Gold Metallodrugs to Fight the Corona Virus: Inhibitory Effects on the Spike-ACE2 Interaction and on PLpro Protease Activity by Auranofin and Gold Organometallics

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Keywords: Auranofin, corona virus, gold complexes, metallodrugs, SARS-CoV-2

Abstract
Gold complexes have a long tradition in medicine and for many examples antirheumatic, anticancer or anti-infective effects have been confirmed. Here we evaluated the lead compound Auranofin and five selected gold organometallics as inhibitors of two relevant drug targets of severe acute respiratory syndrome coronaviruses (SARS-CoV). The gold metallodrugs were effective inhibitors of the interaction of the SARS-CoV-2 spike protein with the angiotensin converting enzyme 2 (ACE2) host receptor and might thus interfere with the viral entry process. The gold metallodrugs were also efficient inhibitors of the SARS-CoV-1 papain-like protease (PLpro), which is a key enzyme in the viral replication. Taken together, the results of this pilot study suggest further evaluation of gold complexes as SARS-CoV antiviral drugs.
The current pandemic outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused an unprecedented global health crisis with to date more than 7 million infected individuals.\cite{1,2} While the world struggles with the control of the fast outspread of this coronavirus and it's enormous impact on healthcare, economy and society, efforts to develop vaccines and therapeutics have been undertaken worldwide at a rate, which modern drug discovery has not witnessed ever. The lack of an effective antiviral drug for the treatment of the Coronavirus disease-2019 (COVID-19) has triggered major drug repurposing efforts, however, to this date no approved therapeutic has proven to have sufficient efficacy in the many ongoing clinical trials. The urgent development of new innovative drug candidates against SARS-CoV-2 is the most important mission that medicinal chemists are currently facing.

Regarding drug activity evaluation, several molecular pathways have been in the focus of the search for a possible COVID-19 treatment based on strategies that had already been considered for the SARS-CoV and Middle East respiratory syndrome MERS-CoV outbreaks.\cite{3}

Amongst others these include the entry of the coronavirus into the host cell (e.g. the interaction of TMPRSS2\cite{4} or ACE2 with spike proteins of the coronavirus\cite{5}), the viral replication process in the host cell (e.g. the proteases 3CLpro\cite{6} and PLpro\cite{3,7,8}), transcription, the nucleocapsid protein, or exocytosis of the new virion.\cite{3,7,9}

Figure 1: left: simplified SARS-CoV-2 life cycle, gold drugs targeting the viral entry and replication are symbolised by golden bars; right: gold metallodrugs used in this study
Gold complexes have a long lasting history in medicine and have been used as disease modifying antirheumatic drugs (DMADs) for the treatment of rheumatoid arthritis. Intensive research on other possible therapeutic applications of the lead compound Auranofin and other gold species has focused on anticancer and anti-infective agents. The application of gold complexes as antiviral drugs has not been studied very intensively, although some promising results suggest a possible future use as human immunodeficiency virus (HIV) therapeutics.\[10\]

Here we report the results of a pilot study, in which we investigated the effects of Auranofin and selected experimental gold metallodrugs (see Figure 1) on two relevant coronavirus targets (SARS-CoV-2 spike protein, SARS-CoV-1 papain like protease, PLpro). Whereas \textbf{Au-1}\[11\], \textbf{Au-3}\[12,13\] and \textbf{Au-5}\[15\] were selected from our previous works on organometallic gold metallodrugs, \textbf{Au-2} and \textbf{Au-4} have not been reported before and their synthesis and characterization are described here. Complexes \textbf{Au-1} to \textbf{Au-5} are organometallics containing either a \textit{N}-heterocyclic carbene (NHC) or an alkynyl ligand. Complexes of these types have demonstrated promising activities in a fast increasing number of recent reports.\[14\]

The entry of SARS-CoV-2 into target cells is facilitated by the spike (S) protein of coronaviruses and mediated by the angiotensin-converting enzyme 2 (ACE2) as the entry receptor.\[1,4\] The S1 subunit of the SARS-CoV-2 spike protein contains the receptor binding domain (RBD). Binding of the RBD to the human ACE2 receptor can be measured by ELISA allowing to evaluate inhibitors of the S protein ACE2 interaction. In this assay, the gold complexes \textbf{Au-1} to \textbf{Au-5} and Auranofin displayed good IC\textsubscript{50} values in the range of 16 - 25 µM and were thus slightly more active than the reference drug Chloroquine (IC\textsubscript{50} value: 31.9 µM).

