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RSC Advances

COMMUNICATION

Supplementary information

Toward anticancer gold-based compounds targeting PARP-1: a new case study

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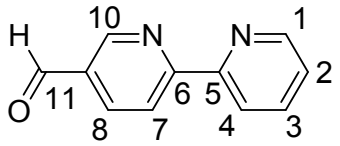
Experimental Section

Synthesis

2,2'-bipyridine-5-carbaldehyde

5-Bromo-2,2'-bipyridine (5.0 g, 21.3 mmol) was dissolved in DMF (29.0 ml). Na_2CO_3 (2.24 g, 21.2 mmol), triethylsilane (7.0 ml, 43.8 mmol) and 1,3-bis(diphenylphosphino)propane (263 mg, 638 μmol) were added. After purging the solution with nitrogen for 10 min, tris(dibenzylideneacetone)dipalladium(0) (390 mg, 426 μmol) was added, the autoclave charged with CO (30 bar), and the reaction stirred at 90 °C for 19 h. After cooling to room temperature and releasing the CO, the black reaction mixture was suspended in water (40 ml) and extracted with Et_2O (4 x 50 ml). The organic phase was concentrated and subjected to silica gel chromatography (*n*-hexane / ethyl acetate 9:1, R_f = 0.31) to provide 2,2'-byridine-5-carbaldehyde (376 g, 20.4 mmol, 96%) as a white solid.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 10.16 (s, 1H, H11), 9.11 (dd, $^4J_{H10,H8}$ = 2.1 Hz, $^5J_{H10,H7}$ = 0.7 Hz, 1H, H10), 8.72 (ddd, $^3J_{H1,H2}$ = 4.8 Hz, $^4J_{H1,H3}$ = 1.7 Hz, $^5J_{H1,H4}$ = 0.9 Hz, 1H, H1), 8.60 (d, $^3J_{H7,H8}$ = 8.2 Hz, 1H, H7), 8.55−8.47 (m, 1H, H4), 8.27 (dd, $^3J_{H8,H7}$ = 8.2 Hz, $^4J_{H8,H10}$ = 2.2 Hz, 1H, H8), 7.93−7.81 (m, 1H, H3), 7.38 (ddd, $^3J_{H2,H3}$ = 7.5 Hz, $^3J_{H2,H1}$ = 4.8 Hz, $^4J_{H2,H4}$ = 1.2 Hz, 1H, H2). ^{13}C-NMR (75 MHz, $CDCl^3$) δ = 190.7 (C11), 160.8 (C6), 154.9 (C5), 151.8 (C10), 149.6 (C1), 137.3 (C3), 137.0 (C8), 131.2 (C9), 124.9 (C2), 122.4 (C4), 121.4 (C7). FT-IR (Film) = 1697 (CO), 1584, 1553, 1448, 1360, 1249, 1203, 1142, 1084, 1034, 997, 841, 790, 738, 698, 626, 396. HR-MS ESI (+) m/z = 207.0531 (207.0529 calculated for $C_{11}H_8N_2ONa$, $[M + Na]^+$).



2-((2,2'-bipyridin)-5-yl)-1H-benzimidazol-4-carboxamide

2,2'-bipyridine-5-carbaldehyde (800 mg, 4.34 mmol) and 2,3-diaminobenzamide (722 mg, 4.78 mmol) and Ce(NH4)2(NO396 (360 mg, 0.66 mmol) were carfully mixed. H_2O_2 (30% aqueous solution, 4.0 ml, 39.2 mmol) was added. **Caution:** the reaction is strongly exothermic and proceeds under gas evolution for several minutes. After cooling to room temperature, the brown reaction mixture was suspended in water (100 ml), the water was removed and the solid again washed with water (100 ml). Afterwards, the solid was washed with acetone (50 ml) and the ligand **1** obtained as a brown solid (1.17 g, 3.81 mmol, 88%).

