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Development of a Method for the Quantification of the Molar Gold Concentration in Tumour Cells Exposed to Gold-Containing Drugs

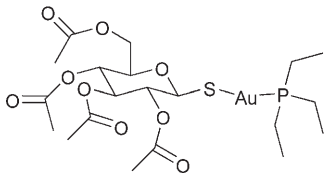
Ingo Ott,* Heike Scheffler, and Ronald Gust[a]

The knowledge of the cellular molar concentration of a drug is an extremely important parameter for the discussion and interpretation of its efficacy and bioavailability. Concerning metal complexes, electrothermal atomic absorption spectroscopy (ETAAS) offers a valuable analytical tool. However, matrix effects often hamper proper quantification of the metal concentration in biological tissues. This paper describes the development of an ETAAS method for the quantification of the molar gold concentration in HT-29 colon carcinoma cells. ETAAS analytical conditions were optimised and a factor was developed which allows the calculation of the molar cellular gold concentration from the measured gold per cellular biomass value. The method was used to quantify the gold content in HT-29 cells after exposure to the gold drug auranofin. Results indicated a strong cellular uptake of auranofin (compared to other metal anticancer drugs), which significantly correlated with the antiproliferative effects triggered by this agent.

Introduction

Gold complexes had been used for the treatment of arthritis some decades ago but most of them disappeared from the drug market because of intolerable side effects, such as gastrointestinal adverse reactions (for example, diarrhoea), nephrotoxicity, and haematological reactions. During the last years, however, the therapeutic interest in gold complexes has been renewed by reports about their antiproliferative activity, suggesting a use for the cure of human cancerous malignancies.

Auranofin, shown below, represents a prominent gold compound, which had found application in the antiarthritic therapy



under the name Ridaura[1,2] but also demonstrated antitumour activity in vitro and in vivo. This led to tremendous efforts to clarify the mode of drug action of auranofin and other gold compounds. In many cases the biological activity seemed to depend on the inhibition of the enzyme thioredoxin reductase and on antimitochondrial activity. These effects are supposed to lead to an enhanced killing of tumour cells.[3,4]

A very important parameter needed for the discussion and interpretation of such pharmacological effects is the uptake of the agents into (tumour) cells. It is very likely that structurally related compounds, which showed a correlation between the biological activities and cellular concentrations acted by a unique mode of action. The lack of such a correlation is an indicator for differing modes of action or differing quality of the biological effects. Another extremely valuable parameter is the cellular molar concentration, which can be calculated from the quotient of metal and cellular protein amounts, the mean cellular volume, and the mean cellular protein content.[5–7]

Cellular uptake studies on various gold containing complexes have been performed by atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectroscopy (ICP–MS) or by using radiolabelled [^{195}Au] derivatives.[8–13] However, most of these studies were done without calculation of the molar cellular concentrations and without determination of the cell biomasses at the end of the experiments. This makes the comparison of data obtained from different experimental setups quite difficult.

Therefore, we developed an assay to perform quantification of the cellular molar gold concentration using electrothermal atomic absorption spectroscopy (ETAAS). In this paper we describe its application for studies on the gold drug auranofin in HT-29 cells.

[a] *Dr. I. Ott, H. Scheffler, Prof. Dr. R. Gust*
Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin (Germany)
Fax: (+49) 30-83856906
E-mail: ottingo@zedat.fu-berlin.de

Results

ETAAS measurements: pyrolysis and atomisation conditions

Gold chloride standard solutions were diluted with aqueous lysates of HT-29 cells (see experimental section) and were used as probes for the optimisation of ETAAS conditions. Triton X-100 was added to a final concentration of 0.1% (*v*/*v*) before the ETAAS measurements to stabilise cellular components (for example, disrupted membrane fractions). After injection into the graphite tube, the samples were dried, pyrolysed, and atomised by heating in the graphite furnace (see experimental section for details). The drying steps were adjusted from a method used for the determination of cobalt containing cellular lysates.[14] Optimal conditions for pyrolysis and atomisation were developed using a method suggested by Welz et al.[15] The pyrolysis temperature is optimised in the first series of experiments with constant atomisation temperature, then the atomisation temperature is evaluated keeping the (optimised) pyrolysis temperature unchanged.

