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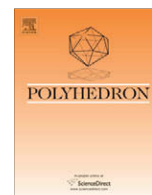

Comparative studies on the cytotoxicity, cellular and nuclear uptake of a series of chloro gold(I) phosphine complexes

Heike Scheffler [a], Ya You [a], Ingo Ott [b,*]

[a] Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, Germany
[b] Institute of Pharmaceutical Chemistry, Technische Universität Braunschweig, Beethovenstr. 55, 38106 Braunschweig, Germany

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ABSTRACT

Gold(I) phosphine complexes have shown promising results as a novel class of anticancer drugs in a variety of biochemical and pharmacological investigations. Studies on pharmacokinetic properties of these species are rare. Here we report the results of a comparative study on the cytotoxicity, cellular and nuclear uptake of a series of chloro gold(I) phosphine complexes (Cl–Au–P(R)$_3$, R = Me, Et, tert-But, Ph) containing different ligands on the phosphor. Cellular and nuclear gold levels in HT-29 colon carcinoma and MCF-7 breast cancer cells were measured by electrothermal atomic absorption spectrometry. All studied complexes exhibited significant antiproliferative effects in both investigated cell lines. Cellular and nuclear gold levels were enhanced especially for Cl–Au–P(Ph)$_3$ indicating a positive influence of larger and more lipophilic substituents.

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1. Introduction

The search for new metal containing anticancer drugs is one of the major interests in bioinorganic or bioorganometallic medicinal chemistry. In order to overcome the disadvantages (e.g. nephrotoxic side effects) of the established platinum antitumor agents the current focus in the development of novel metallodrugs is shifting to the use of non platinum central atoms and to the incorporation of different organic ligands into metal complexes [1–6].

Gold(I) phosphine complexes (see Fig. 1) derived from the antirheumatic drug auranofin as well as other gold compounds have recently gained considerable attention due to some unique pharmacological properties including the inhibition of the enzyme thioredoxin reductase or the triggering of antimitochondrial effects [7–10].

Concerning drug development in general the knowledge of parameters such as the cellular uptake and intracellular biodistribution are of great value to optimize not only the pharmacodynamic but also the pharmacokinetic properties of new agents (Fig. 2).

Early bioanalytical studies on auranofin and the related Cl–Au–P(Et)$_3$ in RAW 264.7 macrophages showed that gold was distributed between the nuclear, cytosolic and membrane fractions [11] and that the uptake of both compounds depended on temperature and time [12]. The gold levels of HT-29 colon carcinoma cells exposed to various concentrations of auranofin were found to be 9.9–49.4 fold increased compared to the extracellular concentrations and the level of accumulation depended on the used incubation concentration [13]. For cationic lipophilic gold(I) phosphine complexes a significant influence of the lipophilicity on the accumulation in CH-1 human ovarian carcinoma cells and on protein binding was observed [14]. Furthermore, the uptake of gold(I) complexes into mitochondria [15–17] and lysosomes [18] was reported in recent studies. These studies altogether indicated that the pharmacodynamic properties of gold agents are strongly influenced by their cellular uptake and biodistribution profiles.

During comparative studies on the novel gold(I) naphthalimide compound Au–Naphth-1 and Cl–Au–P(Et)$_3$ we recently noted that the uptake into the nuclei of tumor cells was 5–25 fold lower for Cl–Au–P(Et)$_3$ than for Au–Naphth-1, in which the chloro ligand of Cl–Au–P(Et)$_3$ was replaced by a thio-naphthalimide ligand [19]. The low uptake of Cl–Au–P(Et)$_3$ into the nuclei of tumor cells agrees well with the agent being a non DNA targeting compound and raised the question if simple modifications not only concerning a replacement of the chloro ligand but also a modification of the substituents on the phosphor might change this behavior.