^{1}H-NMR (300 MHz, DMSO-d_6) δ = 13.68 (s, 1H, H1), 9.52 (s, 1H, H19), 9.30 (s, 1H, H8), 8.74 (d, *J* = 5.3 Hz, 2H, H18/H11), 8.59 (d, $^3J_{H12,H11}$ = 8.3 Hz, 1H, H12), 8.48 (d, $^3J_{H15,H16}$ = 7.9 Hz, 1H, H15), 8.09–7.96 (m, 1H, H16), 7.92 (d, $^3J_{H3,H4}$ = 7.5 Hz, 1H, H3), 7.88–7.67 (m, 2H, H5/H1), 7.51 (dd, $^3J_{H17,H16}$ = 6.8 Hz, $^3J_{H17,H18}$ = 5.0 Hz, 1H, H17), 7.40 (t, J = 7.8 Hz, 1H, H4). ^{13}C-NMR (75 MHz, DMSO-d_6) δ = 166.0 (C1), 156.4 (C13 oder 14), 154.4 (C14 oder 13), 149.5 (C18), 149.3 (C9), 147.6 (C19), 141.4 (C6), 137.5 (C16), 135.4 (C11), 135.3 (C10), 125.3 (C7), 124.7 (C17), 123.3 (C4), 122.9 (C3), 122.7 (C2), 120.9 (C15), 120.6 (C12), 115.2 (C5). FT-IR (Film) = 3184, 1662, 1597, 1503, 1458, 1412, 1352, 1316, 1250, 745, 585, 558. HR-MS ESI (+) m/z = 338.1011 (338.1012 calculated for $C_{18}H_{13}N_5ONa$, $[M + Na]^+$). Elemental Analysis, Calculated: C, 68.56; H, 4.16; N, 22.21; Experimental: C, 68.53; H. 4.14; N, 22.24.

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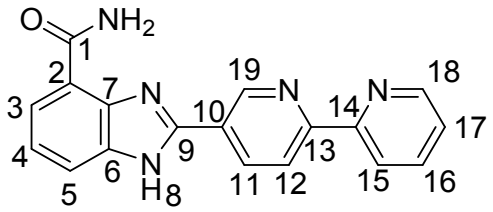

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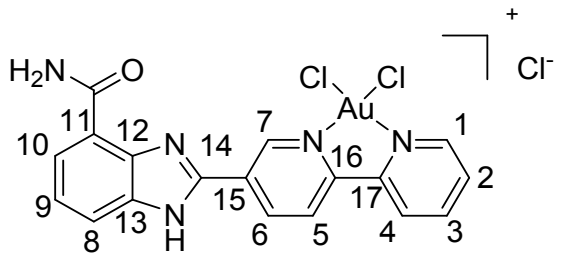
[{2-((2,2'-bipyridin)-5-yl)-1H-benzimidazol-4-carboxamide} $AuCl_2$]Cl [$LAuCl_2$]Cl

A round-bottom flask equipped with a condenser, was charged with 2-((2,2'-bipyridin)-5-yl)-1H-benzimidazol-4-carboxamide (50 mg, 0.16 mmol) in suspension in ethanol (0.5 mL). $HAuCl_4.H_2O$ (1 eq, 54 mg, 0.16 mmol) dissolved in ethanol (0.5 mL) was added to the suspension of the ligand. The reaction mixture was refluxed overnight during which time a brown precipitate was formed. After cooling down, the precipitate was collected by filtration and washed twice with diethylether. The product was obtained as a brown powder (67 mg, 68 % yield). ^{1}H NMR (DMSO-d6, 500.13 MHz): 7.44 (broad s, 1 H, H^9), 7.66 (broad s, 1 H, H^2), 7.80 (broad s, 1 H, NH_2), 7.85 (d, J_{H-H} = 6.0 Hz, 1 H, H^8), 7.93 (d, J_{H-H} = 6.0 Hz, 1 H, H^{10}), 8.18 (broad s, 1 H, H^3), 8.58 (d, J_{H-H} = 6.0 Hz, 1 H, H^4), 8.63 (d, J_{H-H} = 6.0 Hz, 1 H, H^5), 8.80 (broad s, 1 H, H^1), 8.82 (broad s, 1 H, H^5), 9.07 (broad s, 1 H, NH_2), 9.57 (broad s, 1 H, H^7). $^{13}C\{^1H\}$ NMR (DMSO-d6, 125.76 MHz): 116.7 (s, CH^8), 121.7 (s, CH^5), 122.4 (s, CH^4), 122.7 (s, C^{11}), 123.8 (s, $CH^{9/10}$), 124.1 (s, $CH^{9/10}$), 125.6 (s, C^{12}), 125.9 (s, CH^2), 136.3 (s, C^{15}), 136.9 (s, CH^3, CH^6), 140.2 (s, C^{13}), 148.5 (s, CH^7), 148.6 (s, C^{14}), 149.5 (s, CH^1), 153.1 (s, $C^{16/17}$), 155.0 (s, $C^{16/17}$), 166.7 (s, C=O). (DMSO/MeOH), positive mode exact mass for $[C_{18}H_{13}N_5OAuCl_2]^+$ (582.01572): measured m/z 582.01794 $[M-Cl]^+$. Elemental Analysis, Calculated: C, 34.95; H, 2.12; N, 11.32; Experimental: C, 34.90; H, 2.10; N, 11.36.



