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A Spontaneous Gold(I)-Azide Alkyne Cycloaddition Reaction Yields Gold-Peptide Bioconjugates which Overcome Cisplatin Resistance in a p53-Mutant Cancer Cell Line

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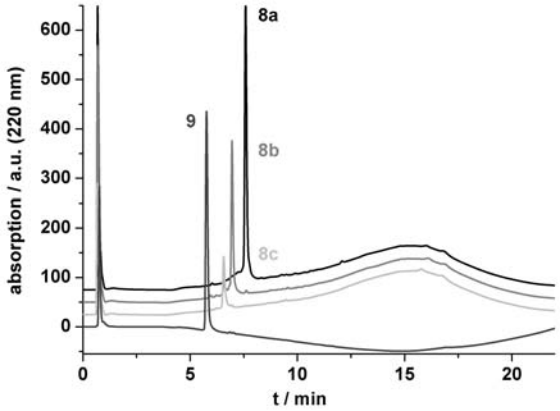

Figure S1. LC traces of purified **9** (dark grey), **8c** (light grey), **8b** (medium grey) and **8a** (black) at λ = 220 nm. RP-HPLC conditions: linear gradient from 0% eluent B to 85% eluent B in 25 min at 5 ml min^{-1}, eluent A: 95% Millipore® water, 5% acetonitrile, 0.1% TFA; eluent B: 95% acetonitrile, 5% Millipore® water, 0.1% TFA).

Compound stability measured by ^{31}P NMR spectroscopy.

Time-dependent stability of **7b** in cell culture medium was tested by mixing 300 µl of a 4 mM DMSO-d^6 stock solution of the gold dipeptide with 300 µl DMEM (Invitrogen, high glucose) containing 10% FCS and 1% PenStrep. The solution was transferred to an NMR tube, incubated at 37 °C in the dark and monitored by $^{31}P\{^1H\}$ NMR spectroscopy after certain time intervals (t = 0, 12, 24 and 72 h). The reactivity of **6b** towards cysteine in aqueous solution was also determined by time-dependent $^{31}P\{^1H\}$ NMR spectroscopy. A 5 mM solution of (Diethlphenylphosphine)gold(I) chloride in DMSO-d^6 served as the internal reference. 100 µl of the reference solution were transferred to a NMR tube, a second NMR tube inlet was inserted, and placed into a schlenk flask flushed with nitrogen. A 2.4 mM DMSO-d^6 stock solution of **6b** and a 6.4 mM aqueous stock solution of cysteine hydrochloride were mixed under inert conditions in a schlenk flask (final concentrations:1.8 mM **6b** and and 1.6 mM cysteine hydrochloride). 150 µl of the solution were transferred into the NMR tube inlet under inert conditions and the tube was incubated at 37 °C in the dark. $^{31}P\{^1H\}$ NMR spectroscopy was performed at certain time intervals (t = 0, 6, 24 and 48 h). Integrated signal intensities of **6b** are referenced to the integral of the resonance of (Diethlphenylphosphine)gold(I) chloride at 37.4 ppm set to 1.

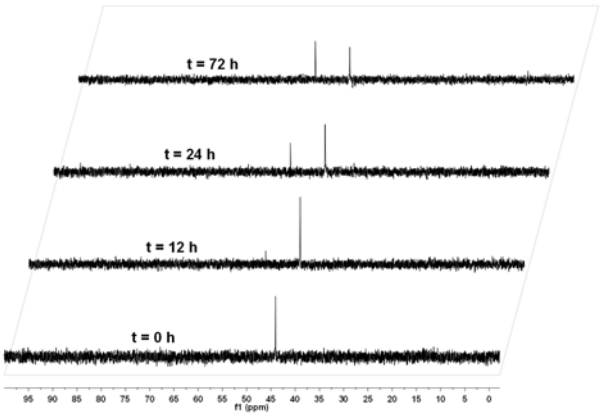

Figure S2. Time-dependent $^{31}P\{^{1}H\}$ NMR spectroscopy of **7b** in a 1:1 (v/v) mixture of DMSO-d^6 and DMEM (containing 10% v/v FCS).

