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In vitro antitumour and hepatotoxicity profiles of Au(I) and Ag(I) bidentate pyridyl phosphine complexes and relationships to cellular uptake

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Abstract

In this study we characterised the *in vitro* antitumour and hepatotoxicity profiles of a series of Au(I) and Ag(I) bidentate phenyl and pyridyl complexes in a panel of cisplatin-resistant human ovarian cancer cell-lines, and in isolated rat hepatocytes. The gold and silver compounds overcame cisplatin-resistance in the CH1-cisR, 41M-cisR and SKOV-3 cell-lines, and showed cytotoxic potencies strongly correlated with their lipophilicity. Complexes with phenyl or 2-pyridyl ligands had high antitumour and hepatotoxic potency and low selectivity between different cell-lines. Their cytotoxicity profiles were similar to classic mitochondrial poisons and an example of this type of compound was shown to accumulate preferentially in the mitochondria of cancer cells in a manner that depended upon the mitochondrial membrane potential. In contrast, complexes with 3- or 4-pyridyl ligands had low antitumour and hepatotoxic potency and cytotoxicity profiles similar to 2-deoxy-D-glucose. In addition, they showed high selectivity between different cell-lines that was not attributable to variation in uptake in different cell-types. The *in vitro* hepatotoxic potency of the series of gold and silver compounds varied by over 61-fold and was closely related to their lipophilicity and hepatocyte uptake. In conclusion, Au(I) and Ag(I) bidendate pyridyl phosphine complexes demonstrate activity against cisplatin-resistant human cancer cells and *in vitro* cytotoxicity that strongly depends upon their lipophilicity.

Keywords: Ovarian cancer cell-lines; Gold(I) and silver(I) phosphines; Antitumour activity; Hepatocytes

1. Introduction

Metal-based drugs have important roles in cancer chemotherapy but their medical applications are often limited by tumour resistance. Ovarian cancer, for instance, is commonly treated using cisplatin but some tumours are intrinsically unresponsive to the drug or become so after an initial response. Several mechanisms of cisplatin-resistance have been identified in human ovarian cancer cell-lines. For example, the 41M-cisR cell-line (a cisplatin resistant sub-line of the 41M ovarian cancer cell-line) has acquired resistance due to reduced cisplatin uptake [1], the CH1-cisR cell-line (a cisplatin resistant sub-line of the CH1 ovarian cancer cell-line) has increased capacity for repair and tolerance of cisplatin-DNA adducts [1], and the SKOV-3 cell-line has intrinsic cisplatin-resistance based in-part upon elevated levels of glutathione [2].

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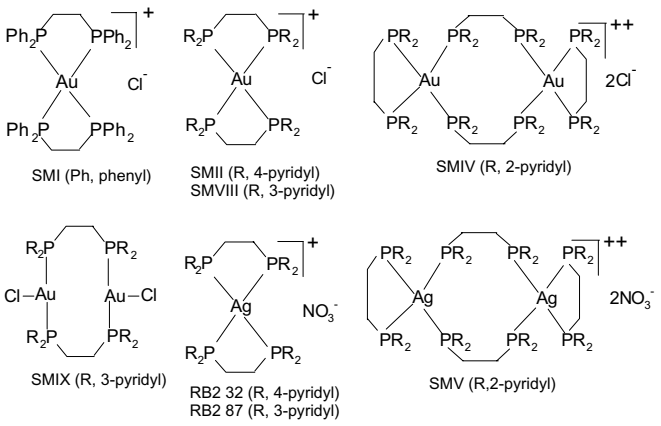

Fig. 1. Chemical structures of Au(I) and Ag(I) bidendate phenyl and pyridyl phosphine complexes.

One potential treatment approach for cisplatin-resistant cancer is with the Au(I) phosphine compounds auranofin [3], or $[Au(dppe)_2]Cl$, where dppe is 1,2-bis(diphenylphosphino)ethane). $[Au(dppe)_2]Cl$ is highly cytotoxic to tumour cells [4] probably due to a mechanism involving disruption of their mitochondria [5–8]. Other Au(I) compounds aurothiomalate [9] and aurothioglucose [10] are being investigated as new agents for the treatment of non-small-cell lung cancer. $[Au(dppe)_2]Cl$ has activity against cisplatin-resistant clones of the P388 murine leukaemia line [6] but its development as an antitumour agent was interrupted by the observation of hepatotoxicity in dogs [11]. In an attempt to achieve greater selectivity for tumour cells versus normal cells, we previously modified the diphosphine ligands of metal complexes related to $[Au(dppe)_2]Cl$ in order to vary the hydrophilic character of the complexes (Fig. 1). We retained aromatic substituents, which appear to be important for antitumour activity [12], but replaced some, or all by hydrophilic pyridyl groups [13–16]. As a result, a series of 1:2 adducts of Au(I) and Ag(I) with bidentate pyridylphosphines compounds were prepared with potential for activity in cisplatin-resistant ovarian cancer and reduced hepatotoxicity relative to $[Au(dppe)_2]Cl$ [17]. In the current study, we aimed to characterise this series of compounds with respect to their *in vitro* antitumour and hepatotoxicity profiles, in cisplatin-resistant human ovarian cancer cell-lines and isolated rat hepatocytes respectively, and to relate these cytotoxicity profiles to those of classic mitochondrial poisons and to their cellular uptake.