UV-Visible Absorption Spectroscopy

The absorption spectra of the complex **2** in the UV-Visible region were recorded on a Cary 5000 UV-Visible NIR spectrophotometer. The hydrolysis experiments were carried out with a solution of compound **2** 10^{-4} M (from a 10 mM stock solution in DMSO) in PBS buffer (pH 7.4) at room temperature by monitoring the electronic spectra of sample over 24 hours. In another experiment, 2 equivalents of GSH (from a 100 mM stock solution in milliQ water) were added to the same solution (ca. 10^{-4} M complex **2**) in PBS buffer (pH 7.4), and the sample was monitored over 24 h at room temperature.

Cell lines

The human lung cancer A549 and human ovarian cancer cell lines SKOV3, A2780 and A2780cisR (resistant to cisplatin) (obtained from the European Centre of Cell Cultures ECACC, Salisbury, UK) were

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cultured respectively in DMEM (Dulbecco's Modified Eagle Medium) or RPMI containing GlutaMaxI supplemented with 10% FBS and 1% penicillin/streptomycin (all from Invitrogen), at 37º C in a humidified atmosphere of 95% of air and 5% of CO_2 (Heraeus, Germany).

Cell growth inhibition studies

Cell viability was evaluated by using a colorimetric method based on the tetrazolium salt MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is reduced by viable cells to yield purple formazan crystals. Cells were seeded in 96-well plates at a density of 7-10x10^3 cells per well (200 µl). After overnight attachment, the medium was replaced by 200 µl of a dilution series of the compounds in the medium, and cells were incubated for further 72 h. Stock solutions of the complexes were prepared in DMSO, made exception for cisplatin which was dissolved in aqueous solution, and Auranofin in EtOH. The percentage of DMSO or ethanol in the culture medium did not exceed 0.2%. At the end of the 72 h incubation period the media was removed and cells were incubated with MTT (0.5 mg/mL in culture medium; 200 µl) for 3-4 h at 37 ºC and 5% CO_2. The purple formazan crystals formed inside the cells were then dissolved in 200 µl of DMSO, and the absorbance was read at 570 nm, using a plate spectrophotometer (Power Wave Xs; Bio-Tek). Each test was performed with at least six replicates and repeated at least 4 times. The IC_{50} value is expressed as percentage of the surviving cells in relation to the control (cells with regular medium).

Additive and Synergistic Cytotoxicity Analysis

The combination index method of Chou and Talaly was used to determine whether the observed interactions between cisplatin and complex 2 were additive or synergistic [Chou, T. C.; Talalay, P. Adv. Enzyme Regulation 1984, 22, 27–55]. If the interaction was additive, the sum of the effects of the two drugs should be equal to the product of their fractional activities. The representative function defined as the expected cell survival rate corresponds to f(u)1,2 = f(u)1 3 f(u)2, where f(u)1 = the fraction unaffected by drug 1, f(u)2 = the fraction unaffected by drug 2, and f(u)1,2 = the fraction unaffected by drugs 1 and 2. The expected and observed cell survival rates obtained from a minimum of six replicates and of at least three repetitions were analyzed by the Student's t test (p); $p < 0.05$ was viewed as significant.

Preparation of cell extracts for PARP-1 activity assays

SKOV3 cells were grown in DMEM GlutaMaxI with 10% FBS (or 3% and 1% when indicated) and incubated with different doses of the compounds. After 48 h, cells were scraped in ice-cold PBS and centrifuged at 10000 g for 10 sec at 4° C. The pellet was re-suspended in 5-10 volumes of lysis buffer (PARP Buffer, Trevigen, Gaithersburg, MD, U.S.A.)) containing protease inhibitor cocktail (Roche, Basel, Switzerland), 0.4 M NaCl, and 1% Triton X-100). After 15 min on ice, lysates were centrifuged at 14000 g for 10 min at 4° C to pellet the cellular debris and the supernatants removed for further use. The total protein content was determined by using the DC Protein Assay Kit (Biorad, Hercules, CA, U.S.A.). Alternatively, cell extracts were incubated with the compounds (different concentrations between 1-40 µM) for 24 hours at room temperature followed by determination of PARP-1 activity as described below.

PARP-1 activity determinations

PARP-1 activity was determined using Trevigen's HT Universal Colorimetric PARP Assay. This assay measures the incorporation of biotinylated poly(ADP-ribose) onto histone proteins in a 96 microtiter strip well format. Either recombinant human PARP-1 (High Specific Activity, purified from *E.coli* containing recombinant plasmid harboring the human PARP gene, supplied with the assay kit) or an aliquot of protein cell extracts (50 µg) was used as the enzyme source. 3-Aminobenzamide (3-AB), provided in the kit, was used as control inhibitor. Purified PARP-1 was incubated with various concentration of

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compounds for 1 h at room temperature prior the assay, while cell extracts were either obtained from cancer cells pre-treated with the compounds (48 h), or directly treated with different amounts of gold complexes (24 h) as described above. Two controls were always performed in parallel: a positive activity control for PARP-1 without inhibitors, that provided the 100% activity reference point, and a negative control, without PARP-1 to determine background absorbance. The final reaction mixture (50 μL) was treated with TACS-Sapphire™, a horseradish peroxidase colorimetric substrate, and incubated in the dark for 30 min. Absorbance was read at 630 nm after 30 min. The data corresponds to means of at least three experiments performed in triplicate ± SD.