For further going studies on the cellular accumulation and biodistribution of gold into the nuclei of tumor cells we chose a series of commercially available chloro gold(I) phosphine complexes with different alkyl/aryl substituents on the phosphine partial structure (see Fig. 1). Some of these compounds, namely Cl–Au–P(Et)$_3$ and Cl–Au–P(Ph)$_3$ have been investigated intensively already in early studies on the antitumor potential of gold complexes and are known inhibitors of TrxR [8,10]. Cl–Au–P(Et)$_3$ and Cl–Au–P(Ph)$_3$ are often used as reference compounds in studies on new gold

* Corresponding author. Tel.: +49 531 391 2743.
E-mail address: ingo.ott@tu-bs.de (I. Ott).

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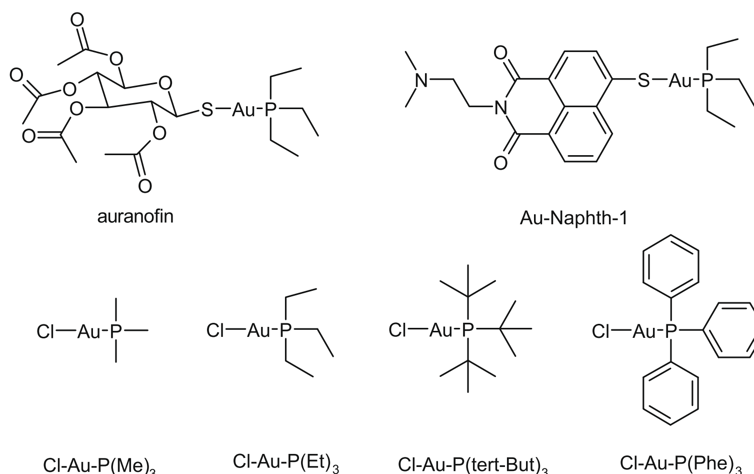

Fig. 1. Bioactive gold(I) phosphine complexes.

species and their gold phosphine partial structures are found in many currently developed gold metallodrugs [8,19]. Thus, a more detailed knowledge on the biodistribution characteristics of these and structurally related gold(I) phosphine complexes could be essential for projects dealing with the development of gold(I) anticancer therapeutics.

2. Experimental

2.1. General

Chemicals and reagents were purchased from Sigma, Aldrich and Fluka; chloro(trimethylphosphine)gold(I), chloro(triethylphosphine)gold(I), chloro(tri-*tert*-butylphosphine)gold(I) and chloro-(triphenylphosphine)gold(I) were from Sigma; PBS: phosphate buffered saline pH 7.4; HT-29 human colon carcinoma cells and MCF-7 breast cancer cells were maintained in 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) containing 10% (V/V) fetal calf serum at 37 °C/5% CO_2 and passaged twice a week according to standard procedures.

2.2. Cell growth inhibitory activity

Cells were suspended in cell culture medium (HT-29: 2850 cells per mL, MCF-7: 10 000 cells per mL), 100 μL aliquots thereof were plated in 96 well plates and incubated at 37 °C/5% CO_2 for 48 h (HT-29) or 72 h (MCF-7). Stock solutions of the compounds in dimethylformamide (DMF) were freshly prepared and diluted with cell culture medium to the desired concentrations (final DMF 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 glutaraldehyde solution (0.5 mL glutaraldehyde + 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 the gold complexes in graded concentrations (six replicates). After further incubation for 72 h (HT-29) or 96 h (MCF-7) 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 3 times with water, and incubated on a softly rocking rotary shaker with 180 μL ethanol (70%) for further 3–4 h. Absorption was recorded in a microplate reader at 590 nm (Flashscan AnalytikJena AG). The mean absorption of the initial cell biomass plate wells was subtracted from the mean absorption of each gold complex treated series of wells and control wells. The corrected control was set 100% and the data of the treated wells were calculated accordingly (T/C_{corr}(%) value). The IC_{50} value was determined as that concentration causing 50% inhibition of cell proliferation (T/C_{corr} = 50%) and calculated as average of two independent experiments.