2. Materials and methods

2.1. Chemicals and reagents

The bidentate pyridyl phosphine ligands [14] and their Au(I) or Ag(I) complexes were synthesised according to published procedures for $[Au(dppe)_2]Cl$ (SMI) [18], $[Au(d4pype)_2]Cl$ (SMII), $[Au(d2pype)_2]_2Cl_2$ (SMIV) and $[Au(d3pype)_2]Cl$ [16], and $[Ag(d4pype)_2]NO_3$ (RB2 32) and $[Ag(d2pype)_2]_2(NO_3)_2$ (SMV)) [15], where dppe is 1,2-bis(diphenylphosphino)ethane and d*n*pype is 1,2-bis(di-*n*-pyridylphosphino)ethane. Their lipophilicity was determined by measuring the $\log k_w$ using an HPLC method described previously [19]. MKT-077 was a gift from Dr. L.B. Chen in Dana-Farber Cancer Institute, Boston, Mass., USA. Cisplatin, rhodamine 123, 2-deoxy-D-glucose, 2,4-dinitrophenol, valinomycin and other chemicals were purchased from Sigma, St Louis, MO, USA, unless stated otherwise. Drug stock solutions were made in 0.9% NaCl (Baxter Healthcare, Auckland, New Zealand) with occasional addition of ethanol (Scharlau Chemicals, Barcelona, Spain) or cremophor/ethanol of <0.3% to assist solubility. Further dilutions were carried out with phenol red-free or supplemented culture medium to make up 100–5000 μM working solutions.

2.2. Cell cultures

CH1, 41M, SKOV-3, CH1-cisR and 41M-cisR human ovarian carcinoma cell-lines were obtained from Dr. L. Kelland at The Institute of Cancer Research, Surrey, UK. Cells were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum, 200 U/mL penicillin, 100 μg/mL streptomycin and 0.3 g/mL glutamate (Invitrogen, Auckland, New Zealand) in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Cells were passaged every 3–7 days using a 0.5% trypsin-5.3 mM EDTA solution (Invitrogen) with routine mycoplasma check using H 33258 staining.

2.3. Cytotoxicity determination by sulforhodamine B assay in tumour cells

The sulforhodamine B (SRB) assay was used to determine the IC_{50} values for each compound as described previously [19]. Briefly, cells in exponential phase of growth were seeded in 96-well plates at the optimal density in triplicate, and 24 h later, they were treated with one of the test compounds at the indicated concentration range for 96 h. Then the medium was removed and cells were fixed by 10% trichloroacetic acid and stained with 0.4% SRB solution in 1% acetic acid (Scharlau Chemicals, Barcelona, Spain). After washing with 1% acetic acid and solubilising the SRB dye with 10 mM Tris-base (pH 10.5), the absorbance of each stained well was measured at 570 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvales, CA, USA). IC_{50} was defined as drug concentration reducing absorbance by 50% of the untreated control wells. MKT-077, rhodamine 123, 2-deoxy-D-glucose, 2,4-dinitrophenol and cisplatin were employed as reference cytotoxic agents.

2.4. Gold uptake and subcellular distribution

For uptake studies, cells were seeded at the optimal density of $2–5 \times 10^4$ cells/well, respectively, and after 24 h trea-

ted with 80 μM of SMIX for up to 8 h. After the drug incubation, cultures were washed with ice-cold PBS and cells collected in 500 μL Milliq water. Concentration of gold was measured using an inductively coupled plasma mass spectrometry (ICP-MS) method and normalised by the cellular protein level (ng Au/mg protein) measured by a bicinchoninic acid (BCA) assay as previously reported [19]. For gold analysis, samples (300 μL) were digested in 70% nitric acid (500 μL) at 70 °C for 2 h then diluted 1:10 in Milliq water for ICP-MS analysis. A Hewlett-Packard HP4500 ICP-MS was used with a Babington (v-groove) nebuliser and a Scott double-pass spray chamber maintained at 2 °C. Gold was read at 197 amu and the sensitivity limit for detection was 0.05 ng/mL.