For the evaluation of the gold pyrolysis conditions the temperatures were raised stepwise starting with 600 °C. The temperature for atomisation was kept at 1700 °C or 2500 °C, respectively. As shown in Figure 1 a (open symbols) increasing the pyrolysis temperature to 700 °C or 800 °C afforded an increase in signal intensity. Any further enhancement of the temperature led to a dramatic loss in absorption making pyrolysis at higher temperatures not useful.

Interestingly, atomisation at 2500 °C led to lower absorption signals than atomisation at 1700 °C (compare circular open and corresponding square open symbols in Figure 1 a). This result was confirmed when the temperature for atomisation was optimised (see below).

Addition of ascorbic acid (0.1%) as modifier allowed pyrolysis temperatures to be set to 1000–1200 °C (compare corresponding closed and open symbols in Figure 1 a). Thus, the ascorbic acid modifier was used in all the following experiments.

Background measurements using a deuterium lamp showed similar results under all the described conditions and afforded minimal values using pyrolysis temperatures of 1000 °C and higher.

The pyrolysis temperature was kept constant at 1000 °C and the atomisation temperature was incremented stepwise to optimise the atomisation conditions. A maximum of signal intensity was observed around 1600 °C followed by a slight but continuous decrease in absorption intensity towards higher atomisation temperatures (see Figure 1 b).

For the following experiments the pyrolysis temperature was set to 1000 °C and the atomisation temperature to 1700 °C.

Stability of the probes

The stability of a 100 ng mL^{-1} gold sample in cellular lysate was investigated over a period of 4 h. No loss of signal intensity could be noted (recovery 103% of the initial value after 4 h).

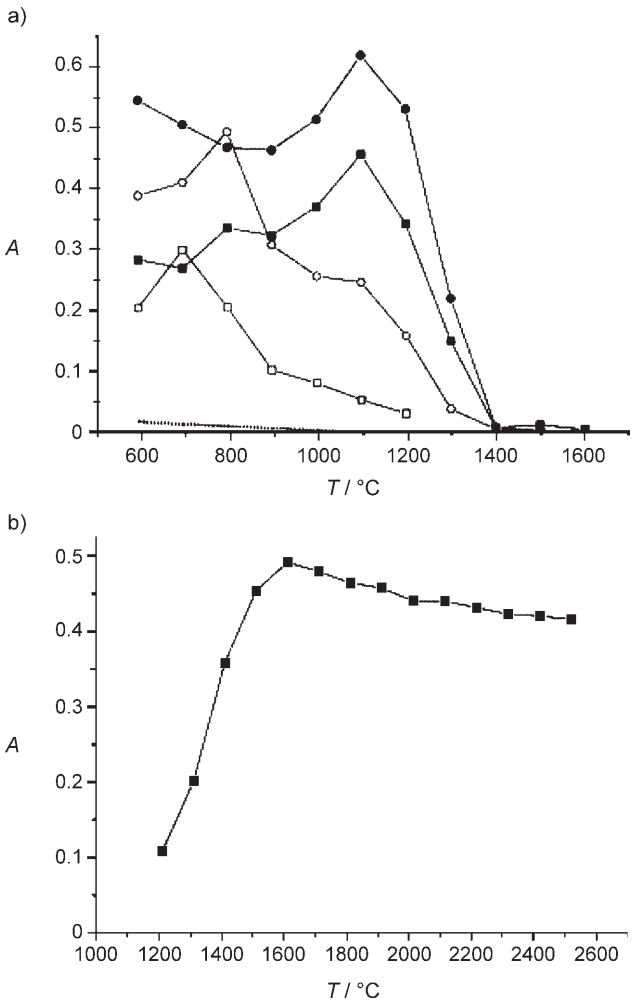


Figure 1. a) Variation of pyrolysis temperature; sample gold content: 200 ng mL^{-1}; sample matrix (protein) content: 0.83 mg mL^{-1} for atomisation at 1700 °C and 0.68 mg mL^{-1} for atomisation at 2500 °C; legend: □: without ascorbic acid, atomisation at 2500 °C; ○: without ascorbic acid, atomisation at 1700 °C; ■: with ascorbic acid, atomisation at 2500 °C; ●: with ascorbic acid, atomisation at 1700 °C; background: dotted line. b) Variation of atomisation temperatures; sample gold content: 200 ng mL^{-1}; sample matrix (protein) content: 0.83 mg mL^{-1}.