ESI-MS experiments

The PARP-1 model peptide (GRASCKKCSESIPKDSLRMAIMVQSPMFDGKVPHWYHFSCFWKV) was purchased from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). The apo-zinc-finger peptide was dissolved in milliQ water to a stock solution of 1 mM. The disulphide bonds were reduced using 3 molar equivalents of dithiothreitol (DTT) for 2 hours at room temperature. The complex **2** stock solution (10 mM) was prepared in DMSO and stored at -20°C. A solution of 150 μM in milliQ water was then freshly prepared. Zn^{2+}-reconstituted peptide-**2** adducts were prepared by diluting 10 μL of the peptide stock solution (1 mM in water) with 200 μL of complex **2** (150 μM) in 1.790 mL of milliQ water. This allowed reaching of a gold compound:peptide ratio of 3:1 in each sample (5 μM peptide + 15 μM **2** complex). After 10 minutes incubation, samples were analysed using a Waters Synapt G2-Si TOF mass spectrometer. The samples were infused directly into the MS at 5 μL/min in ES+ve mode. The source was set up at 3.2 kV with a nitrogen gas flux at a pressure of 6.5 bar (1000 L/h). Data analysis and isotope modelling were performed using the Mass Lynx software provided by Waters at a resolution setting of 31.000.

ICP-MS studies

For the evaluation of the cell uptake, cells were seeded in 6-well plates and grown to approximately 70% confluency and incubated with compound **2** at 70 μM for 24 h. At the end of the incubation period, cells were rinsed cells with 5 mL of PBS, detached by adding 0.4 mL enzyme free cell dissociation solution (Millipore) and collected by centrifugation. Cellular extracts were prepared according to established procedures.[C. Bresson et al, *Metallomics* 2013, 5, 133-143] All samples were analysed for their protein content (to establish the number of cells per sample) prior to ICP-MS determination using a BCA assay (Sigma Aldrich). Samples were digested in ICP-MS grade concentrated hydrochloric acid (Sigma Aldrich) for 3 h at room temperature and filled to a total volume of 8 ml with ultrapure water. Indium was added as an internal standard at a concentration of 0.5 ppb. Determinations of total metal contents were achieved on an Elan DRC II ICP-MS instrument (Perkin Elmer, Waltham, M, U.S.A.). The ICP-MS instrument was tuned daily using a solution provided by the manufacturer containing 1 ppb each of Mg, In, Ce, Ba, Pb and U. External standards were prepared gravimetrically in an identical matrix to the samples (with regard to internal standard and hydrochloric acid) with single element standards obtained from CPI International (Amsterdam, The Netherlands). The results are expressed as mean ± SE of at least three determinations.

Thioredoxin reductase and glutathione reductase inhibition studies in vitro

Cytosolic thioredoxin reductase (TrxR1) was prepared from rat liver according to Luthman and Holmgren.[1] The protein content of isolated enzyme was estimated according to Lowry *et al.*.[2] Thioredoxin reductase activity was measured at 25 °C in 0.2 M Na, K-phosphate buffer (pH 7.4) with 5 mM EDTA and 0.25 mM NADPH in presence of **1** and **2**. Reaction was started by the addition of 1 mM DTNB (DNTB = 5,5'-dithiobis- (2-nitrobenzoic acid Ellman's reagent) and followed spectrophotometrically at 412 nm. Yeast Glutathione reductase activity was measured in 0.2 M Tris-HCl

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buffer (pH 8.1), 1 mM EDTA, 0.25 mM NADPH in presence of **1** and **2**. The assay was initiated by the addition of 1mM GSSG and followed spectrophotometrically at 340 nm.

Thioredoxin reductases and glutathione reductase assays in SKOV3 cell lysates

SKOV3 cells (6×10^5) were incubated with **1** and **2** for 48 h with refresh after 24 h. After incubation cells were trypsinized and washed with PBS buffer. Each sample was lysed with a modified RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% TRITON, 0.1% SDS, 0.5% DOC, 1 mM NaF, 0.1 mM PMSF and an antiprotease cocktail ("Complete" Roche, Mannheim, Germany). After 40 min of incubation at 0 °C, lysates were centrifuged at 14000 g for 5 min. The supernatants were tested for enzyme activities. Aliquots of lysates (50 µg) were subjected to thioredoxin reductase determination in a final volume of 250 µl of 0.2 M Na, K-phosphate buffer (pH 7.4) with 5 mM EDTA, containing 2 mM DTNB. After 2 min the reaction was started with 0.300 mM NADPH. Glutathione reductase activity (80 µg of cell lysates) was measured in 0.2 M Tris-HCl buffer (pH 8.1), 1 mM EDTA, and 0.25 mM NADPH. The assay was initiated by addition of 1 mM GSSG and followed spectrophotometrically at 340 nm as described above.