To investigate the subcellular distribution of gold, cells were homogenized in 5 volumes of a homogenisation buffer containing 0.25 M sucrose, 10 mM pH 7.4 HEPES, 1 mM EGTA and 0.5% bovine serum albumin using a homogenizer with a Teflon pestle in glass tube by 20 up-down strokes (Glas-Col, Terre Haute, IN, USA). After centrifuging the homogenate at 377*g* for 10 min, the pellets were reconstituted with the buffer and kept as the nuclear fraction. The supernatant was centrifuged at 156, 055*g* for 2.5 h and resulting supernatants and pellets were taken as the cytoplasmic and mitochondrial fractions, respectively. Lactate dehydrogenase (LDH) activity, gold content and protein level were determined using the LDH assay, ICP-MS method and BCA assay described elsewhere. Measured values for each fraction were expressed as a percentage to the total sample. To study the effect of valinomycin on gold uptake, cells were exposed to a range of concentrations of valinomycin concurrently with the test gold compound.

2.5. Primary culture of hepatocytes

Male Wistar rats weighing 200–250 g were obtained from the animal resource unit at the University of Auckland and all procedures were approved by the local animal ethics committee. Hepatocytes were isolated using a collagenase-perfusion method as described [20] with slight modifications. Briefly, under the terminal anaesthesia induced by an intraperitoneal dose (90 mg/kg) of sodium pentobarbitone (Chemstock Animal Health, Christchurch, New Zealand), the inferior vena cava of animal was exposed and cannulated with a 20G×11/4'' Surflo I.V. catheter (Terumo Australia Pty. Ltd. Melbourne). Livers were perfused at a rate of 10–20 mL/min with the superior vena cava clamped and the portal vein cut. The liver was firstly perfused with 200 mL of carbogen (95% oxygen and 5% CO_2)-saturated modified Krebs-Henseleit buffer (20 mM HEPES, 20 mM $NaHNO_3$, 26 mM NaCl, 1.16 M KCl, 54 mM $MgSO_4$, 8 mM KH_2HPO_4, 40 mM Na_2HPO_3, 30 mM NaOH and 0.1 mM EDTA), then with the same buffer supplemented with 0.12 mg/mL collagenase (Boehringer Mannheim, Germany) and 1 mM $CaCl_2$. After perfusion, the liver was excised and gently teased apart in 40 mL calcium containing buffer in a Petri dish. The cell suspension was filtered through two layers of nylon mesh (250 μm and 200 μm) and then centrifuged at 50*g* for 2 min at 4 °C. Cell pellets were resuspended in DMEM following two more washes. Cell preparations with a viability of >80%, measured by trypan blue exclusion, were used in this study. Hepatocytes were seeded in a six-well Nunc plates pre-coated with collagen solution (type IV, 6 mg/mL) at a density of 5×10^5 viable cells/mL/well and cultured in above-mentioned supplemented DMEM at 37 °C. After 1 h, the medium was replaced with that containing different concentration of compounds of interest.

2.6. Toxicity and uptake in primary hepatocytes

The viability of the isolated rat hepatocytes after drug exposure was determined by measuring LDH leakage into the culture medium [21]. Rat primary hepatocytes were exposed to test or reference compounds dissolved in the DMEM at a range of concentrations for up to 24 h. At each time point, 50 μL of culture medium was removed. At the final time point, 2% Triton-X was added and hepatocytes were scraped off the bottom of the well. Four volumes of LDH-NAD^+ solution (40.8 mM lactic acid, 6.5 mM acid free NAD^+, 100 mM Tris base, pH 8.9) was added and absorbance at 340 nm was measured every 5 s over a 3 min period on the SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Hepatocyte viability was expressed as the percentage of LDH leakage relative to the total enzymatic activity of the well. The IC_{50} for *in vitro* hepatotoxicity was expressed in units of μM h, i.e. equivalent to an one-hour drug exposure.

To study uptake, hepatocytes were exposed to gold complexes for 3 h at increasing concentrations (0–750 μM). At the end of incubation, cells were washed several times with ice-cold phosphate buffered saline before suspension in 500 μL Milliq water. Concentration of gold and protein were determined using an ICP-MS method and a BCA assay respectively as previously reported [19]. Data were expressed as ng Au/mg protein.

2.7. Statistical analysis

The significance of relationships between experimental parameters was assessed using linear regression and rank correlation. A two-sided *P*-value of less than 0.05 or non-overlapping 95% confidence intervals was regarded as statically significant.

