Supporting Information

A gold(I) biscarbene complex with improved activity as TrxR inhibitor and cytotoxic drug: comparative studies with different gold metallodrugs

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1.) NMR spectra of biscarbene 3

**Figure SI1:** $^1$H-NMR spectrum of biscarbene 3 (400 MHz in CDCl$_3$)

**Figure SI2:** $^{13}$C-NMR spectrum of biscarbene 3 (400 MHz in CDCl$_3$)
**Figure SI3:** $^{13}$C-NMR-DEPT135 spectrum of biscarbene 3 (400 MHz in CDCl$_3$)

**Figure SI4:** $^{19}$F-NMR spectrum of biscarbene 3 (400 MHz in CDCl$_3$)
2.) ESI-MS stability tests of monocarbene 2

![Figure SI5](image)

**Figure SI5:** Preliminary stability tests of monocarbene 2 (100 µM) in water (1% DMF) after 75 min. Positive scan mode: m/z 200-750; Retention time: 0.066 min (top), 0.672 min (bottom).

<table>
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<th>m/z</th>
<th>Abundance [%]</th>
<th>Structure</th>
<th>m/z</th>
<th>Abundance [%]</th>
<th>Structure</th>
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<td>219.20</td>
<td>45.9</td>
<td>NHC⁺ (1)</td>
<td>219.80</td>
<td>7.9</td>
<td>NHC⁺ (1)</td>
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<td>431.50</td>
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<td>100.0</td>
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<td>39.4</td>
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<td>(NHC)₂Au⁺ (3)</td>
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**Table SI1:** Intensities of selected masses of preliminary stability tests of monocarbene 2 (100 µM) in water (1% DMF) after 75 min, positive scan mode: m/z 200-750.
37°C: mono-carbene 2 10µM in DMF  

25°C: mono-carbene 2 10µM in DMF

Figure SI6: Stability of monocarbene 2 (concentration: 10 µM) in water (1% DMF) time points: 1min, 15 min, 75 min, masses of interest (m/z: 219.15, 432.10, 450.05, 488.15 or 493.10, 633.20) in positive SIM mode.

<table>
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<th>m/z</th>
<th>Structure</th>
<th>Abundance at 37 °C</th>
<th>Abundance at 25 °C</th>
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<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>15 min</td>
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<tr>
<td>219.15</td>
<td>NHC⁺ (1)</td>
<td>342</td>
<td>369</td>
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<td>432.10</td>
<td>NHC-Au-OH</td>
<td>633</td>
<td>1452</td>
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<tr>
<td>450.05</td>
<td>NHC-Au-Cl</td>
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<td>203</td>
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<tr>
<td>488.15</td>
<td>NHC-Au-DMF</td>
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<td>232</td>
</tr>
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<td>633.20</td>
<td>(NHC)₂Au⁺ (3)</td>
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<td>274</td>
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Table SI2: Intensities of MS stability studies of monocarbene 2 in 1 % DMF

Figure SI7: Intensities of MS stability studies of monocarbene 2 in 1 % DMF (left at 37 °C, right at 25 °C)
37°C: mono-carbene 2 10μm in DMSO

25°C: mono-carbene 2 10μm in DMSO

Figure SI8: Stability of mono-NHC complex 2 in water (1% DMSO) time points: 1min, 15 min, 75 min.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Structure</th>
<th>1 min</th>
<th>15 min</th>
<th>75 min</th>
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<th>15 min</th>
<th>75 min</th>
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<td>633.20</td>
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<td>298</td>
<td>273</td>
<td>437</td>
<td>336</td>
<td>257</td>
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Table SI3: Intensities of MS stability studies of complex 2 in 1 % DMSO

Figure SI9: Intensities of MS stability studies of complex 2 in 1 % DMSO (left at 37 °C, right at 25 °C)
3.) Photometric Measurements (UV-Vis)

UV/VIS measurements were performed on a Specord 40 Photometer (Analytik Jena) and the software Winspect was used for calculations (range: 200 – 600 nm in 0.5 nm steps and a scan speed of 5 nm/s).

All test compounds were solved in DMF and diluted with DMEM cell culture medium (without phenolred or any other supplements) to a final compound concentration of 50 µM (0.1% v/v DMF) and incubated at 37 °C throughout the whole experiment. 1.0 mL of these solutions were transferred to a quartz cuvette and measured time-dependently (0, 1, 2, 4 and 8 hours). A reference spectrum of a blank, which contains only DMF (0.1% v/v) in DMEM was measured before and automatically subtracted by the software.