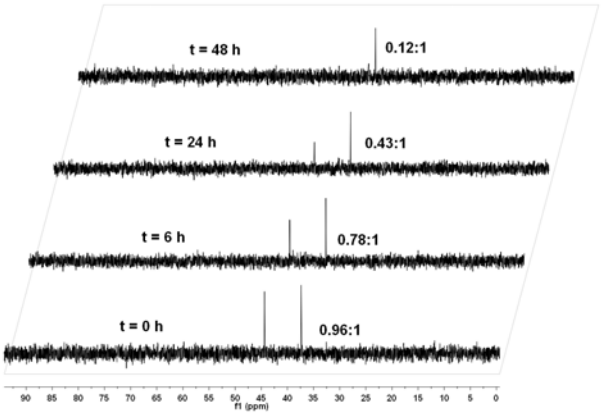

Figure S3. Time-dependent $^{31}P\{^{1}H\}$ NMR spectroscopy of an equimolar mixture of **6b** and cysteine hydrochloride under inert conditions in aqueous solution. (Diethylphenyl-phosphine)gold(I) chloride served as an internal reference ($\delta \sim 37$ ppm).

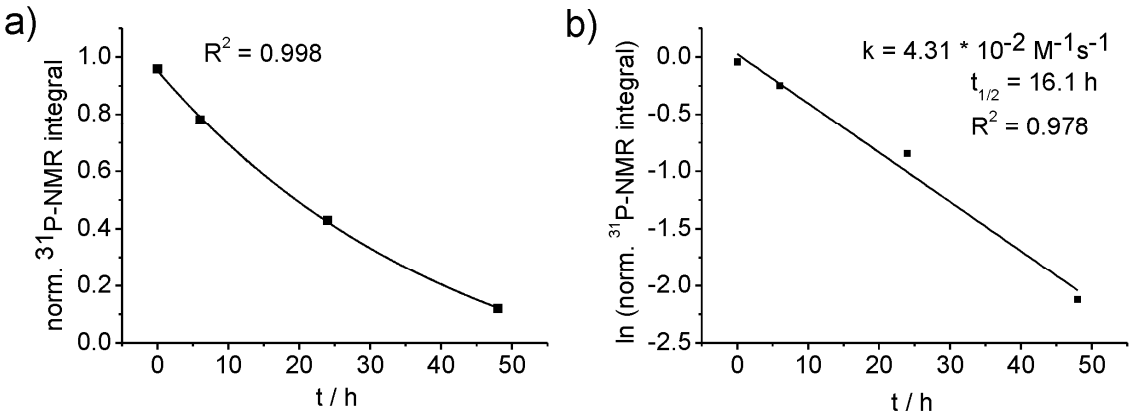

Figure S4. a) Exponential fit curve and b) rate constant plot of the first-order decay of **6b** in the presence of cysteine measured by ^{31}P-NMR.

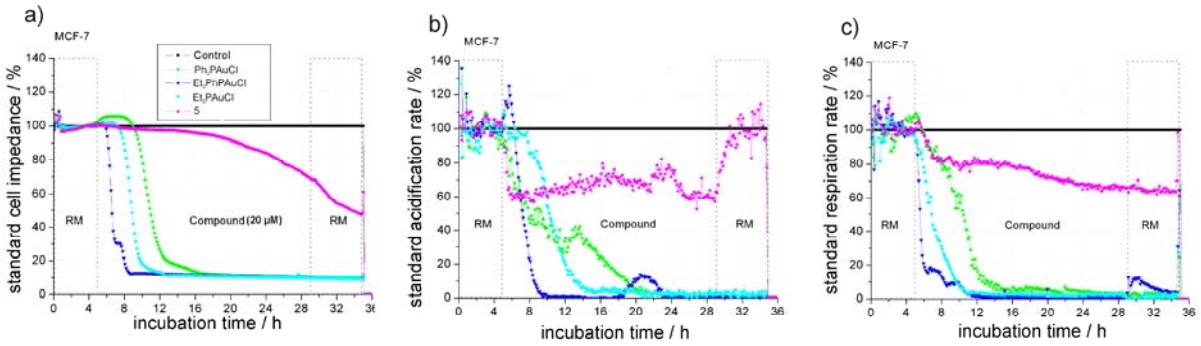

Figure S5. a) Standard cell impedance, **b)** standard acidification rate and **c)** standard respiration rate of MCF-7 cells incubated with each 20 µM Ph_3PAuCl (green line), $Et_2PhPAuCl$ (blue line), Et_3PAuCl (turqois line) and **5** (pink line). "RM" ("running medium") denotes compound free cell culture medium. "Exposure/ 20 µM" indicates the window of incubation with the respective compound and concentration.