2.3. Sample preparation for cellular uptake studies

The cellular uptake was measured according to a previously described procedure [13]. In short: cells were grown until at least 70% confluency in 175 cm^2 cell culture flasks. Stock solutions of the gold complexes in DMF were freshly prepared and diluted with cell culture medium to the desired concentration (final DMF concentration: 0.1% V/V, final gold complex concentration: 5.0 μM). The cell culture medium of the cell culture flasks was replaced with 10 mL of the cell culture medium solutions containing the compounds and the flasks were incubated at 37 °C/5% CO_2 for 6 h. The cell pellets were isolated by trypsinisation and centrifugation (room temperature, 2000 g, 5 min), resuspended in twice distilled water, lysed by using a sonotrode and appropriately diluted using twice distilled water. An aliquot was removed for the purpose of protein quantification by the Bradford method. The determination of the gold content of the samples was performed by electrothermal atomic absorption spectroscopy (AAS, see Section 2.5). Results were calculated from the data of 2–3 independent experiments and are given as nmol gold per μg cellular protein.

2.4. Sample preparation for nuclear uptake studies

The nuclei of the tumor cells were isolated according to previously described procedures [20,21] with some minor modifications: cells were grown in 175 cm^2 cell culture flasks until at least 70% confluency. The medium was removed and replaced with 10 mL of medium containing 5.0 μM drug. After 6 h of incubation at 37 °C in humidified atmosphere, the drug containing medium was removed, cells were trypsinized, resuspended in 10 mL of cell culture medium, isolated by centrifugation (1500 rpm, 5 min) and 0.5–1.0 mL of 0.9% NaCl solution were added. After centrifugation (1500 rpm, 5 min) pellets were resuspended in 300 μL of RSB-1 (0.01 M Tris–HCl, 0.01 M NaCl, 1.5 mM $MgCl_2$, pH 7.4) and left for

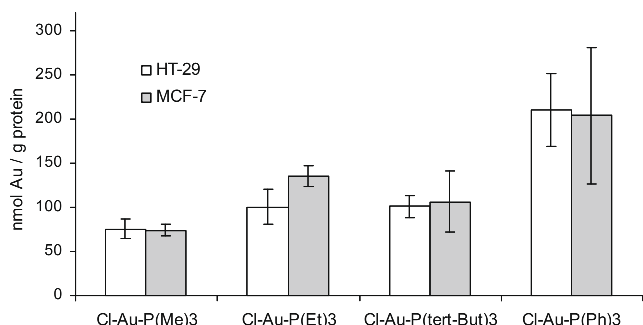

Fig. 2. Cellular uptake of chloro gold(I) phosphine complexes after 6 h in HT-29 and MCF-7 cells.

10 min in an ice-bath. Swollen cells were centrifuged (2000 rpm, 5 min), resuspended in 300 μL of RSB-2 (RSB-1 containing each 0.3% V/V Nonidet-P40 and sodium desoxycholate) and homogenised by 10–15 up/down-pushes in an 1 mL syringe with needle. Aliquots of 50 μL of the homogenisate were removed for determination of the total gold content and mixed with 500 μL water. The homogenisate was centrifuged at 2500 rpm for 5 min and the resulting crude nuclei were taken up in 150 μL of 0.25 M sucrose containing 3 mM $CaCl_2$. The suspension was underlayed with 150 μL of 0.88 M sucrose and centrifuged 10 min at 2500 rpm. The nuclei pellets were stored at −20 °C or immediately dissolved in 500 μL water and disrupted by use of a sonotrode. The gold content of the samples was measured by electrothermal AAS (see Section 2.5) and the protein content by the Bradford method. Results are expressed as means of two independent experiments as nmol gold per g nuclear protein or as percentage of the total gold content.

2.5. Atomic absorption spectroscopy (AAS)

Electrothermal AAS measurements were performed according to a previously published standard addition procedure [13] with some minor modifications. In short: to 100 μL aliquots of the diluted lysates (see Sections 2.3 and 2.4) increasing amounts of aqueous gold standard solutions were added. All probes were adjusted to a final volume of 200 μL using twice distilled water, each 20 μL triton X-100 (1%) and ascorbic acid (1%) were added and the probes were measured as described below. The gold content of the lysates was accessed by the linear extrapolation method. A Vario 6 electrothermal 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 regular graphite wall tubes. Drying, atomisation and tube cleaning steps were performed as outlined in more detail in the literature [13]. The temperature for pyrolysis was set to 1200 °C. The mean AUC (area under curve) absorptions of duplicate injections were used throughout the study. The limit of gold detection using biological samples as described above was 1.7 μg/L.