3. Results

3.1. Activity against cisplatin-resistant cancer cells

The series of Au(I) and Ag(I) compounds showed *in vitro* antitumour activity against cisplatin-resistant human ovarian cancer cell-lines (Table 1). The IC_{50} for cisplatin in the CH1-cisR cell-line was 5.2-fold higher than in the CH1 cell-line. In contrast, the IC_{50} values for the Au(I)

and Ag(I) compounds were on average only 1.2-fold higher in the CH1-cisR relative to the CH1 cell-line. Cisplatin had an IC_{50} in the 41M-cisR cell-line that was 2-fold higher than in the parent 41M cell-line. In contrast, the 41M-cisR cell-line, on average, had similar sensitivity (1.0-fold) to the Au(I) and Ag(I) complexes as the 41M cell line. The SKOV-3 cell-line was intrinsically-resistant to cisplatin with an IC_{50} of 5.23 μM while three of the Au(I) and Ag(I) compounds had IC_{50} values less than 1.5 μM. These results show that the Au(I) and Ag(I) complexes were active against some cisplatin-resistant human ovarian cancer cells.

The lipophilicity ($\log k_w$) of the series of Au(I) and Ag(I) compounds correlated with their *in vitro* antitumour activity against human ovarian cancer cell-lines (Table 1, Fig. 2). Across the whole series of compounds, antitumour IC_{50} values varied by five orders of magnitude, and lipophilicity varied by over three $\log k_w$ units. Ranking of the compounds according to their lipophilicity significantly correlated with their cytotoxic potency in the CH1 ($t = 0.7143$; $P < 0.046$), CH1-cisR ($t = 0.5238$; NS), 41M ($t = 0.9048$; $P < 0.01$), 41M-cisR ($t = 0.9048$; $P < 0.01$) and SKOV-3 ($t = 0.8095$; $P < 0.046$) cell-lines. In each cell-line there was a trend for decreasing cytotoxic potency with increasing hydrophilicity.

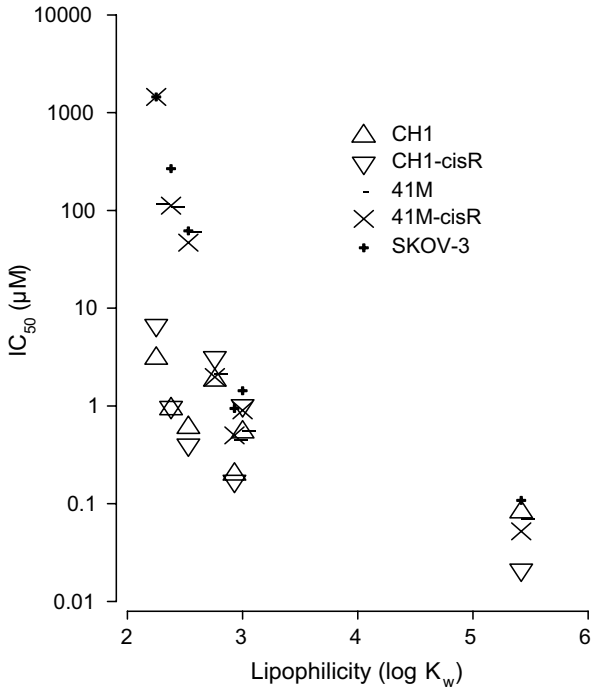

Fig. 2. Compound lipophilicity ($\log k_w$) correlated with cytotoxicity (IC_{50}) against human ovarian cancer cell-lines.

3.2. Activity profiles of phenyl and 2-pyridyl complexes

Complexes with phenyl or 2-pyridyl phosphine ligands had antitumour profiles characterised by high cytotoxic potency and low selectivity between different cell-lines. Compared to other compounds in the series, the phenyl and 2-pyridyl complexes had low IC_{50} values, ranging from 0.021 to 1.43 μM. In addition, the IC_{50} values for each compound varied by less than 5.5-fold between the five cancer cell-lines. A similar lack of selectivity was displayed by the 2-pyridyl ligand, dinitrophenol, rhodamine 123, MKT-077 and SMI, whose IC_{50} values varied across the panel of cell-lines by 5.2-, 1.4-, 2.0-, 3.5- and 5.1-fold, respectively. Since dinitrophenol [22], rhodamine 123 [23],

Table 1
In vitro antitumour and hepatotoxicity profiles of Au(I) and Ag(I) bidentate phenyl and pyridyl phosphine complexes