DNA fragmentation and mitochondrial inner membrane potential ΔΨm. Measurement of DNA fragmentation and mitochondrial inner membrane potential ΔΨm in BJAB lymphoma cells by flow cytometry was conducted as reported elsewhere.[1]

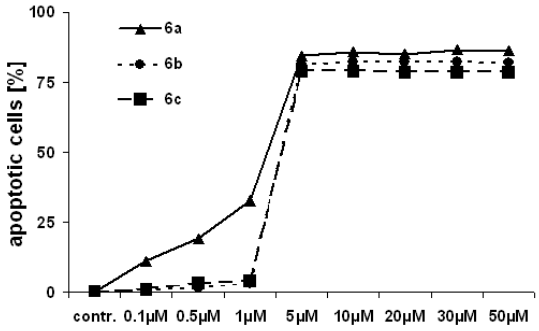

Figure S6. DNA fragmentation in BJAB lymphoma cells after incubation with **6a-c** for 72 h.

Determination of lipophilicity.

The *n*-octanol/water partition coefficients were determined by the shake-flask method as described previously[2] with minor modifications. PBS (1x, $pH_{7.4}$) and *n*-octanol were mixed on a laboratory shaker for > 72 h to allow saturation of the phases. Stock solutions of the gold compounds were freshly prepared in DMSO and diluted with the PBS-phase to a final volume of 2 ml (10-50 µM). 3 x 600 µl were added to 1.5 ml Eppendorf vials, followed by 600 µl of the *n*-octanol phase to each vial. The vials were mixed vigorously on a laboratory vortexer for 30 min., followed by centrifugation of the resulting suspension (5 min, 3000 g) to separate the phases. 3 x 150 µl of each phase and each vial were pipetted into a 96-well plate and absorbance at 260 nm was read on a Tecan safire[2] microplate reader. The partition coefficient, expressed as $logD_{7.4}$, was defined as the decadic logarithm of the ratio of the compound concentration in the *n*-octanol and the PBS phase ($log\ D_{7.4} = log([compound_{(n\text{-}octanol)}]/[compound_{(PBS)}])$.

Cellular gold uptake measurement by Electrothermal Atomic Absorbtion Spectroscopy (ET-AAS). Cellular uptake experiments were performed as previously described.[3] HT-29 cells were grown in 75 cm^2 cell culture flasks (Sarstedt) until subconfluency was reached (< 90%). Stock solutions of the gold compounds were freshly prepared in DMSO (dipeptides) or PBS, pH7.4 (tetrapeptides) and diluted to the final assay concentrations of 20 µM using cell culture medium (final conc. DMSO v/v 0.1%). Cells were incubated with 10 ml cell culture medium containing the gold conjugates at 37 °C/ 5% CO_2 for 2 h. The medium was removed,

the cells were washed with PBS, trypsinised, collected and pelleted by centrifugation (5 min, 2000g, r.t.). Cell pellets were suspended in 1ml ultrapure water and lysed in an ultrasonic bath (1 h). An aliquot was used for protein quantification by the Bradford method.[4] The gold content of the cellular lysates was determined by electrothermal atomic absorbtion spectroscopy (ET-AAS)**.** For the measurements a contrAA 700 graphite furnace atomic absorbtion spectrometer (Analytic Jena) was used and gold was detected at λ = 242.8 nm with a 0.8 nm bandpass and a deuterium lamp as background correction. Drying, pyrolysis, atomisation and tube cleaning steps were employed according to Ott *et al* [3]. To 200 µl of the compound-containing cell lysates, 20 µl of Triton X-100 (1%) are added and 25 µl of this mixture are injected into the graphite furnace tubes. The mean AUC (area under curve) absorbtions of duplicate injections were used for calculations. Determination of the gold content of the lysates was done by the matrix calibration method. Blank cell lysates were adjusted to the protein content of a given compound-containing lysate and increasing concentrations of the respective gold compound were added to the blank lysates and after addition of Triton X-100 (1%), the samples were measured by AAS. Results, given as ng Au/ mg protein, were calculated from two independent experiments.

