m = multiplet; coupling constants are reported in Hz. IR spectra were run as KBr pellets on a Bruker Tensor 27 instrument. Elemental analyses were performed by staff of the microanalytical laboratory of the University of Wuppertal. All Reactions were carried out under an atmosphere of dry dinitrogen using standard Schlenk techniques. All chemicals and solvents (anhydrous quality) were sourced commercially and used as received. 7-Chloro-(4-propargyloxy)quinoline, as well as [AuCl(PPh$_3$)] were prepared as described in the literature.[23,24]

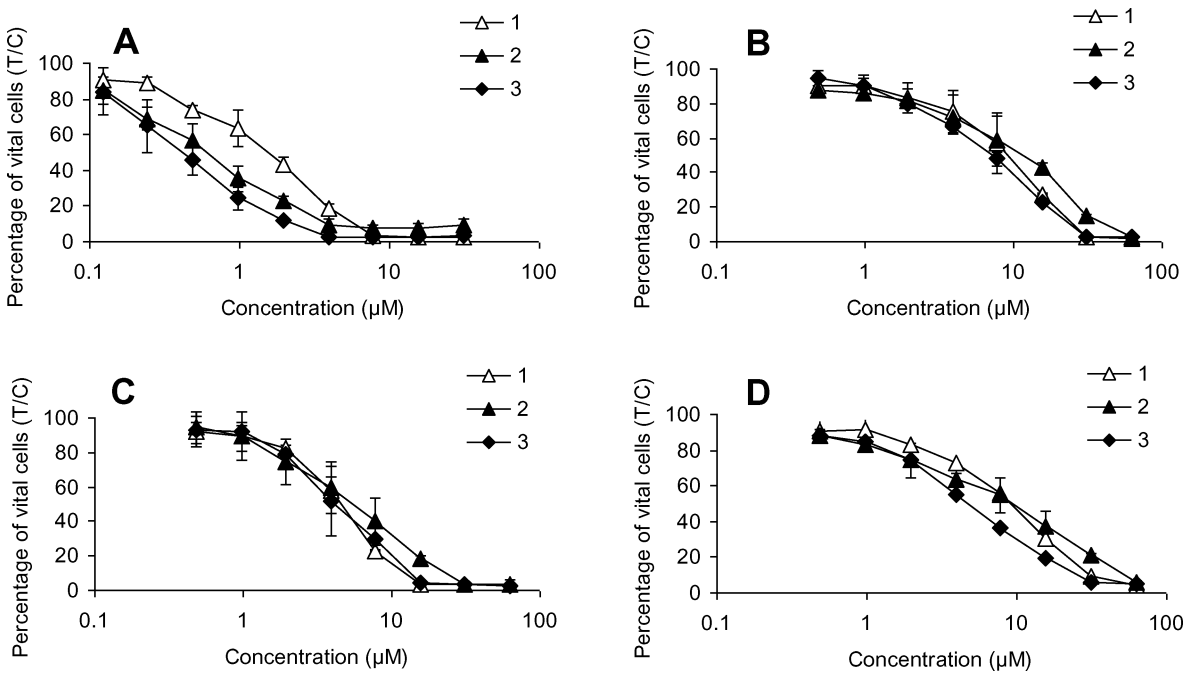


Fig. 1 Concentration-effect curves of complexes **1-3**, obtained by the MTT assay (96 h exposure) in four human cancer cell lines: CH1 (A), SK-OV-3 (B), HeLa (C) and SW480 (D).

Preparation of propargyl ethers

To a solution of the appropriate phenol in acetone (60 mL) was added K_2CO_3 (3 eq.) followed by propargyl bromide (1.2 eq.). The mixture was heated to reflux for *ca.* 18 h. The suspension was subsequently filtered and the filtrate was taken to dryness in vacuum. The resulting solid was purified by flash chromatography (SiO_2, CH_2Cl_2) to give the propargyl ethers as oils in high yields.

1-Propargyloxynaphthalene

This was prepared as described above from 1-naphthol (2.0 g, 14 mmol), K_2CO_3 (5.9 g, 42 mmol) and propargyl bromide (2.2 mL, 20 mmol). Yield: 2.2 g (87%) red-brown oil. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 2.56 (t, J = 2.3 Hz, 1 H, C≡CH), 4.90 (d, J = 2.2 Hz, 2 H, OCH_2), 6.96 (d, J = 7.5 Hz, 1 H H2), 7.41 (t, J = 7.9 Hz, 1 H, H3), 7.49-7.54 (m, 3 H, H4, 6, 7), 7.83 (m, 1 H, H5), 8.31 (m, 1 H, H8). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 56.1 (OCH_2), 75.5 (C≡*C*H), 78.6 (*C*≡CH), 105.5 (C2), 121.2 (C4), 121.9 (C8), 125.4 (C7), 125.7 (C4a), 125.5 (C3), 126.4 (C6), 127.4 (C5), 134.5 (C8a), 153.3 (C1). IR (cm^{-1} CH_2Cl_2) 3302 ν(C≡CH), 2124 ν(C≡C)[1].