Compound	Formula	Lipophilicity $\log k_w$	IC_{50} (μM h) for hepatotoxicity	IC_{50} values for human ovarian cancer cell-lines (μM for 96 h exposure)					
				CH1	CH1-cisR	41M	41M-cisR	SKOV-3	Fold[b]
SMI	$[Au(dppe)_2]Cl$	5.42[a]	62	0.082	0.021	0.070	0.052	0.108	5.1
SMV	$[Ag_2(d2pype)_4](NO_3)_2$	3.0	92	0.54	1.01	0.55	0.9	1.43	3.6
SMIV	$[Au_2(d2pype)_4]Cl_2$	2.93	147	0.2	0.17	0.45	0.50	0.94	5.5
RB2 87	$[Ag(d3pype)_2]NO_3$	2.76	–	1.830	3.13	2.1	1.97	>100	>55
SMIX	$[Au_2(d3pype)_2]Cl_2$	2.53	681	0.6	0.4	60	46.7	61.7	154
SMVIII	$[Au(d3pype)_2]Cl$	2.38	–[c]	0.93	0.97	108	112	267	287
SMII	$[Au(d4pype)_2]Cl$	2.25	3752	3.06	6.68	116	1450	1450	473
RB2 32	$[Ag(d4pype)_2]NO_3$	–	241	4.28	4.43	2.25	1.53	>100	>65
RB2 16	d2pype	−1.1	>6000	3.95	8.88	1.7	3.3	4.1	5.2
RB2 48	d4pype	−1.1	>6000	16	16	260	200	333	21
DNP	–	–	–	111	145	115	132	158	1.4
2DG	–	–	–	180	159	1414	1664	3159	20
Cisplatin	–	−1.1	–	0.14	0.73	0.47	0.95	5.23	37.3
MKT-077	–	–	221	1.1	4.7	2.53	1.97	3.87	3.5
Rh-123	–	3.02	174	0.67	1.0	1.87	1.25	0.61	2

See Fig. 1 for structures. Abbreviations: dppe, 1,2-bis(diphenylphosphino)ethane; d*n*pype, 1,2-bis(di-*n*-pyridylphosphino)ethane; DNP, 2,4-dinitrophenol; 2DG, 2-deoxy-D-glucose; Rh-123, Rhodamine 123.

[a] Data values were expressed as mean of at least three replicates with %CV (coefficient of variation) ⩽ 20%.

[b] Fold of difference between the greatest and lowest IC_{50} values across a panel of human ovarian carcinoma cell-lines.

[c] Not applicable or not determined.

MKT-077 [24] and SMI [5–8] preferentially accumulate and/or interfere with mitochondria, the phenyl and 2-pyridyl complexes may also have an antimitochondrial mechanism of cytotoxicity.

Further evidence for their antimitochondrial mechanism of cytotoxicity came from studies of the cellular uptake of the 2-pyridyl digold complex SMIV in CH1 cells (Fig. 3a). During exposure to SMIV, gold became preferentially accumulated in the mitochondrial fraction of CH1 cells, compared to nuclear and cytoplasmic fractions. In addition, the cellular accumulation of SMIV was inhibited by valinomycin (Fig. 3b), a potassium ionophore that, by depolarising mitochondrial membranes, inhibits the mitochondrial uptake of cationic compounds [25].