Cell culture.

HT-29 colon carcinoma and MCF-7 breast adenocarcinoma cell were cultured in DMEM (high glucose, PAA or Gibco) supplemented with 10% (v/v) fetal calf serum (FCS), 1% sodium pyruvate, 100 U/ml Penicillin/Streptavidin. Human skin cell fibroblasts (GM5756) were cultured in DMEM (low glucose, PAA) supplemented with 20% (v/v) FCS, 1% sodium pyruvate, 100 U/ml Penicillin/Streptavidin and were only used from passage 12-16. The cells were maintained at 37 °C/ 5% CO_2 in a humidified incubator.

Proliferation assays on HT-29 and MCF-7 cancer cells and normal human skin cell fibroblasts GM5756.

Cell growth inhibition of cancer cells after 72h (HT-29) and 96 h (MCF-7) and fibroblasts after 72 h of incubation with the gold peptide conjugates was performed following an established procedure.[5] Fresh stock solutions of the compounds were prepared in DMF, DMSO or ultrapure water and diluted to the final assay concentrations using cell culture medium (0.1% v/v DMF; 1% v/v DMSO). IC_{50} values were calculated from the average (± SE) of at least two independent experiments and represent the concentration causing 50% cell growth inhibition compared to an untreated control.

Annexin V/ PI assay.

Jurkat cells were incubated with the gold conjugates at the indicated concentrations for 48 h, collected and stained with Annexin V-FITC (eBioscience). Some 5 x 10^5 cells were resuspended in 50 µl of Annexin V staining buffer (10 mM Hepes, 140 mM NaCl and 2.5 mM CaCl (ph 7.4)). 2.5 µl of Annexin V conjugate and 1.25 ml of PI solution (1 mg/ml) were added and the samples were incubated at r.t. for 15 min. under the exclusion of light. Samples were analysed with a FACSCalibur (Becton Dickinson) and CellQuest Pro (BD) software (FL1 channel: Exc. 488 nm, Em. 515-545 nm for Annexin V-FITC; FL2 channel: Exc. 488 nm, Em. 564-606 nm for PI).

DNA interactions.

Stock solutions of the gold dipeptides **6a-c** in DMSO were diluted with 10 mM Tris-EDTA buffer (pH 7.4) to a concentration of 125 µM (2.5% v/v DMSO). A 125 µM stock solution of cisplatin (Sigma-Aldrich) was prepared by directly dissolving the complex in 10 mM Tris-EDTA buffer. The 125 µM stock solutions were mixed with 100 bp quick load DNA ladder (New England Biolabs) and 10 mM Tris-EDTA buffer to a final compound concentration of 25 µM. The mixture was incubated at 37 °C for 4 h in the dark. Gel electrophoresis was carried out in Tris-acetate/EDTA buffer on a 1% (w/v) agarose gel (Gold Universal agarose, peqlab) at 100 V for 2 h in a PerfectBlue Gelsystem Mini M (peqlab) electrophoresis chamber. The gel was stained for 20 min in an aqueous ethidium bromide solution (50 µg/ml) and the DNA bands were visualised and documented with a CN3000 Biovision gel scanner (peqlab) equipped with VisionCapture Software (Vers. 14.2).

Cell metabolism and morphology.

A Bionas 2500 sensor chip system (Bionas, Rostock, Germany), comprising metabolic sensor chips (SC 1000) with ion-sensitive field-effect transistors to monitor pH changes, oxygen sensitive electrodes to control oxygen consumption and interdigitated electrode structures for impedance measurement of the cell layer, was employed to detect changes in cellular metabolism and morphology.[6] 10^5 cells in DMEM (PAA, E15-883) with penicillin/streptomycin and 10% (v/v) FCS (PAA) were seeded onto the sensor chip and incubated at 37 °C/ 5% CO_2 until 90% confluency. The sensor chip was transferred to the Bionas 2500 analyser in which the medium is exchanged continuously in 8 minute cycles (4 min medium exchange and 4 min without flow), during which the respective parameters were

measured. DMEM without carbonate but with 1 mM Hepes buffer and reduced FCS (0.1%) was used as "running medium" (RM) during the analysis. Drugs were tested with the following scheme: 1) Equilibration for 5 h with RM; 2) incubation with substances at indicated concentrations and times; 3) Regeneration of the cells with substance-free RM; 4) Addition of 0.2% Triton X-100 to generate a basic signal without living cells on the sensor surface as negative control.