2-Propargyloxybenzophenone

This was prepared as described above from 2-hydroxybenzophenone (1.0 g, 5 mmol), K_2CO_3 (2.1 g, 15 mmol) and propargyl bromide (0.8 mL, 8 mmol). Yield: 1.1 g (89.7%) brown oil. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 2.43 (t, J = 2.3 Hz, 1 H, C≡CH), 4.59 (d, J = 2.3 Hz, 2H, OCH_2), 7.09 (t, J = 7.6 Hz, 1 H, H5), 7.14 (d, J = 8.2 Hz, 1 H H3), 7.38-7.43 (m, 3 H, H3′, 4, 5′), 7.47 (td, J = 8.2 Hz, J = 1.7 Hz, 1 H, H6), 7.54 (t, J = 7.3 Hz, **1** *H H4′*), 7.81 (dd, J = 7.1 Hz, J = 1.0 Hz, 2 H, H2′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 56.1 (OCH_2), 75.7 (C≡*C*H), 78.0 (*C*≡CH), 113.3 (C3), 121.5 (C5), 128.1 (C3′, C5′), 129.6 (C2), 129.7 (C1′), 129.8 (C2′, C6′), 131.6 (C6), 132.8 (C4′), 137.6 (C4), 155.1 (C1), 195.9 (C=O). IR (cm^{-1} CH_2Cl_2) 3302 ν(C≡CH), 2124 ν(C≡C), 1711 ν(C=O).

Preparation of the gold(I) alkynyl complexes [Au(C≡COCH$_2$Ar)(PPh$_3$)]

To a solution of the propargyl ether (0.34 mmol) in EtOH and MeOH (1:1 10 mL) was added a solution of 2 M KOH in MeOH (0.2 mL, 0.4 mmol). After stirring the mixture for *ca.* 5 min, solid [AuCl(PPh_3)] (0.100 g, 0.20 mmol) was added and the mixture was stirred at 50 °C for 1 h. The resulting solution was cooled and concentrated in vacuum to a volume of *ca.* 5 mL. Addition of hexane led to the precipitation of the complexes. Complete removal of KCl and excess base was achieved by dissolving the crude material in CH_2Cl_2, filtration through Celite and precipitation of the complexes by addition of hexane.

Using this procedure the following compounds were prepared:

[Au(C≡COCH$_2$Ar)(PPh$_3$)] (Ar = 7-chloro-(4-propargyloxy)-quinoline) 1. Yield: 0.087 g (87%) colourless solid. Found: C, 52.9; H, 3.3; N, 1.85; Calc. for $C_{30}H_{22}AuClNOP$: C, 53.3; H, 3.3; N, 2.1%. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 4.89 (s, 2H, OCH_2), 6.26 (d, J = 7.8 Hz, 1 H, H3), 7.31 (dd, ^3J = 8.6 Hz, ^4J = 1.7 Hz, 1 H, H6), 7.44–7.52 (m, 15H, PPh_3), 7.65 (s, 1 H, H8), 7.92 (d, J = 7.8 Hz, 1 H, H5), 8.37 (d, J = 8.6 Hz, 1 H, H2). ^{13}C-NMR (101 MHz, $CDCl_3$, 25 °C): δ 43.8 (OCH_2), 93.7 (d, J = 26.8Hz, *C*≡C-Au), 110.6 (C3), 115.6 (C8), 124.3 (C6), 125.6 (C4a), 128.6 (C5), 129.2 (d, J = 11.6Hz, *m*-PPh_3), 129.4 (d, J = 56.5Hz, *ipso*-PPh_3), 131.5 (d, J = 141.9 Hz, C≡*C*Au), 131.7 (d, J = 1.7Hz, *p*-PPh_3), 134.2 (d, J = 13.7 Hz, *o*-PPh_3), 138.5 (CCl), 140.6 (C8a), 142.4 (C2), 177.7 (C-O). Atom numbering as shown in Scheme 1.

$^{31}P\{^{1}H\}$ NMR (162 MHz, $CDCl_3$, 25 °C): δ 42.98. IR (cm^{-1}, KBr disk) 2115 ν(C≡C).