Measurement of the gold content in cellular lysates

Applying the above described measurement conditions, various amounts of gold were added to blank cellular lysates and the recovery rates were determined. A significant decrease of gold signal intensity compared to aqueous gold solutions could be noted in the probes containing cellular matrix (see Figure 2). The slopes of the calibration functions of the matrix containing probes were lower than that of the aqueous probes and the detection limits (characteristic concentrations) were significantly increased (see caption of Figure 2). Moreover, the signal intensities depended on the concentration of the matrix and decreased with increasing matrix contents. The matrix content was determined by measurement of the protein concentration of the samples using the Bradford method. Thus, for the 100 ng mL^{-1} gold samples the recovery (compared to the corresponding aqueous standard) was 60% at a matrix protein

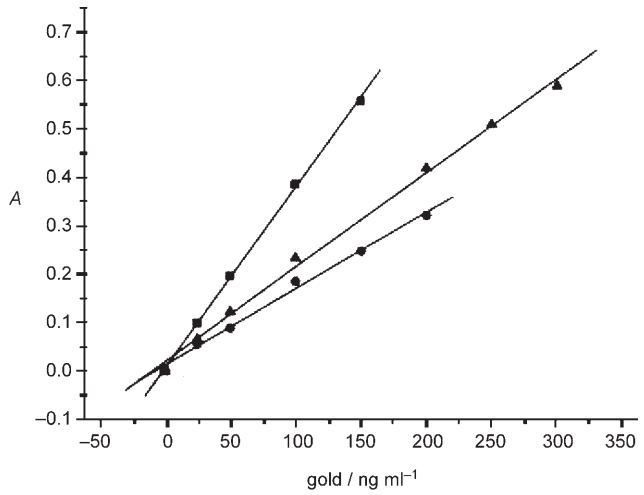


Figure 2. Measurement of the gold content of standard samples; characteristic concentrations were expressed as (ng gold ml^{-1})/1% absorption and are given between brackets; legend: -■-: aqueous standards (1.17), -▲-: matrix standards 0.42 mg mL^{-1} (2.25), -●-: matrix standards 1.28 mg mL^{-1} (2.78).

concentration of 0.42 mg mL^{-1} and 48% at 1.28 mg mL^{-1}. Interestingly, all chosen conditions allowed linear calibration functions (with $r^2 > 0.997$) up to absorbance values of approximately 0.6.

Based on these observations a proper analysis of cellular gold contents seemed to be possible if the matrix content of the standard samples used for calibration was adjusted to that of the probes under question (matrix calibration). However, from a practical point of view this treatment was inappropriate for the determination of a larger number of samples and routine use.

Therefore, cellular lysates containing different amounts of gold were analysed using the standard addition method (see Table 1). This procedure allowed recoveries in the range of 97.7–106.3% for samples containing different matrix protein and gold concentrations (matrix: 0.81 and 1.28 mg mL^{-1}; gold: 25–100 ng mL^{-1}). Slopes and correlation coefficients were similar in all the experiments.

Table 1. Analysis of gold containing samples using the standard addition method.

matrix (protein)[a] [mg mL^{-1}]	gold[a] [ng mL^{-1}]	slope[b] (*A*/ng)	correlation (r^2)	recovery
0.81	25	0.133	0.9941	106.3%
0.81	50	0.125	0.9935	98.2%
0.81	100	0.113	0.9940	99.9%
1.28	25	0.115	0.9904	98.9%
1.28	50	0.106	0.9911	102.0%
1.28	100	0.102	0.9984	97.7%

[a] content in sample [b] slopes were calculated as follows: slope = integrated absorption value/ng of gold added to the samples (*A*/ng).