Effects on respiration of isolated mouse liver mitochondria.

Mitochondrial oxygen consumption was measured with OxoPlate (PreSens, Germany) 96-well plates. A two-point calibration with oxygen-free (1% Na_2SO_3) and air-saturated water with oxygen partial pressures corresponding to 0% and 100% was performed. Fluorescence was read at λ_{em} = 650 nm (λ_{ex} = 540 nm, λ_{em}(ref) = 590 nm). Signal ratio 650/590 nm equals oxygen partial pressure. 18 µg freshly isolated mitochondria[7] were suspended in respiration buffer (25 mM sucrose, 100mM KCl, 75 mM mannitol, 5 mM MgCl2, 10 mM KH_2PO_4, 0.5 mM EDTA, 10 mM TRIS, 0.1% fatty acid-free BSA, pH 7.4) containing 10 mM pyruvate, 2 mM malate, 2 mM ADP, 0.5 mM ATP and test compounds. Continuous measurement for 400 min at intervals of 5 min was done on a Tecan Safire[2] microplate reader (Tecan, Maenneddorf, Switzerland) at 37 °C, with plates sealed by a breathable membrane (Diversified Biotech, Boston, MA, USA). 5 µM rotenone and 1 µM CCCP (both Sigma-Aldrich) were used as controls.

Effects on enzymatic activity of TrxR and GR.

The TrxR and GR inhibition assay was carried out using a modified microplate reader based assay. Rat liver TrxR and baker's yeast GR were aquired from Sigma-Aldrich. 2.0 U/ml concentrations were prepared with distilled water. To each 25 µl aliquots of the enzyme solution, 25 µl of potassium phosphate buffer (pH 7.0) containing the compound in graded concentrations, or DMF without compound (vehicle control), were added and the resulting solutions (final DMF concentration: 0.5% v/v) were incubated with moderate shaking at 37 °C in a 96-well plate for 75 min. To each well, 225 µl of the reaction mixture (1000 µl reaction mixtures consisted of 500 µl potassium phosphate buffer pH 7.0, 80 µl of a 100 mM EDTA solution pH 7.5, 20 µl of a 0.05% BSA solution, 100 µl of a 20 mM NADPH solution and 300 µl distilled water) were added and the reaction was started by the addition of 25 µl of an 20 mM ethanolic DTNB solution. After proper mixing, the formation of 5-TNB was monitored with a microplate reader (Perkin-Elmer Victor X4) at 405 nm in 10 s intervals for 6

min. The increase of 5-TNB concentration over time followed a linear trend ($R^2 \geq 0.99$) and the enzymatic activities were calculated as the slopes (increase of absorbance per second) thereof. For each tested compound, the non-interference with the assay components was confirmed by a negative control experiment using an enzyme-free solution. The EC_{50} values were calculated aas the concentrations of compound decreasing the enzymatic activity of the untreated control by 50% and are given as the means (± SE) of at least two independent experiments.[7]

Formation of ROS.

Jurkat cells were incubated with the gold conjugates at the concentrations indicated for 24 and 48 h. Following incubation, the cells were collected, centrifuged at 0.2 g, resuspended in FACS buffer (DPBS (Gibco) + 1% BSA (PAA)). The cell suspensions were treated with DHE (dihydroethidium (Sigma Aldrich), 5 µl of a 5 mM stock solution per 1 ml of cell suspension containing 10^6 cells) at room temperature in the dark for 15 min., washed once with with FACS buffer and were immediately analysed with a FACSCalibur cell counter (Becton Dickinson) and CellQuest Pro (BD) analysis software. Excitation and emission filters were set to 488 nm and 564 - 606 nm (FL2 filter), respectively. Of note: even though DHE is known to interact with the superoxife anion only, the intensity of fluorescence is commonly considered to reflect total intracellular ROS.[7]

References.

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