Figure SI10: Photometric measurements in DMEM cell culture medium without phenolred) a) 1 (λmax 237 nm), 2 (λmax 252 nm) and 3 (λmax 237 nm/272 nm) after 0 hours b) 1 (λmax 237 nm), 2 (λmax 248 nm/268 nm) and 3 (λmax 252 nm/272 nm) after 8 hours and c) 2 over 8 hours (λmax 252 nm → λmax 248 nm/268 nm)

The spectra at the beginning of the experiment (0 h, Fig. SI10a) showed that it is to some extent possible to compare compounds 1, 2 and 3 by UV-Vis spectroscopy. Importantly, 3 showed an additional maximum at 272 nm, which was not present in 1 and 2. After 8 hours of exposure the spectra of 1 and 3 did not show significant changes, whereas the spectrum of 2 had experienced major alterations, which could be explained with formation of 1 and 3 (increase of absorbance and appearance of a shoulder at approx. 272 nm, Fig. SI10b). The changes in the spectrum of 2 appeared in a time dependent manner (Fig. SI10c).
4.) HPLC-MS (ESI) stability tests for precursor 4-phenylimidazolium iodide 1

**Figure SI11:** Stability of NHC ligand 1 in DMF; grey line: chromatogram after 0 hour; black line: chromatogram after 96 hours; Peaks: m/z (0.22 min) = 74.1 (DMF), m/z (1.01 min) = 219.1 (cation of ligand 1)

**Figure SI12:** Stability of NHC ligand 1 in DMSO; grey line: chromatogram after 0 hour; black line: chromatogram after 96 hours; Peaks: m/z (0.23 min) = 79.1 (DMSO), m/z (1.00 min) = 219.1 (cation of ligand 1)
Figure SI13: Stability of NHC ligand 1 in water; grey line: chromatogram after 0 hour; black line: chromatogram after 96 hours; Peaks: m/z (0.19 min) = 74.1 (DMF), m/z (1.01 min) = 219.1 (cation of 1)

Figure SI14: Stability of NHC ligand 1 in PBS buffer; grey line: chromatogram after 0 hour; black line: chromatogram after 96 hours; Peaks: m/z (0.20 min) = 74.1 (DMF), m/z (0.53 min) = 219.1 (cation of ligand 1)
Figure SI15: Stability of NHC ligand 1 in DMEM cell culture medium (without supplements); grey line: chromatogram after 0 hour; black line: chromatogram after 96 hours; Peaks: m/z (0.20 min) = 74.1 (DMF) and 203.0 (DMEM matrix), m/z (1.01 min) = 219.1 (cation of ligand 1)

Figure SI16: Stability of NHC ligand 1 in 1-Octanol; grey line: chromatogram after 0 hours; black line: chromatogram after 96 hours; Peaks: m/z (0.31 min) = 74.1 (DMF), m/z (0.36 min) = various masses (octanol impurities), m/z (1.15 min) = 219.1 (cation of ligand 1)
5.) HPLC-MS (ESI) stability tests for biscarbene 3

**Figure SI17:** Stability of bis-NHC complex 3 in n-Octanol at 0 hours; black line: 96 hours; Peaks: m/z (0.20 min) = DMF, m/z (0.25 min) = various masses (octanol impurities); m/z (1.04 min) = 219.1 (cation of ligand 1), m/z (2.41 min) = 633.2 (cation of biscarbene 3)

**Figure SI18:** Stability of bis-NHC complex 3 in DMF; grey line: chromatogram at 0 hours; black line: 96 hours; Peaks: m/z (0.24 min) = 74.1 (DMF) m/z (2.39 min) = 633.2 (cation of biscarbene 3)
Figure SI19: Stability of bis-NHC complex 3 in DMSO: grey line: chromatogram at 0 hours; black line: 96 hours; Peaks: m/z (0.23 min) = 79.1 (DMSO) m/z (2.37 min) = 633.2 (cation of biscarbene 3)

Figure SI20: Stability of bis-NHC complex 3 in water; grey line: chromatogram at 0 hours; black line: 96 hours; Peaks: m/z (0.23 min) = 74.1 (DMF) m/z (2.37 min) = 633.2 (cation of biscarbene 3)
**Figure Si21:** Stability of bis-NHC complex 3 in PBS buffer; grey line: chromatogram at 0 hours; black line: 96 hours; Peaks: m/z (0.22 min) = 74.1 (DMF) m/z (2.32 min) = 633.2 (cation of biscarbene 3)

**Figure Si22:** Stability of bis-NHC complex 3 in DMEM cell culture medium (without any supplements); grey line: chromatogram at 0 hours; black line: after 96 hours; Peaks: m/z (0.23 min) = 74.1 (DMF) and 203.0 (DMEM matrix), m/z (2.46 min) = 633.2 (cation of biscarbene 3)
6.) Protein binding