Determination of the cellular molar gold concentration

After measurement of the gold content by ETAAS and protein quantification by the Bradford method the cellular gold concentration could be expressed as ng gold per mg cellular protein. A factor describing the relationship of the protein content and the cell volume was required for the calculation of the cellular molar concentration using this ng mg^{-1} value. The mean volume of HT-29 cells (0.92 pL) could be determined under assumption of a spherical shape of the cells based on the knowledge that the average cellular diameter is 12.07 $\pm$1.00 μm, which was determined microscopically (see Figure 3). The mean protein content of HT-29 cells (0.18 $\pm$ 0.04 ng per cell) was measured with the Bradford method for protein quantification and a Neubauer cell chamber for cell counting. Accordingly, 1.0 ng gold found in a cell matrix containing 1.0 mg of protein corresponded to a cellular gold concentration of 1.0 μM.

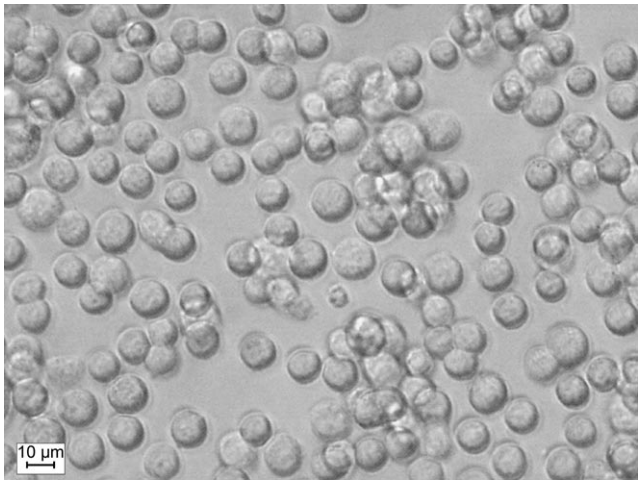


Figure 3. HT-29 cells suspended in PBS.

Quantification of cellular gold after exposure to auranofin and cell growth inhibitory activity

For the purpose of quantification of cellular gold after exposure to auranofin at least 70% confluent cell monolayers were incubated with various concentrations of auranofin. The cells were isolated after incubation for 6 h and investigated for their molar gold content according to the procedures described above.

Cellular gold concentrations between 11.3 and 494.3 μM could be determined (see Figure 4), if auranofin was used in the range of 1.0 to 10.0 μM. A polynomial regression analysis revealed that the data could be fitted with good correlation ($r^2 = 0.9955$) according to Equation (1).

$$c_g = 0.949 - 1.205\, m_g + 5.134\, m_g^2 \quad (1)$$

c_g = cellular gold (μM); m_g = gold (auranofin) in the culture media at the beginning of the experiment (μM)

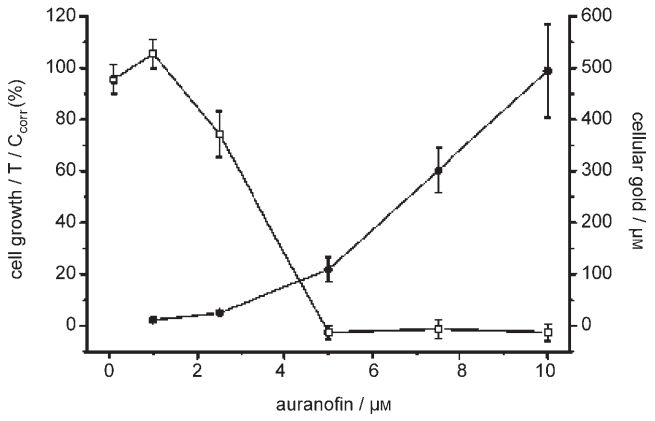


Figure 4. Cellular uptake (exposure period: 6 h, $n=2$–3) and cell growth inhibitory activity (exposure period: 72 h, a representative example of a dose response curve ($n=6$) is shown) of auranofin in HT-29 cells; in some cases the error bars are hidden behind the symbols; legend: □: cell growth, ●: cellular gold.