3. Results and discussion

3.1. Antiproliferative effects

Initially we evaluated the antiproliferative effects of the gold phosphine complexes in two human tumor cell lines (see Table 1). For this purpose we selected MCF-7 breast cancer cells and HT-29 colon carcinoma cells as these represent highly relevant and widespread tumoral malignancies and originate from different unrelated tissues.

Table 1
Antiproliferative effects of chloro gold(I) phosphine complexes.

	HT-29 (μM)	MCF-7 (μM)
Cl–Au–P(Me)$_3$	5.2 ± 0.6	3.9 ± 0.9
Cl–Au–P(Et)$_3$	5.3 ± 1.9	3.2 ± 1.3
Cl–Au–P(tert-But)$_3$	5.2 ± 2.1	3.1 ± 0.4
Cl–Au–P(Ph)$_3$	4.2 ± 0.9	2.6 ± 0.1

IC_{50} values were obtained in the range of 2.6–5.3 μM, which is well within the range of different established cytostatic agents (e.g. cisplatin exhibits IC_{50} values of 7.0 μM in HT-29 cells and 2.0 μM in MCF-7 cells [22]) or the gold phosphine lead compound auranofin (IC_{50} values of 2.6 μM in HT-29 cells [13] and 1.1 μM in MCF-7 cells [23]) in the same assay.

It is of interest to note that despite the different alkyl/aryl substituents on the phosphor, which lead to large differences in lipophilicity and molecular volumes, the antiproliferative activity of the complexes was comparable for all the complexes. The lowest IC_{50} values were found for the phenyl containing Cl–Au–P(Ph)$_3$ in both studied cell lines. However, as indicated this improve in cytotoxic activity compared to the other gold phosphine agents is rather small.

3.2. Cellular uptake and uptake into nuclei

Next we evaluated the cellular uptake of the complexes and their biodistribution into the nuclei of the tumor cells. The knowledge of these parameters is of great value in modern drug design and drug development as in many cases the ineffectiveness of certain experimental agents could be related to an insufficient cellular accumulation or an inappropriate intracellular distribution. However, for these measurements a bioanalytical method with high selectivity and sensitivity is required. For metal compounds several methods such as inductively coupled plasma mass spectrometry (ICP-MS) [24,25] atomic absorption spectroscopy (AAS) [13,22,26–28] or fluorescence microscopy [18,19,29–32] (for fluorescent complexes) exist, which fulfill these requirements. In the present study we used a recently described electrothermal AAS method, which was developed for the bioanalysis of gold phosphine complexes and is based on the standard addition method to correct for influences of the biological sample matrix [13]. Experiments were performed using 5.0 μM solutions of the agents and a short exposure period (6 h). Under these conditions no significant loss of cell biomass occurs due to toxic effects. Results were corrected for the respective protein contents of the samples and values are accordingly presented as nmol gold per g cellular protein.

As expected the lowest uptake values were observed for Cl–Au–P(Me)$_3$, which exhibits the lowest lipophilicity of the complexes under study. Somewhat higher values were found for the more lipophilic Cl–Au–P(Et)$_3$ and Cl–Au–P(tert-But)$_3$ but the differences between these 3 agents were in general not high. In contrast Cl–Au–P(Ph)$_3$ led to a significantly increased gold uptake in both investigated cell lines indicating a correlation with the stronger antiproliferative effects noted for this compound.

Based on certain biophysical parameters of HT-29 cells the nmol Au/g protein values obtained in HT-29 cells can be used to estimate the respective cellular molar concentrations [13]. For Cl–Au–P(Me)$_3$, Cl–Au–P(Et)$_3$, Cl–Au–P(tert-But)$_3$ and Cl–Au–P(Ph)$_3$ the results correlate to 15, 20, 21 and 41 μM, respectively. Thus, the cellular concentrations exceeded the exposure concentration (5 μM) approximately 3–8 fold. This is comparable to data obtained with the platinum antitumor drugs cisplatin or carboplatin [24] but well below the accumulation of the gold(I) phosphine lead compound auranofin, which reached 109 μM

Table 2
Uptake of chloro gold(I) phosphine complexes into the nuclei of HT-29 and MCF-7 cells after 6 h; n.d.: not detectable.