Table 1: Inhibition of the spike-ACE2 interaction and PLpro activity (mean values and standard deviations, n=3-4); n.d. not determined. Benzimidazole was used as a negative reference in both assays.

<table>
<thead>
<tr>
<th></th>
<th>spike-ACE2 (IC\textsubscript{50}, µM)</th>
<th>PLpro (IC\textsubscript{50}, µM)</th>
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</thead>
<tbody>
<tr>
<td>benzimidazole</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>31.9 ± 5.4</td>
<td>n.d.</td>
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<tr>
<td>Disulfiram</td>
<td>n.d.</td>
<td>6.5 ± 0.4</td>
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<tr>
<td>Auranofin</td>
<td>22.2 ± 2.8</td>
<td>25.5 ± 1.2</td>
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<tr>
<td>\textbf{Au-1}</td>
<td>19.4 ± 5.7</td>
<td>6.3 ± 1.6</td>
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<tr>
<td>\textbf{Au-2}</td>
<td>20.0 ± 2.3</td>
<td>5.5 ± 0.5</td>
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<tr>
<td>\textbf{Au-3}</td>
<td>21.3 ± 6.8</td>
<td>14.2 ± 0.3</td>
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<tr>
<td>\textbf{Au-4}</td>
<td>25.0 ± 4.2</td>
<td>14.1 ± 2.1</td>
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<tr>
<td>\textbf{Au-5}</td>
<td>16.2 ± 2.4</td>
<td>6.7 ± 0.9</td>
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An essential step in the replication of coronaviruses is the processing of the replicase polyprotein by proteases, such as the papain-like protease (PLpro), resulting in a number of non-structural proteins (nsps) that are involved in downstream binding and replication events.\[^{3,7,8}\] SARS-CoV-1 PLpro shares 83% sequence identity with PLpro from SARS-CoV-2, structural components of the active sites of the enzymes do not substantially differ. As a cysteine protease PLpro is a likely target for gold-based drugs based, which generally are known to interact with sulfur-containing molecular targets. The inhibitory activity of the gold compounds towards SARS-CoV-1 PLpro was determined by an enzymatic FRET assay. In these experiments **Au-1, Au-2** and **Au-5** exhibited IC\(_{50}\) values in the range of 5 - 7 µM matching the potency of the reference inhibitor Disulfiram. Complexes **Au-3** and **Au-4** were less active with IC\(_{50}\) values of 14 µM. Auranofin remained the lowest active gold compound with an IC\(_{50}\) value of 25.5 µM.

In conclusion, we have demonstrated that gold complexes can target two relevant pathways in the life cycle of corona viruses. Strong activity of the organometallic gold complexes **Au-1** to **Au-5** was observed against the cysteine protease PLpro. Auranofin was a weaker inhibitor of this enzyme, however, regarding the inhibition of the spike-ACE2 interaction it showed comparable activity compared with the organometallic gold compounds. The differing activities observed in the two used assays and among the individual gold drugs suggest further studies aiming at an optimization of the inhibitory effects and at developing structure-activity relationships. Notably, during writing up of this manuscript the evaluation of the FDA approved Auranofin for COVID-19 treatment was suggested by other authors and the inhibition of the replication of SARS-COV-2 in human cells at low micro molar concentration was confirmed.\[^{16}\] The screening of gold and other metal-based drugs towards relevant SARS-CoV-2 molecular targets is definitely warranted and is ongoing in our laboratories.

**Experimental**

**General**

Chemicals and reagents were obtained from Sigma-Aldrich, TCI, Alfa Aesar and ACROS unless otherwise noted. NMR spectra were recorded on a Bruker DRX-400 AS NMR spectrometer; Positive-ion ESI (electrospray ionization) mass spectra were recorded on a LTQ-OrbitrapVelos linear iontrap coupled with orbitrap mass analyser (ThermoFisher Scientific). Elemental analyses were conducted in Flash EA1112. A VictorTM X4 Pekin Elmer 2030 multilabel reader was used for the inhibitor assays. Complexes **Au-1, Au-3** and **Au-5** were prepared as previously reported.\[^{11,13,15}\]
Chlorido(1,3-diethyl-benzimidazol-2-ylidene)gold(III) (Au-2)