Glutathione redox state estimation in SKOV3 cell lysates

SKOV3 cells (5×10^5) in complete medium were incubated for 18 h in presence of **1** and **2**. Cells were trypsinized and washed twice with cold PBS and then lysed and deproteinized with 6% meta-phosphoric acid. After 10 minutes at 4° C, samples were centrifuged and supernatants were neutralized with 15% Na_3PO_4 and assayed for total glutathione.[3] Sample aliquots were derivatized with 2-vinylpyridine in order to block reduced glutathione, and oxidized glutathione was then estimated.[4] Protein concentration was determined by the Lowry *et al.* assay in deproteinized samples washed with 1 ml of ice-cold acetone, centrifuged at 11000 g, dried and then dissolved in 62.5 mM Tris-HCl buffer (pH 8.1) containing 1% SDS.

Determination of mitochondrial membrane potential in cancer cells

Mitochondrial membrane potential of SKOV3 cells was analyzed using flow cytometry. 5×10^5 SKOV3 cells in complete medium were incubated for 18 h with different concentrations of compounds **1** and **2**. The changes of the membrane potential induced by the compounds were estimated with a FACSCanto™ II (Becton Dickinson) flow cytometer with an argon laser at 585 nm, using tetramethylrhodamine (TMRM) as a fluorescent dye.

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Figure S1. Hydrolysis profiles of the gold(III) complex **2** dissolved in PBS, pH 7.4, over time at room temperature. Concentration of the complex is 9 x 10^{-5} M.

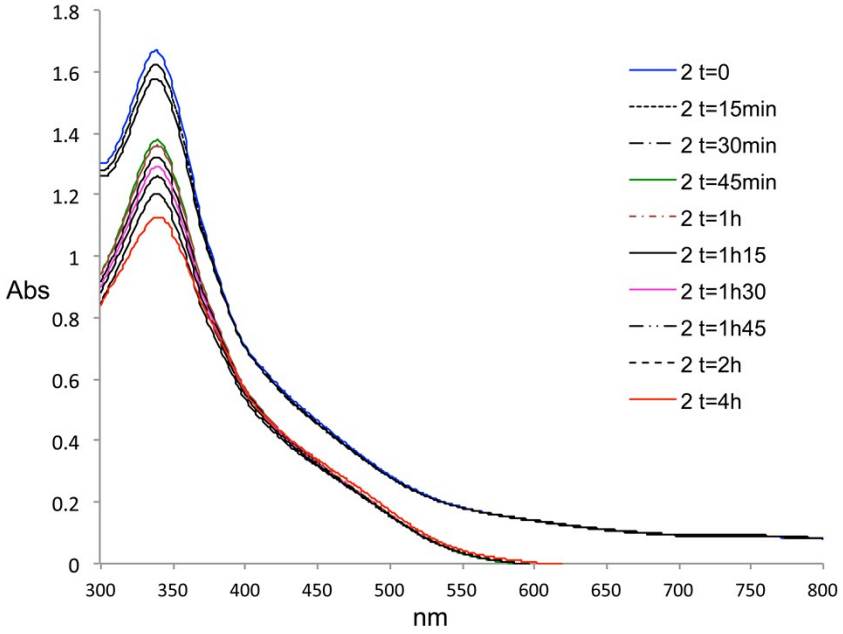

Figure S2. Interaction of the gold(III) complex **2** dissolved in PBS, pH 7.4, with GSH 1:2 over time at room temperature. Concentration of the complex is 1 x 10^{-4} M.