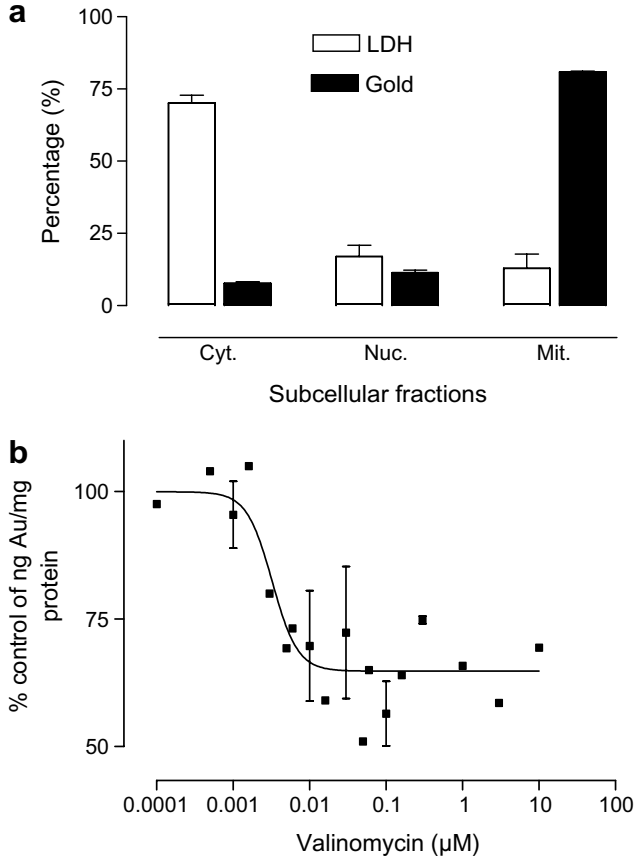

Fig. 3. Uptake studies of SMIV in the CH1 cell line. (a) Subcellular distribution of Au in CH1 cells after 4 h exposure to SMIV. Cytoplasmic (Cyt.), nuclear (Nuc.) and mitochondrial (Mit.) fractions of CH1 cell homogenates were prepared by differential centrifugation. Separation of subcellular fractions was confirmed by measuring lactate dehydrogenase (LDH) activity, a cytoplasmic marker. The Au content of each subcellular fraction was measured by ICP-MS and expressed as a percentage of the total gold content of CH1 cells. (Mean and standard error of 2–4 determinations). (b) Inhibition by valinomycin of the cellular uptake of SMIV. CH1 cells were exposed concurrently to SMIV (1 μM) and valinomycin for 4 h. The Au content of CH1 cells was measured by ICP-MS and expressed as a percentage of the Au content of CH1 cells not exposed to valinomycin. The solid line represents a sigmoidal E_{max} model fitted by nonlinear regression ($r^2 = 0.63$) with maximal inhibition of 65% of control and valinomycin concentration for half maximal inhibition of 3.25 nM.

3.3. Activity profiles of 3- and 4-pyridyl complexes

Complexes with 3- and/or 4-pyridyl phosphine ligands had antitumour profiles characterised by low cytotoxic potency and high selectivity between different cell-lines. Compared to other compounds, the 3- and 4-pyridyl compounds had high IC_{50} values, ranging from 0.4 to >100 μM. In addition their IC_{50} values varied by greater than 50-fold between the five cancer cell-lines. SMIX, for example, had IC_{50} values of 0.93, 108 and 267 μM in the CH1, 41M, and SKOV-3 cell-lines, respectively. During exposure to SMIX, CH1, 41M and SKOV-3 cells accumulated gold at an initial rate of 1.86, 2.07 and 1.27 ng Au/μg protein per hour, respectively (Fig. 4). There was no statistically significant difference in these values for the initial rate of SMIX accumulation between CH1, 41M and SKOV-3 cells despite their differing sensitivity to this compound.

The antitumour profiles of 3- and 4-pyridyl complexes were similar to 2-deoxy-D-glucose and unlike those of mitochondrial poisons. Like 3- and 4-pyridyl compounds, 2-deoxy-D-glucose displayed low cytotoxic potency and greater than 20-fold variation in IC_{50} values between the cell-lines. Similarly, the 4-pyridyl ligand alone showed low cytotoxic potency and greater than 20-fold variation in IC_{50} values between the cell-lines.

3.4. In vitro hepatotoxicity and hepatocyte uptake

In primary cultures of isolated rat hepatocytes, the gold and silver compounds showed *in vitro* hepatotoxicity that was closely related to their lipophilicity (Table 1). Across the series of compounds there was 61-fold variation in *in vitro* hepatotoxic potency. Ranking of the compounds according to their hepatotoxic potency correlated with their lipophilicity ($t = 1.00$; $P < 0.046$), with lipophilic compounds being more hepatotoxic than hydrophilic compounds. Rhodamine 123 and MKT-077 were also

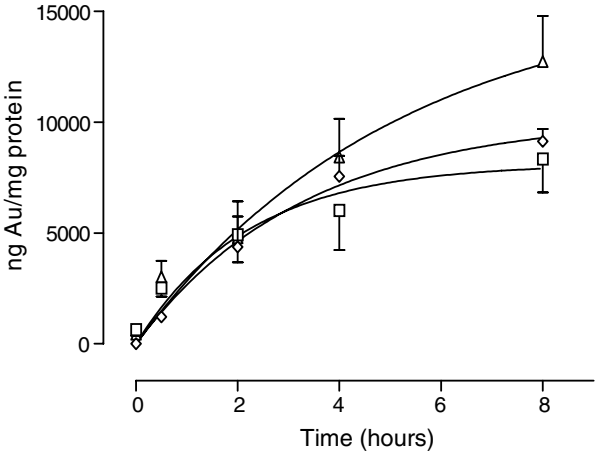

Fig. 4. Gold uptake in CH1 (□), 41M (△) and SKOV-3 (◇) ovarian tumour cell-lines exposed to 80 μM of SMIX for up to 8 h. Solid lines were fitted by non-linear regression and showed no statistically significant difference in the initial rate of uptake of Au between the three cell-lines.

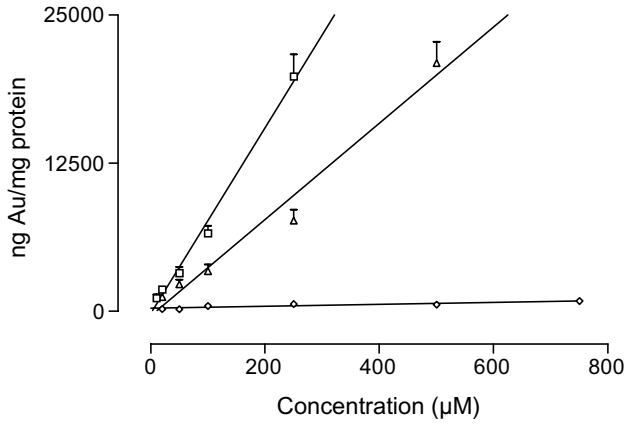

Fig. 5. Hepatocyte uptake of Au during exposure to SMI (□), SMIV (△) or SMII (◇) by concentration. Isolated hepatocytes were exposed to Au compounds for 4 h, then Au content determined by ICP-MS. Solid lines were fitted by linear regression and showed significant differences in the slopes of regression lines for different Au compounds.

hepatotoxic but with potencies less than the most toxic gold and silver compounds.