[Au(C≡COCH$_2$Ar)(PPh$_3$)] (Ar = 1-propargyloxy-naphthalene) 2. This was prepared as described above, except that the mixture was refluxed for one hour. Yield: 0.078 g (60%) colourless solid. Found: C, 58.1; H, 3.6; Calc. for $C_{31}H_{24}AuOP$: C, 58.1; H, 3.8%. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 5.05 (s, 2H, OCH_2), 7.05 (dd, ^{3}J = 6.9 Hz, ^{4}J = 1.6 Hz, 1 H, H2), 7.36-7.54 (m, 19H, H3, H4, H6, H7, PPh_3), 7.73 (dd, ^{3}J = 7.1 Hz, ^{4}J = 2.1 Hz, 1 H, H5), 8.34 (dd, ^{3}J = 6.9 Hz, ^{4}J = 2.4 Hz, 1 H, H8). ^{13}C-NMR (101 MHz, $CDCl_3$, 25 °C): δ 57.3 (OCH_2), 98.2 (*C*≡CAu), 105.5 (C2), 120.3 (C3), 122.6 (C8), 124.9 (C-naph), 125.9 (C-naph), 125.9 (C8a), 126.1 (C-naph), 127.2 (C5), 129.1 (d, *J* = 11.1Hz, *m*-PPh_3), 130.0 (d, *J* = 52.5Hz, *ipso*-PPh_3), 131.4 (d, *J* = 2.1 Hz, *p*-PPh_3), 134.2 (d, *J* = 14.0 Hz, *o*-PPh_3), 134.5 (C4a), 154.06 (C1), C≡*C*Au not observed. Atom numbering as shown in Scheme 1. $^{31}P\{^{1}H\}$ NMR (162 MHz, $CDCl_3$, 25 °C): δ 43.3. IR (cm^{-1}, KBr disk) 2134 ν(C≡C).

[Au(C≡COCH$_2$Ar)(PPh$_3$)] (Ar = 2-propargyloxy-benzophenone) 3. This was prepared as described above, except that the mixture was stirred at room temperature over night. Yield: 0.129 g (92%) colourless solid. Found: C, 58.8; H, 3.5; Calc. for $C_{34}H_{26}AuO_2P$: C, 58.8; H, 3.8%. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 4.75 (s, 2H, OCH_2), 7.03 (t, *J* = 7.4 Hz, 1 H, H5), 7.36-7.53 (m, 21H, H3, H3′, H4, H4′, H5′, H6, PPh_3), 7.84 (d, *J* = 7.0 Hz, 2 H, H2′, H6′). ^{13}C-NMR (101 MHz, $CDCl_3$, 25 °C): δ 57.4 (OCH_2), 97.7 (d, *J* = 2.2 Hz, *C*≡CAu), 113.8 (C3), 120.7 (C5), 128,1 (C3′, 5′), 128.7 (d, *J* = 57.2 Hz, *ipso*-PPh_3), 129.2 (d, *J* = 11.3 Hz, *m*-PPh_3), 129,3 (C2), 129.6 (C4), 129.9 (C1′), 129.9 (C2′, 6′), 131.6 (d, *J* = 2.2 Hz, *p*-PPh_3), 131.8 (C6), 132.0 (d, *J* = 129.0 Hz, C≡*CAu*), 132.7 (C4′), 134.3 (d, *J* = 13.8Hz, *o*-PPh_3), 156.1 (C1), 196.4 (C=O). Atom numbering as shown in Scheme 1. $^{31}P\{^{1}H\}$ NMR (162 MHz, $CDCl_3$, 25 °C): δ 43.2. IR (cm^{-1}, KBr disk) 2152 ν(C≡C), 1700 ν(C=O).

Cell lines and culture conditions

CH1 and SK-OV-3 cells (both ovarian carcinoma, human) were kindly provided by Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, U.K.) and Evelyn Dittrich (Department of Medicine I, Medical University of Vienna, Austria), respectively. HeLa cells (cervical carcinoma, human) and SW480 cells (colon carcinoma, human) were kindly provided by Thomas Czerny (Institute Of Genetics, University of Veterinary Medicine Vienna, Austria) and Brigitte Marian (Institute of Cancer Research, Medical University of Vienna, Austria), respectively. Cells were grown in 75 cm^2 culture flasks (Iwaki/Asahi Technoglass) as adherent monolayer cultures in complete culture medium, *i.e.*, Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 1% non-essential amino acids (100×) (Sigma-Aldrich) without antibiotics. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cytotoxicity in cancer cell lines