The cell growth inhibitory effects of auranofin in HT-29 cells were evaluated after an incubation period of 72 h. In agreement with the results from the gold uptake studies exposure to higher auranofin concentrations led to stronger antiproliferative effects. In the experiments using auranofin concentrations of 5–10 μM even the cells, which were present at the start of the incubation period, were terminated (as was illustrated by negative T/C_{corr} (%) values). Four independent experiments afforded an IC_{50} value of 2.6 ± 0.4 μM for auranofin in HT-29 cells.

Discussion

To quantify the gold amount in cellular lysates an ETAAS based procedure had to be developed. Besides sample preconcentration and element specific sensitivity the most critical parameters for optimal ETAAS measurements are generally the addition of chemical modifiers and proper thermal sample pretreatment in the graphite furnace before atomisation and absorption reading. In this context ascorbic acid as modifier for gold samples has been reported to allow elevated pyrolysis temperatures.[16,17] In our experiments the differences in signal intensities between samples containing ascorbic acid and modifier free probes were most marked at temperatures between 1000 °C and 1200 °C. Pyrolysis above 1200 °C led to a dramatic decrease in absorbance, which can be attributed to vaporisation and subsequent loss of analyte before absorption reading in the atomisation phase. The effectiveness of ascorbic acid as a modifier is considered to be the consequence of the products formed during its decomposition in the pyrolysis phase. Thus, the formation of reductive gaseous compounds (such as CO) and of active and (at higher temperatures) thermally stable carbon species was observed.[18] Iwamoto et al. reported that pyrolysed ascorbic acid forms a carbon film on the surface of graphite tubes.[17]

Increasing stepwise the atomisation temperature from 1200 °C initially to 2500 °C revealed a steep increase of signal intensity between 1200 °C and 1600 °C because of higher amounts of atomised gold. The maximum at 1600 °C was followed by a slight decrease of absorbance, which is probably the consequence of partial ionisation of gold at higher temperatures leading to undetectable species.

In the following experiments the pyrolysis temperature was set to 1000 °C, atomisation was performed at 1700 °C and ascorbic acid was added to all samples as a modifier.

Using these analytical conditions the gold recovery from matrix (cellular lysate) containing samples was determined and found to be significantly lowered by higher matrix concentrations. Sufficient recoveries of added gold were observed when the standard addition method was applied. Thus, the gold amount of the samples could be quantified by ETAAS and be correlated to the cellular protein concentration, which was measured by the Bradford method (ng gold per mg protein (ng mg^{-1})value).

Based on the knowledge of the mean cellular diameter and of the protein content a factor was determined, which allowed the conversion of the ng mg^{-1} value to the molar cellular gold concentration. Thus, it could be stated that 1.0 ng gold found in cell matrix containing 1.0 mg of protein corresponded to 1.0 μM cellular gold.

A practical application of the described method was performed on HT-29 cells exposed to varying concentrations of the gold drug auranofin for 6 h. According to Figure 4 and Equation (1) the cellular uptake of gold did not show a linear correlation to the amount of auranofin used for incubation. The observed enhanced cellular uptake at exposure to higher drug concentrations indicated a disintegration of the cellular membranes or perturbed transport processes as a consequence of initial toxic effects.

The onset of cytotoxicity of auranofin within the first hours of incubation had already been observed and reported. In these studies the cellular association of auranofin in melanoma cells or macrophages followed a linear trend during the first two hours of exposure.[11–13] However, in such short time exposure experiments the cell biomasses at the end of the incubation periods were usually not determined, which makes a correction of the measured gold values for possible cell loss impossible. Other metal complexes (for example, platinum or hexacarbonyldicobalt species) showed a linear accumulation trend even after longer (6 h or more) periods of incubation.[7,19]

The accumulation grade of a drug is defined as the quotient of the cellular and the exposure concentration (initial concentration in the cell culture medium). Accordingly, for incubation with auranofin accumulation grades between 9.9 (exposure to 2.5 μM) and 49.4 (exposure to 10 μM) could be determined. This result is of special interest because established anticancer platinum complexes such as cisplatin or carboplatin usually reached accumulation grades up to 6.[5,7]