This *in vitro* hepatotoxicity appeared related to gold uptake by cultured hepatocytes and to the lipophicility of gold compounds (Fig. 5). SMI, the most lipophilic ($\log k_w$ 5.42) and most hepatotoxic gold compound, was taken up comparatively rapidly by cultured hepatocytes. In contrast, SMII, the least lipophilic ($\log k_w$ 2.25) and least hepatotoxic gold compound, was taken up comparatively slowly. SMIV, which had intermediate hepatotoxicity and lipophilicity ($\log k_w$ 2.93), displayed hepatocyte uptake between these extremes. The 2- and 4-pyridyl ligands alone lacked *in vitro* hepatotoxicity suggesting that this toxicity was unrelated to the inherent properties of these pyridyl ligands.

4. Discussion

We previously noted that the homologous series of Au(I) bidentate phosphine derivatives utilised in this study provides an excellent opportunity to investigate the relationship between drug lipophilicity and activity because the hydrophilic nature of these compounds can be varied over a very large range without losing aromatic character [19]. The degree of hydrophilicity depends critically on the position of the N atom on the pyridyl substituent [16]. The current study includes a series of Ag(I) analogs which have been characterised in the solid state and solution [15]. An essential consideration for biological evaluation of metal-based compounds is whether the structural integrity is maintained under the testing conditions. For bis-chelated Au(I) phosphine complexes the high thiol reactivity typical of linear Au(I) complexes is considerably reduced and previous studies have shown that the parent compound $[Au(dppe)_2]Cl$ (SMI) is stable in the presence of thiols and remains intact in blood plasma [6,26]. Similarly ^{31}P NMR studies showed that all the 1:2 adducts of Au(I) and Ag(I) with the ligands d*n*pype ($n = 2$, 3 or 4) are stable after prolonged incubation in RPMI 1640 cell culture medium containing 5% foetal calf serum [27] with the complexes SMIV, SMII and SMV shown also to remain intact in blood plasma [28]. Thus, it is a valid exercise to investigate the relationship between the drug lipophilicity and biological activity for this series of compounds.

The *in vitro* antitumour and hepatotoxicity profiles of Au(I) and Ag(I) bidentate pyridyl phosphine complexes, determined in the current study, suggested that these compounds may have more than one mechanism of cytotoxicity. One group of compounds, with phenyl or 2-pyridyl ligands and $\log k_w$ values ranging from 2.93 to 5.42, had *in vitro* activity profiles characterised by high cytotoxic potency and low selectivity between different cell-types. $[Au(dppe)_2]Cl$ (SMI), the prototype compound of this group, is known to interfere with oxidative phosphorylation [6–8]. The similarity of their *in vitro* antitumour profiles to those of classic mitochondrial poisons, such as 2,4-dinitrophenol and rhodamine 123, provided further evidence of an antimitochondrial mechanism of cytotoxicity. In addition, an example of this group of compounds (SMIV) was shown in this study to accumulate preferentially in the mitochondrial fractions of cancer cells in a manner that depended upon the mitochondrial membrane potential.

A second group of compounds, with 3-pyridyl or 4-pyridyl ligands and $\log k_w$ values ranging from 2.25 to 2.76, had distinctive *in vitro* antitumour profiles characterised by low cytotoxic potency and high selectivity between different cell-types. Each of these compounds showed greater than 50-fold range in IC_{50} values between the five cancer cell-lines that was not attributable to variation of their uptake between different cell-types. Their *in vitro* activity profiles were unlike classic mitochondrial poisons, but similar to the glycolysis inhibitor 2-deoxy-D-glucose. Overall, these findings suggest that these 3- and 4-pyridyl compounds have a different cytotoxicity mechanism compared to the antimitochondrial toxicity of Au(I) and Ag(I) phosphine complexes with phenyl and/or 2-pyridyl ligands. It is noted, however, that mitochondria may be a possible target for the 3- and 4-pyridyl compounds. Both the 2- and 3-pyridyl Au(I) complexes (SM IV and SMVIII) have been shown to induce cyclosporin-A sensitive swelling in isolated rat liver mitochondria, indicative of the induction of the membrane permeability transition [29]. The 2- and 4-pyridyl ligands alone also showed distinctive cytotoxicity profiles, which were generally similar to those of their respective metal complexes, suggesting that inherent properties of the pyridyl phosphine ligands may be key determinants of the mechanism of antitumour activity. Interestingly, the 2-pyridyl ligand (d2pype) was reported to be inactive ($IC_{50} > 100\ \mu M$) when evaluated against mouse B16 melanoma cells *in vitro* (2 h exposure) [30] but activity data for the phosphine ligands need to be interpreted with caution as they may be readily oxidised under