Cytotoxicity in the cell lines mentioned above was determined by a colorimetric microculture assay (MTT assay). Cells were harvested from culture flasks by trypsinization and seeded in 100 µL aliquots into 96-well microculture plates (Iwaki/Asahi Technoglass) in the following densities, in order to ensure exponential growth of untreated controls throughout the experiment: 1.5×10^3 (CH1, HeLa), 2.5×10^3 (SW480) and 3.5×10^3 (SK-OV-3) viable cells per well. Cells were allowed to settle and resume exponential growth in drug-free complete culture medium for 24 h, followed by the addition of dilutions of the test compounds in 100 µL/well complete culture medium. For this purpose, the poorly water-soluble compounds were dissolved in dmso first and then diluted in the medium such that the effective dmso content did not exceed 0.3%. After exposure for 96 hours, medium was replaced by 100 µL/well RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine) plus 20 µL/well solution of MTT in phosphate-buffered saline (5 mg/mL) (all purchased from Sigma-Aldrich). After incubation for 4 h, medium/MTT mixtures were removed, and the formazan product formed by vital cells was dissolved in dmso (150 µL/well). Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic), using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of vital cells was expressed as percentage of untreated controls, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration-effect curves by interpolation. Evaluation is based on means from at least three independent experiments, each comprising three replicates per concentration level.

Malaria studies

Continuous culture of laboratory strains of *P. falciparum* Protozoa were grown in RPMI 1640 medium supplemented with gentamycin and 7.5% sodium bicarbonate and 10% human serum at a hematokrit of 5% of blood group 0 rhesus positive red blood cells following the protocol for continuous culture by Jensen and Trager.[25,26] The parasites were cultured in cell culture flasks incubated in a tissue culture incubator at 37 °C in a gas mixture composed of 5% CO_2, 5% O_2, and 90% N_2. The culture medium was changed every 24 to 48 hours. Fresh erythrocytes were added whenever the parasite density reached more than 1%. Cultures with a parasitemia of 6-10% were used for growth assays.

Drug screening and drug sensitivity testing

A highly sensitive HRP2 ELISA was employed for drug sensitivity assays. Stock solutions of the compounds as well as of chloroquine were prepared as previously described.[27] Chloroquine was dissolved in 70% ethanol and gold complexes were dissolved in DMSO (final concentration of solvent not to exceed 0.1%). The resulting stock solutions were diluted with RPMI 1640 to achieve the desired final concentrations.

Sample preparation

After synchronization the samples from continuous culture were diluted with RPMI 1640 containing 10% serum to obtain a hematocrit of 1.5%, and with uninfected red blood cells (blood group 0) to obtain 0.05% parasitemia. 200 µl of the cell medium mixture were added to each well. The plates were then incubated at 37 °C for 72 hours in a gas mixture containing 5% CO_2, 5% O_2, and 90% N_2.

HRP2 double-site antigen capture ELISA[28]

High binding 96-well ELISA plates (Costar® 3590, Corning Inc., NY) were coated with 100 µl/well of a 1.0 µg/ml solution of anti-HRP2 IgM antibody solution (MPFM-55A, Dunn Labortechnik, Asbach, Germany) in phosphate buffered saline (PBS). Subsequently plates were sealed and incubated overnight at 4 °C. The supernatant was discarded and plates saturated for 2 hours with 200 µl/well of a 2% solution of bovine serum albumin (BSA, Sigma-Aldrich, A9647) in PBS. The supernatant was again discarded and plates were washed 3 times with 200µl/well of PBS/Tween washing solution (0.5% Tween®20 in PBS). The plates were then sealed and stored at or below –20 °C.

Samples were hemolyzed by two cycles of freeze-thawing and samples with an initial parasite density of >0.1% were diluted directly in the precoated ELISA plates with distilled water to an equivalent of approximately 0.05% starting parasitemia. The ELISA plates were then incubated with the samples for one hour at room temperature and subsequently washed three times with PBS/Tween.

The antibody conjugate (MPFG-55P, Dunn Labortechnik, Asbach, Germany) was diluted in a solution of 2% BSA and 1% Tween 20 in PBS to 0.05 µg/ml. After addition of 100 µl of the diluted conjugate, the plates were incubated for another hour at room temperature and washed three times with PBS/Tween solution.

100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) were added to each well and the plates were incubated in the dark for 5 to 10 min. The reaction was stopped by addition of 50 µl of 1 M sulfuric acid. Spectrophotometric analysis was performed using a standard ELISA plate reader. Absorbance readings were obtained at 450 nm.

Hyperbolic concentration response curves were fitted to data points using the method of nonlinear least squares. IC_{50} values for growth inhibition were calculated from these concentration response curves.

Acknowledgements

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Notes and references

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