However, stronger accumulation of other metal species has also been reported. For example, for cobalt complexes of the salen type accumulation grades up to 25 were found in breast cancer cells whereas strongly lipophilic hexacarbonyldicobalt complexes were accumulated up to 150-fold in the same cell line.[19,20] An osmium cluster compound reached accumulation

grades of approximately 50 (exposure to 0.1 μM) and approximately 25 (exposure to 1 μM) during experiments with long incubation times (72 h).[21] Exceptionally strong uptake was reported for titanocene dichloride, which achieved an accumulation grade of almost 200 in HCT-116 cells after 4 h.[22]

In comparison with these data obtained from experiments on metal species containing different metal atoms in different cell lines the gold uptake caused by auranofin can be considered as strong. However, the measured values are still within the range of other metal drugs that are considered good accumulating drugs.

Interestingly, the shape of the cellular uptake curve (see Figure 4) correlated very well with the dose response curve obtained in the proliferation experiments. The steep increase in the cellular molar gold concentrations at exposure to 5 μM or higher concentrations of auranofin led to a complete termination of cell proliferation and survival. Incubation with lower concentrations was significantly less effective correlating with lower cellular gold levels.

Experiments on other cytotoxic gold species than auranofin are the subject of ongoing research. The knowledge of the cellular molar gold concentration caused by exposure to these agents is considered to be a valuable parameter for explanation of the biological effects of the new gold compounds under study. Thus, the application of the presented method will be crucial for understanding and interpreting the pharmacological effects of gold drugs.

Experimental Section

General: chemicals and reagents were purchased from Sigma, Aldrich, and Fluka. Auranofin was from MP Biomedicals, Inc.; PBS: phosphate buffered saline pH 7.4; Bradford reagent: 250 mg Serva Blue G dissolved in 250 mL EtOH (96%)/500 mL H_3PO_4 (85%)/250 mL H_2O (this reagent was diluted fivefold with H_2O directly before use); cell culture medium: minimum essential medium eagle supplemented with 2.2 g $NaHCO_3$, 110 mg L^{-1} sodium pyruvate, and 50 mg L^{-1} gentamicin sulfate adjusted to pH 7.4

ETAAS measurements: a Vario 6 graphite furnace atomic absorption spectrometer (AnalytikJena AG) was used for the gold measurements. Gold was detected at a wavelength of 242.8 nm with a bandpass of 0.8 nm. A deuterium lamp was used for background correction. Probes were injected at a volume of 25 μL into graphite wall tubes. Drying, pyrolysis, atomisation, and tube cleaning steps were performed as outlined in Table 2. The mean AUC (area under curve) absorptions of duplicate injections were used throughout the study.

Cell culture and preparation of cell pellets: HT-29 human colon carcinoma cells were maintained in cell culture medium (see above) at 37 °C/5% CO_2 and passaged twice a week according to standard procedures. Cell pellets for method optimisation purposes were prepared and isolated as follows: cells were grown until at least 70% confluency in 175 cm^2 cell culture flasks. The culture medium was removed, the cell layer washed with 10 mL PBS, treated with 2–3 mL trypsin solution (0.05% trypsin, 0.02% EDTA in PBS) and incubated for 2 min at 37 °C/5% CO_2 after removal of the trypsin solution. Cells were resuspended in 10 mL PBS and cell pellets were isolated by centrifugation (RT, 2000 g, 5 min).

Sample preparation for method optimisation purposes: cellular lysates were prepared by resuspending an isolated cell pellet (see above) in 1.0 or 5.0 mL double distilled water followed by treatment with a sonotrode. Matrix containing standards and probes were prepared by addition of gold (obtained from a 1 mg mL^{-1} gold standard solution in 0.5 N HCl (Fluka)) to the cellular lysate and appropriate dilution to the final concentrations using blank cellular lysate. Aqueous standards were prepared analogously using double distilled water. Unless stated otherwise 20 μL of a solution containing Triton X-100 (1%) and ascorbic acid (1%) were added to each 200 μL of the samples. Probes for experiments using the standard addition method were prepared according to the procedure described in the below section.