Au-2 was prepared according to a reported procedure with modifications.[17] Au-1 (100.0 mg, 0.246 mmol, 1.0 equiv) and dichloroiodobenzene (81.1 mg, 0.295 mmol, 1.2 equiv) were dissolved in dichloromethane (4 mL) and the mixture was stirred for 24 h at room temperature under protection from light. Afterwards the solvent was removed under vacuum, the residue was washed with n-hexane and diethylether and two times with cold chloroform. The complex Au-2 was dried under vacuum at 40°C. Yield: 107.6 mg (0.225 mmol, 92 %), yellow-orange powder; \(^1\)H NMR (500 MHz, DMSO-d_6): \(\delta= 8.05 \) (dd, \(^3J_{H,H} = 6.3 \) Hz, \(^4J_{H,H} = 3.2 \) Hz, 2H, ArH), 7.61 (dd, \(^3J_{H,H} = 6.3 \), \(^4J_{H,H} = 3.2 \) Hz, 2H, ArH), 4.72 (q, \(^3J_{H,H} = 7.3 \) Hz, 4H, CH\(_2\)), 1.53 (t, \(^3J_{H,H} = 7.3 \) Hz, 6H, CH\(_3\)); \(^1^3\)C NMR (126 MHz, DMSO-d_6): \(\delta= 147.2 \) (ArC\(_2\)quat), 132.7 (2C, ArC\(_3a\)quat, ArC\(_7a\)quat), 125.7 (2C, ArC\(_4\) + ArC\(_7\)), 113.0 (2C, ArC\(_5\), ArC\(_6\)), 43.3 (2C, CH\(_2\)), 14.4 (2C, CH\(_3\)); Elemental analysis: calcd. (%) for C\(_{11}\)H\(_{14}\)AuCl\(_3\)N\(_2\): C 27.67, H 2.95, N 5.87; found: C 27.55, H 2.89., N 5.61; MS(ESI): \(m/z\) 473.0 [\(M+\text{MeOH}\)]\(^+\), 209.1 [\(M+\text{AuCl}_2\)]\(^+\). Notably, upon oxidation of gold(I) to gold(III) a significant upfield shift of the carbene carbon can be observed in the \(^{13}\)C-NMR spectra.[18] Here the carbene carbon was shifted from 177 ppm (Au-1)[11] to 147.2 ppm.

(1,3-diethyl-benzimidazol-2-ylidene)((4-methoxyphenyl)ethynyl)gold(I) (Au-4)

1-ethynyl-4methoxybenzene (81.3 mg, 0.615 mmol, 1 equiv) and potassium hydroxide (207.0 mg, 3,689 mmol, 6 equiv) were dissolved in methanol (20 mL) and the mixture was stirred for 10 minutes at 50°C. Chlorido(1,3-diethyl-benzimidazol-2-ylidene)gold(I) Au-1 (250.0 mg, 0.615 mmol, 1 equiv) was dissolved in dichloromethane (1 mL) and added to the mixture. The mixture was stirred for 4 h at 65°C and for further 60 h at room temperature under protection from light. The solvent was removed under vacuum, the residue was dissolved in dichloromethane and filtered. The solution was washed with a potassium hydroxide solution (20g / L), evaporated and dried under vacuum at 40°C. Yield: 181.2 mg (0.361 mmol, 58.3 %), yellowish powder; \(^1\)H NMR (400 MHz, DMSO-d_6): \(\delta = 7.87-7.78 \) (m, 2H, ArH), 7.55-7.43 (m, 2H, ArH), 7.27-7.19 (m, 2H, ArH), 6.88-6.80 (m, 2H, ArH), 4.53 (q, \(^3J_{H,H} = 7.2 \) Hz, 4H, CH\(_2\)), 3.74 (s, 3H, OCH\(_3\)), 1.46 (t, \(^3J_{H,H} = 7.2 \) Hz, 6H, CH\(_3\)). Elemental analysis: calcd. (%) for C\(_{20}\)H\(_{21}\)AuN\(_2\)O: C 47.82, H 4.21, N 5.58; found: C 47.32, H 4.23., N 5.33; MS(ESI): \(m/z\) 504.1 [\(M+\text{H}\)]\(^+\), 875.2 [\(M+\text{NHC-Au}\)]\(^+\).