![Protein binding graph](image)

Figure SI23: Protein binding of 2 [12 µM], 3 [3 µM], Auranofin [3 µM], Et3PAuCl [3 µM] and gold chlorides [12 µM] to fetal calf serum proteins

7.) Permeability studies and cellular uptake in Caco-2 cells

Permeability studies with Caco-2 cells were performed in order to investigate if the gold complexes are able to passage through intestinal cells, which is an important aspect for the absorption and distribution by oral administration. In this assay the drug is placed on the "apical" side of an insert, which is separated from the "basolateral" side by a permeable filter membrane on which the CaCo-2 cells were grown as a cell layer. After 6 hours the gold
binding to BSA [%] binding to FCS proteins [%] content at both sides of the permeable filter membrane can be measured by HRCS-AAS (see experimental setup in Fig. SI25). However, in these experiments no gold could be detected neither in the apical aliquots nor in the basolateral aliquots with 2, 3, Et$_2$PAuCl or Auranofin. On the other hand, concentration- and time-dependent cellular uptake studies with the complexes in Caco-2 cells confirmed the accumulation of gold after exposure to the compounds (see Figs SI26 and SI27).

The detection limit of gold in the HRCS-AAS measurements were 2.0 μM in MEM cell culture medium / distilled water [2:1] as well as the used KRB buffer solution. For comparison, the detection limit of gold in pure water was 0.031 μM and 0.125 μM in MCF-7 cell lysates as used for cellular uptake studies (protein concentration 1.0 mg/mL).

(For determining the detections limits, stock solutions (16 μM) of the respective compounds in either KRB or MEM cell culture medium/distilled water [2:1] were diluted and measured via HRCS-AAS until no gold signals could be detected. The last concentration where a signal could reliably be identified was defined as the detection limit.)

Based on the higher detection limits in the KRB buffer solution, a final conclusion on gold permeability could not be drawn. Further method development and a modified experimental setup is necessary to lower the thresholds of quantification for AAS in the respective medium and buffer-based matrices to investigate the permeability and accumulation behavior.

Figure SI25: Experimental setup of permeability tests in Caco-2 cells
8.) Permeability tests in Caco-2 cells (Experimental)

For permeability studies an established in vitro barrier model derived from human epithelial colorectal adenocarcinoma Caco-2 cells was used, which simulates the absorption of compounds in the gastrointestinal tract from the intestinal lumen into the blood stream. For these studies in a 6-well plate 400,000 cells/well were seated on a filter membrane of an insert and incubated (37 °C / 5 % CO₂) in modified MEM cell culture medium until the transepithelial electrical resistance (TEER) parameter was between 250 and 300 Ω*cm² indicating that an appropriate cell layer barrier was formed (Fig. SI26). The cell culture medium was removed and the cells washed with a mixture of MEM cell culture
medium/distilled water (2:1). The compounds were solved in DMF and diluted with the same mixture to final concentrations of either 3.0 μM (Auranofin / 3) or 6.0 μM (Et₃PAuCl / 2) in non-toxic concentrations (comment: the toxicity of the compounds against Caco-2 cells had been determined by MTT assays, data not shown). A volume of 2.0 mL of the respective dilutions were added on the apical site (on the insert membrane) and 3.0 mL of the medium/water mixtures without compounds were pipetted directly into the wells (basolateral site). The plates were incubated for 6 hours (37 °C / 5 % CO₂) under smooth shaking and aliquots (120 μL) were taken from the apical as well as basolateral side of the cell layer after the desired time. The gold content of the aliquots was determined by HR-CS AAS.

9.) Cellular uptake studies in Caco-2 cells (Experimental)
For each time point or test concentration a 75 cm² flask was prepared with 10 mL of Caco-2 cells (from DSMZ) in MEM (120,000 cells/mL) and cultivated for 72 hours (37 °C / 5 % CO₂). Test compounds were dissolved in DMF and diluted with distilled water. These solutions were diluted 1+9 with KRB (final DMF concentration 0.5% v/v). Cell culture medium was removed, the cells were washed with 5 mL KRB and the solution exchanged by 10 mL of the before mentioned mixture, containing the compounds in the desired concentrations. After incubation the medium was removed, the cells were washed with 5 mL PBS buffer and 10 mL fresh PBS buffer was added. The cells were scratched from the flask bottom; the suspension transferred into new tubes, centrifuged (5 min, 1096 g) and the supernatant was decanted. The obtained cell pellets were stored at –20 °C for further use. For metal and protein quantification the pellets were resuspended in demineralized water (0.5 mL) and lysed 30 min by ultra-sonication. The protein content of lysates was determined by the Bradford method and the metal content was determined by AAS as described in the main text.

10.) Reference