		HT-29	MCF-7
Cl–Au–P(Me)$_3$	nmol Au/g protein	21.6 ± 0.8	n.d.
	%	12 ± 1	n.d.
Cl–Au–P(Et)$_3$	nmol Au/g protein	62.4 ± 3.0	n.d.
	%	6 ± 1	n.d.
Cl–Au–P(tert-But)$_3$	nmol Au/g protein	84.3 ± 25.9	109.4 ± 39.3
	%	14 ± 4	16 ± 7
Cl–Au–P(Ph)$_3$	nmol Au/g protein	181.8 ± 14.2	57.6 ± 17.0
	%	10 ± 1	6 ± 2

(correlating to an approx. 22-fold accumulation) under identical experimental conditions [13].

As mentioned above we previously found only marginal amounts of gold in nuclei of tumor cells exposed to Cl–Au–P(Et)$_3$ [19]. Therefore, it was of interest to study if a variation of the substituents on the phosphor center might lead to an increase in accumulation of gold in the nuclei. Accordingly, the nuclei of HT-29 and MCF-7 cells treated with 5.0 μM of the gold complexes for 6 h were isolated and investigated for their gold content. The results of these experiments are expressed as nmol gold per g nuclear protein and as percentage of the overall gold uptake.

As expected exceptionally low amounts of metal per nuclear protein were determined after exposure to Cl–Au–P(Me)$_3$ and Cl–Au–P(Et)$_3$. In MCF-7 cells gold levels were below the limit of detection of the used AAS method. Comparison with results found for Cl–Au–P(Et)$_3$ after longer incubation during our previous study (76 nmol/μg in HT-29 cells and 52 nmol/g in MCF-7 cells after 24 h [19]) indicated that the nuclear gold content could be somewhat increased over time.

In both HT-29 and MCF-7 cells the nmol/μg levels were significantly incremented after exposure to Cl–Au–P(tert-But)$_3$ and Cl–Au–P(Ph)$_3$.

The nmol/μg values obtained within nuclear uptake experiments describe the amount of gold found in a certain nuclear biomass. However, they do not reflect the uptake of gold in the nuclei in relation to the overall uptake of the metal. Therefore, we also determined the ratio of cellular gold (overall uptake) and nuclear gold (see percentage values in Table 2). For this purpose an aliquot was taken from the cell suspensions before isolation of the nuclei (see Section 2.4 for details). The measured values (if detectable) corresponded to 6–16% of gold taken up into the cells and were highest for Cl–Au–P(t-But)$_3$ (14% and 16%, respectively). This demonstrates that the relative biodistribution of gold into the nuclei is slightly enhanced for Cl–Au–P(tert-But)$_3$ and that the elevated nmol/g values noted for Cl–Au–P(Ph)$_3$ can be mainly attributed to the higher overall cellular uptake of this compound. From these experiments it can be also concluded that the majority of gold reaching the cells is not transported into the nuclei, which agrees well with the high relevance of non DNA related properties of this class of metal species. However, this can not completely rule out an involvement of DNA interactions as also the nuclear platinum levels found with cisplatin correlate to a low percentage of overall cellular platinum and are in general low [33,34].

4. Conclusion

Gold(I) phosphine complexes showed appreciable antiproliferative effects in the two cultured human tumor cell lines HT-29 and MCF-7. The cytotoxic activities were most marked for Cl–Au–P(Ph)$_3$. This could be in part attributed to an enhanced uptake of this compound into the cells correlating also with higher levels of gold in the nuclei isolated from the cells. In relation to the overall cellular uptake somewhat enhanced nuclear gold levels were noted for Cl–Au–P(tert-But)$_3$. As an overall result it can be stated that the cytotoxicity, cellular and nuclear uptake values of various chloro gold(I) phosphine complexes indicated a slightly positive influence of larger and more lipophilic substituents such as phenyl.

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