Spike / ACE2 Interaction Assay

The inhibition of the spike-ACE2 interaction was measured using the SARS-Cov2 Inhibitor Screening Assay kit (Adipogen, Cat. N° AG-44B-0007-KI01). All reagents were used from the same kit during individual experiments and the experiment was performed using the manufacturer’s protocol. Briefly, the SARS-CoV-2 Spike S receptor binding domain (RBD):Fc
(human) (rec.) (SPIKE) was reconstituted to 0.1 mg/ mL with deionized water. This was further diluted to a working concentration of 1 μg/ mL in phosphate buffered saline (PBS) and used freshly. The assay plate was coated with 100 μL/ well of SPIKE, covered with a plastic film and kept at 4 °C overnight. The liquid was aspirated and any remaining liquid was removed by blotting against clean absorbent papers. The plate was blocked using 200 μL of Blocking Buffer per well for 2 h at room temperature. The liquid was aspirated and the wells were washed with 1X Washing Buffer (300 μL x 3 times). All liquid was aspirated and excess liquid was removed by blotting against clean absorbent papers. The inhibitors (gold complexes, controls, reference) were diluted in Inhibitor Mix Solution (IMS) which was prepared using ACE2 (human) (rec.) (Biotin) (ACE2) (0.1 mg/ mL) to the working concentration of 0.5 μg / mL in 1X ELISA Buffer. The stock solution of the inhibitors was made in DMSO and the final DMSO concentration in the wells was 0.5%. The IMS-diluted inhibitors were added to the wells (100 μL/ well). The negative control wells were also treated with 0.5% DMSO in IMS. The plate was covered with a plastic film and incubated at 37 °C for 1 h after which the aspiration/ wash step was repeated. Next, horseradish peroxidase-labeled streptavidin (HRP) was reconstituted with 100 μL of 1X ELISA Buffer and further diluted to a working concentration by adding 50 μL in 10 mL of 1X ELISA Buffer (1:200 dilution). It was covered with a plastic film and incubated at RT for 1 h. Following this, the aspiration/ wash step as described earlier was repeated. Substrate development was conducted by the addition of 100 μL of ready-to-use 3,3′,5,5′-tetramethylbenzidine (TMB) to each well for 5 minutes at RT. The reaction was stopped by adding 50 μL of a stop solution. The OD was measured at 450 nm using a Perkin Elmer Victor X4 microplate reader. The individual absorbance value of the blank well was subtracted from the other absorbance values and the percentage of the remaining activity was calculated with respect to the untreated control values. Data fitting was done using Origin 2018 using sigmoidal fitting with Hill1 fitting curve. All treatments were done in duplicates and two independent experiments were performed.

SARS-CoV-1 PLpro Inhibition

The inhibition of SARS-CoV-1 was determined according to reported protocols with minor modifications.[19] The inhibitor compounds were prepared as stock solutions in DMSO and diluted hundredfold with HEPES buffer (50 mM HEPES, pH 7.5, 0.1 mg/mL bovine serum albumin, 0.1 % Triton-X100) to micromolar concentrations. Volumes of 50 μL of 350 nM His$_6$-SARS-CoV-1 PLpro (SouthBayBio) in HEPES buffer or blank HEPES buffer (negative control) were added to the wells of a black 96-well microtiter plate (Nunclon, Nunc). Volumes of 50 μL of the inhibitor solutions or 1% DMSO in HEPES buffer (positive control) were added. The resulting solutions (175 nM PLpro, 0.5% DMSO, 1 - 100 μM test compound or blank HEPES buffer) were mixed and incubated at 37°C for one hour. A volume of 100 μL of 100 μM Z-Arg-
Leu-Arg-Gly-Gly-AMC (Bachem Bioscience) was added to all wells. The resulting solutions were mixed and the fluorescence emission was measured immediately every 30 s for 10 min ($\lambda_{\text{exc}} = 355 \text{ nm}$; $\lambda_{\text{em}} = 460 \text{ nm}$) at 37°C using a Victor™ X4 Pekin Elmer 2030 multilabel reader. The increase of emission over time followed a linear trend ($r^2 > 0.97$) and the enzymatic activities were calculated as the slope thereof. The IC$_{50}$ values were calculated as the concentration of the inhibitor that was required to decrease the enzymatic activity to 50% of the positive control. The wells containing the negative control were used to confirm the absence of false positive results by reaction of the inhibitor compound with the fluorogenic substrate.

References