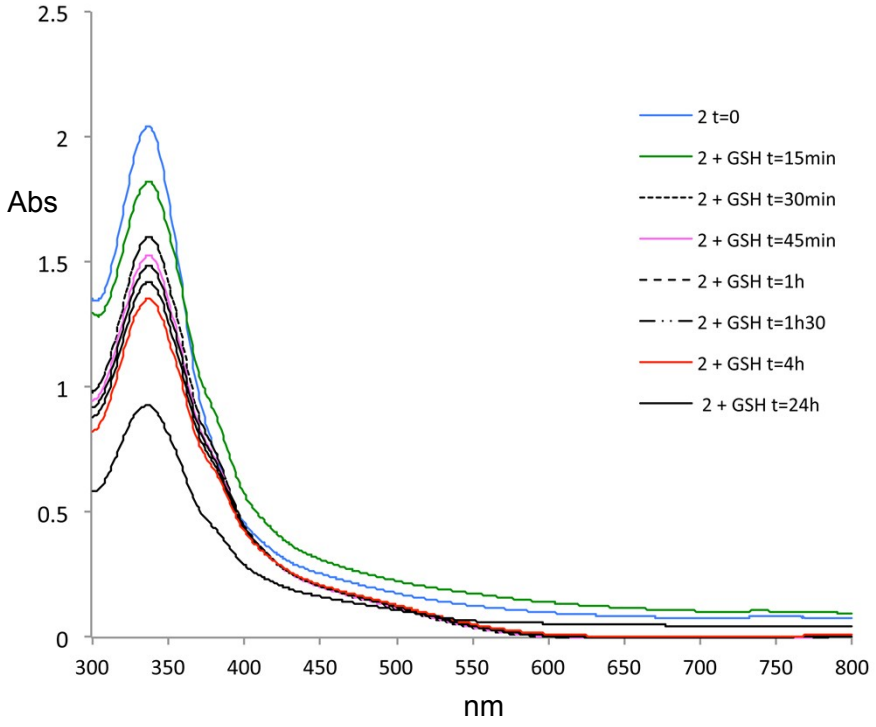

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Figure S3. ESI Orbitrap mass spectrum of the ZF–**2** adduct recorded after 5 min incubation with Zn^{2+} followed by 10 min incubation with **2**.

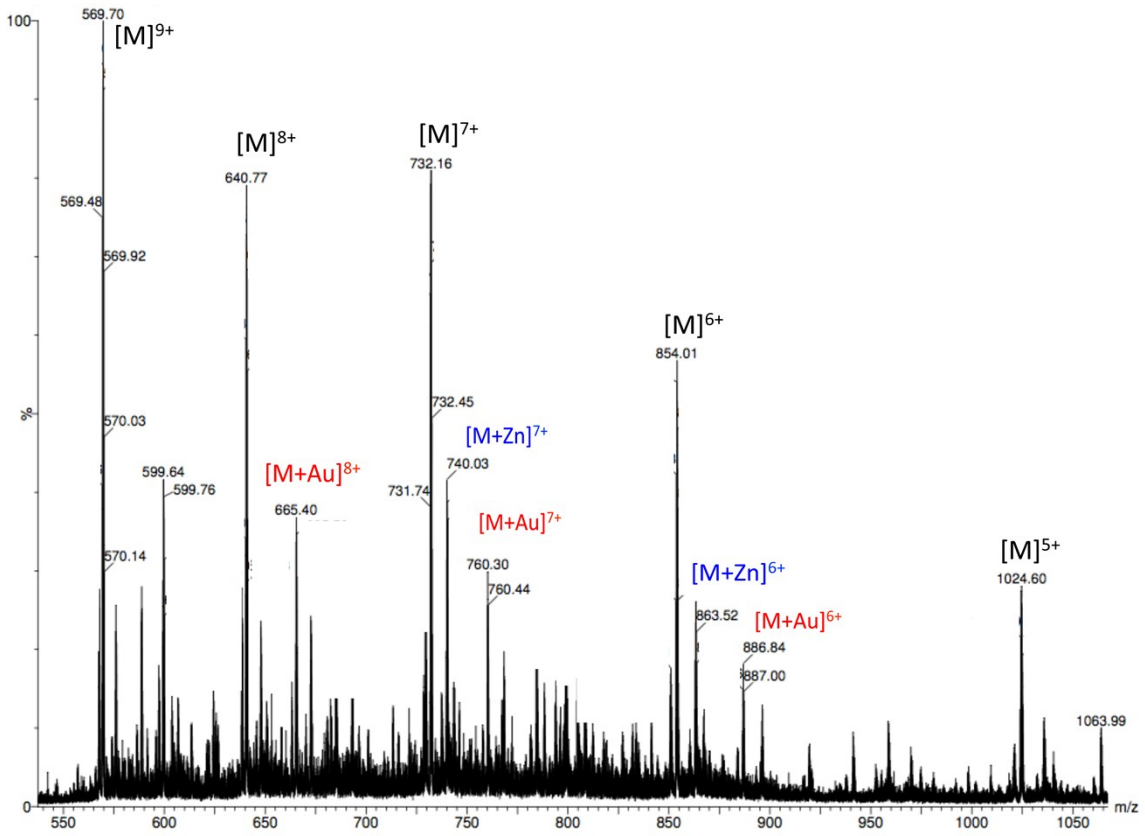

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Figure S4. A: Thioredoxin reductase inhibition by compounds **1, 2** and auranofin (AF). Aliquots of highly purified TrxR1 (60 nM) were incubated in the presence of increasing concentrations of compounds **1, 2** and AF (used as benchmark) and the reaction was followed at 412 nm, as indicated under experimental methods.
B: GR (15 nM) was tested in presence of increasing concentrations of **1**, **2** and AF. NADPH oxidation was followed at 340 nm.