Table 2. Graphite furnace program.

step	*T* [°C]	rate [°C s^{-1}]	hold [s]
drying	0	10	40
drying	105	7	30
drying	120	15	20
drying	500	50	30
pyrolysis	various	200	20
AZ (zeroing)	= pyrolysis	0	3
atomisation	various	max.	6
tube cleaning	2100 or 2550	1000	5

Sample preparation for cellular uptake studies: for cellular uptake studies cells were grown until at least 70% confluency in 175 cm^2 cell culture flasks. Stock solutions of auranofin in dimethylsulfoxide (DMSO) were freshly prepared and diluted with cell culture medium to the desired concentrations (final DMSO concentration: 0.1% *v/v*, final auranofin concentrations: 1.0 to 10.0 μM). The cell culture medium of the cell culture flasks was replaced with 10 mL of the cell culture medium solutions containing auranofin and the flasks were incubated at 37 °C/5% CO_2 for 6 h. The cell pellets were isolated as described above, resuspended in double distilled water, lysed by using a sonotrode, and appropriately diluted using double distilled water. An aliquot was removed for the purpose of protein quantification. The determination of the gold content of the samples was performed using the standard addition method. In short: increasing amounts of aqueous gold standard solutions were added to 100 μL aliquots of the diluted lysates. All probes were adjusted to a final volume of 200 μL using double distilled water, 20 μL Triton X-100 (1%) and ascorbic acid (1%) were added to each and the probes were measured by ETAAS. The gold content of the lysates was assessed by the linear extrapolation method. Results were calculated from the data of 2–3 independent experiments.

Determination of the mean cellular diameter and cell counting: the mean cellular diameter was determined microscopically (Axiovert 40 CFL microscope, Zeiss, software: EasyMeasure) by measuring the diameters of 35–55 cells obtained from a suspension of a cell pellet in 5 or 10 mL PBS. Cell counting was performed microscopically using a Neubauer cell counting chamber. The results were calculated from the data obtained from eight independent experiments.

Quantification of the matrix protein content: the protein content of cellular lysates was determined by the Bradford method.[23] In short, the lysates were appropriately diluted with double distilled water and 20 μL thereof were added to each 200 μL Bradford reagent (see above) in 96 well plates. After proper mixing and incubation for 20 min at room temperature the absorbances at 595 nm were read in a microplate reader (FlashScan AnalytikJena AG). Aqueous solutions of human serum albumin were used for calibration purposes. Probes were measured in duplicate.

Cell growth inhibitory activity: HT-29 cells were suspended in cell culture medium (2850 cells per mL), 100 μL aliquots thereof were plated in 96 well plates and incubated at 37 °C/5% CO_2 for 48 h. Stock solutions of auranofin in DMSO were freshly prepared and diluted with cell culture medium to the desired concentrations (final DMSO concentration: 0.1% *v/v*). One 96 well plate was used for the determination of the initial cell biomass and was processed in the following way: the medium was removed, cells were fixed by a 20–30 min incubation with 100 μL glutardialdehyde solution (0.5 mL glutardialdehyde + 12.5 mL PBS), the wells were emptied, 180 μL PBS were added and the plate was stored at 4 °C until further processing. In the treated plates the medium was replaced with medium containing auranofin in graded concentrations (six replicates). After further incubation for 72 h these plates were processed as described above. The cell biomass was determined by crystal violet staining according to the following procedure: PBS was removed, 100 μL 0.02 M crystal violet solution were added, the plates were incubated for 30 min at room temperature, washed three times with water, and incubated on a softly rocking rotary shaker with 180 μL ethanol (70%) for a further 3–4 h. Absorption was recorded in a microplate reader at 590 nm (FlashScan Analytik-Jena AG). The mean absorption of the initial cell biomass plate wells was subtracted from the mean absorption of each auranofin treated series of wells and control wells. The corrected control was set as 100% and the data of the treated wells were calculated accordingly (T/C_{corr}(%) value). The IC_{50} value was determined as the concentration causing 50% inhibition of cell proliferation ($T/C_{corr} = 50\%$) and calculated as the average of four independent experiments.

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Keywords: atomic absorption spectroscopy · auranofin · bioinorganic chemistry · bioorganometallic chemistry · cellular uptake

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