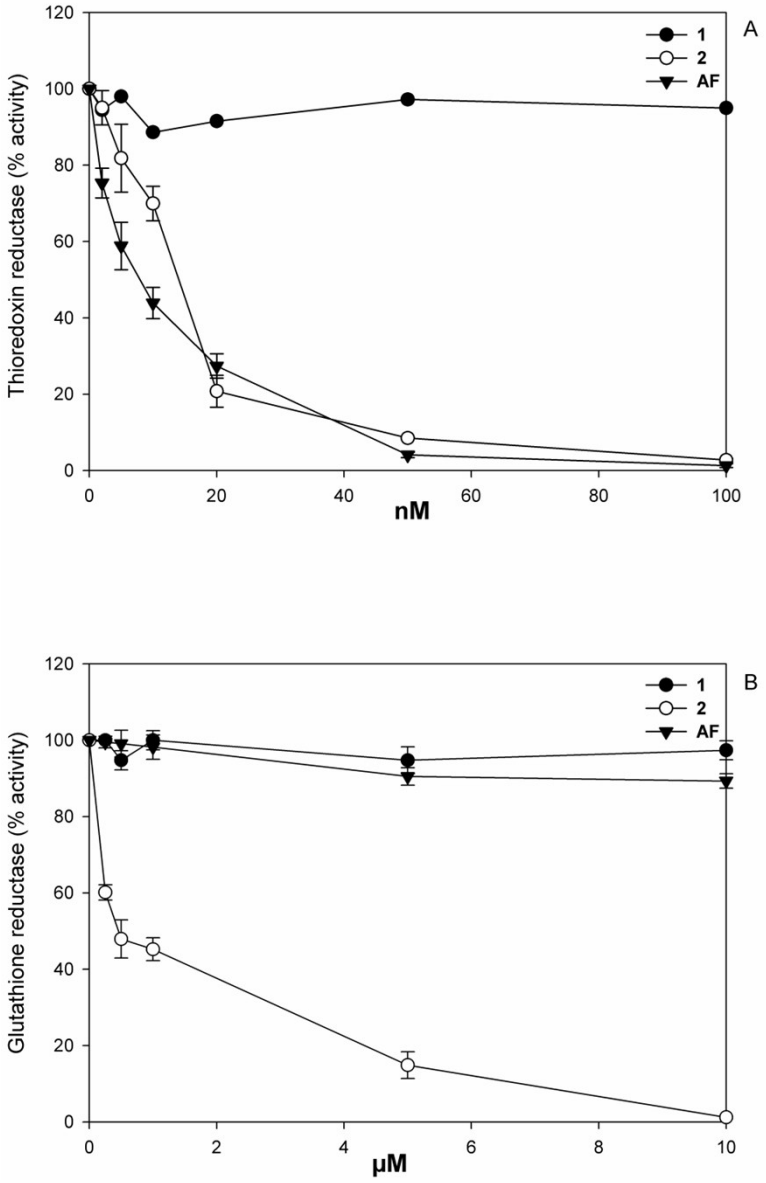

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Figure S5. Thioredoxin reductase (A) and glutathione reductase (B) activities in A2780 cell lysates after the treatment with **1** and **2**. 6 x10^5 cells were treated for 48 h with 25 μM **1** and **2**, with a refresh after 24 h.

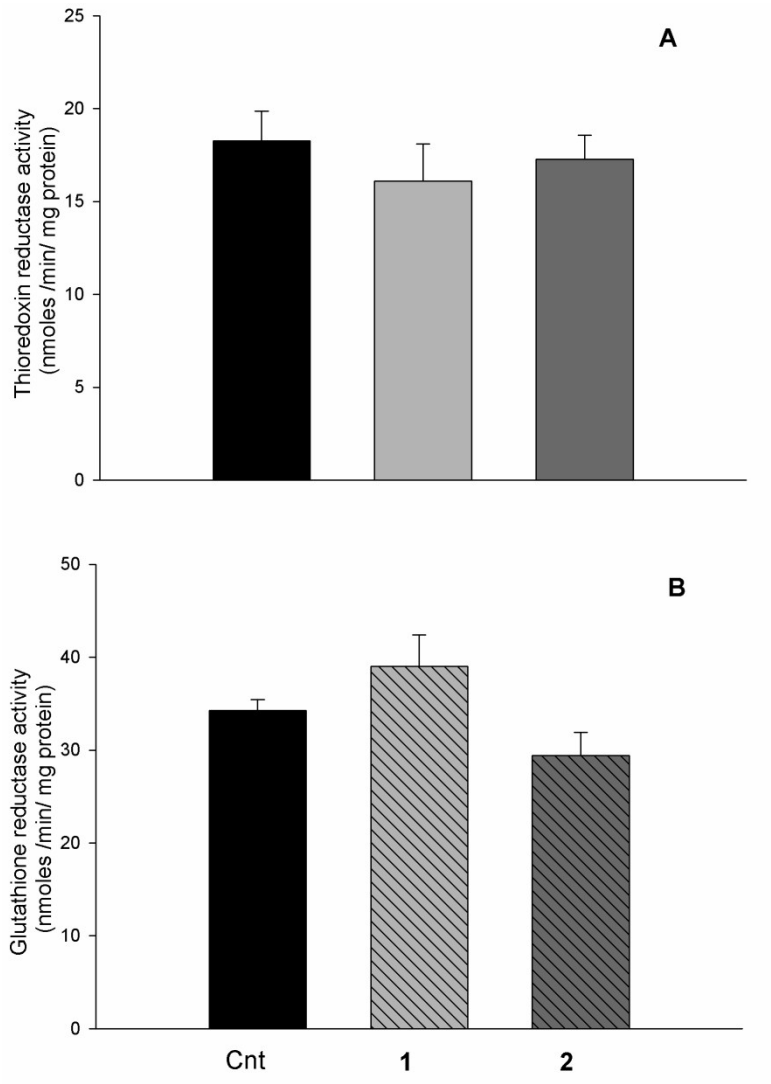

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Figure S6. Total glutathione and oxidized glutathione levels in the presence of **1** and **2**.
Total glutathione and oxidized glutathione were determined in SKOV3 cells, after incubation with the indicated concentrations of **1** and **2** for 48 h.
Statistical Analysis. Multiple comparisons were made by one-way analysis of variance followed by the Tukey−Kramer multiple comparison test. *= p<0.05

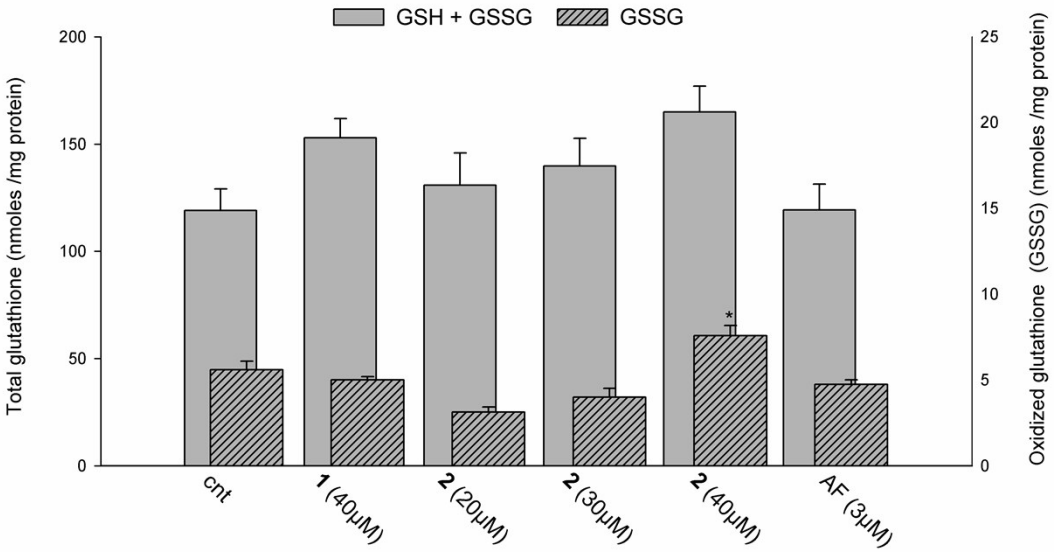

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Table S1. Comparison of the Expected Survival Rates (based on an assumption that the Combined Drug Activities are additive) and the Experimentally Determined Values after treating cells for 72 h with cisplatin (7.5 μM) and different concentrations of **2** (10, 20, 30 μM). Calculation of the predicted survival rates is described in the Experimental Section.

	Survival rate	
Drug treatment	*Expected*	*Observed*
Cispt 7.5μM		0.55
Cispt 7.5μM + **2** 10μM	0.445	0.465
Cispt 7.5μM + **2** 20μM	0.24	0.36
Cispt 7.5μM + **2** 30μM	0.165	0.36

References

1. M. Luthman and A. Holmgren, *Biochem.*, 1982, 21, 6628-6633.
2. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, 193, 265-275.
3. F. Tietze, *Anal Biochem*, 1969, 27, 502-522.
4. M. E. Anderson, *Methods Enzymol*, 1985, 113, 548-555.

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