the testing conditions. Also, the pK_a values of the pyridyl *N* atoms are not known, and the protonation state of the pyridyl *N* atoms could vary between different samples of these ligands and for different testing conditions and this would influence cellular uptake and hence cytotoxicity.

We also showed in the current study that the lipophilicity of these Au(I) and Ag(I) bidendate pyridyl phosphine complexes was strongly correlated with their cytotoxic potency in five cancer cell-lines and in isolated rat hepatocytes. This confirms our previous observation of a similar relationship in the CH1 cell-line [19], and extends this earlier finding to a larger series of compounds and to different cell-types. These strong correlations may be due to lipophilicity determining the rate of cellular uptake and extent of binding to extra-cellular proteins under culture conditions, such that lipophilic compounds have more extensive binding to extracellular proteins but faster rates of uptake into cancer cells and hepatocytes than hydrophilic compounds of this general type. In keeping with this, the hepatocyte uptake of three gold compounds (SMI, SMIV and SMII) was shown, in the current study, to vary in close relationship to their lipophilicity and *in vitro* hepatotoxicity. In addition, we previously showed that altering the lipophilicity of gold phosphine complexes greatly affected both their uptake into CH1 cancer cells and their binding to plasma proteins present in the culture medium [19].

While our studies show the importance of the ligand in the overall lipophilicity, comparison of the cytotoxicity data for the Au(I) and Ag(I) complexes (Table 1) suggests that the metal ion also plays an important role. For example, the lipophilicity of the 2-pyridyl complexes SMV (Ag(I)) and SMIV (Au(I)) are nearly identical and yet the Au(I) has a higher cytotoxic potency in all of the ovarian cancer cell-lines tested, with the difference for the CH1-cisR line being particularly notable. Similarly, a different profile of activity is observed for the isostructural Au(I) and Ag(I) 3-pyridyl complexes (SMVIII and RB87). The Au(I) complex is less lipophilic than the Ag(I) analog but has a higher cytotoxic potency in the CH1 pair; on the other hand the Ag(I) complex is active (IC_{50} ca 2 μM) against the 41M pair whereas the Au(I) complex is inactive. While these bis-chelated complexes have been shown to be stable under the testing conditions, ligand exchange reactions via a ring-opening mechanism may allow the metal ion to react with critical protein targets within cells. Recent studies on the 3-carbon bridge analog of the Au(I) 2-pyridyl complex have shown that this complex induces apoptosis in breast cancer cells via the mitochondrial pathway, and inhibits the activities of the thiol and selenol containing antioxidant proteins thioredoxin and thioredoxin reductase [31]. While we have not investigated possible anion effects it is unlikely that the different anions (Cl^- and NO_3^-) in the Au(I) and Ag(I) complexes will account for the differences in activity. Early studies investigated the parent complex $[Au(dppe)_2]^+$ with a wide variety of different anions and showed that while the anion influenced the solubility of the complex, antitumour activity in animal models was retained [32].

Fine-tuning the hydrophilic-lipophilic balance of Au(I) and Ag(I) bidendate pyridyl phosphine complexes may be important for maximising their therapeutic potential, but the most optimal logk_w for this is unknown. We showed in the current study that reducing lipophilicity decreased their *in vitro* hepatotoxic potency by up to 61-fold. This finding has important implications for their potential as antitumour drugs since the earlier development of a lipophilic compound ($[Au(dppe)_2]Cl$ (SMI)) was interrupted by the observation of liver damage in dogs [11]. The therapeutic potential of compounds related to $[Au(dppe)_2]Cl$ (SMI) therefore depends at least in part upon improving their selectivity for cancer cells relative to hepatocytes and other normal cells. However, decreasing the lipophilicity of these gold and silver compounds reduced their *in vitro* antitumour potency as well as their hepatotoxicity. Ultimately, improved therapeutic selectivity must be demonstrated in animal tumour models after systemic administration of the test compounds. Our preliminary *in vivo* studies of the least hepatotoxic and least lipophilic compound (SMII) showed no significant antitumour activity after intraperitoneal administration to mice bearing transplanted subcutaneous Colon 38 [19] or CH1 xenografted tumours (unpublished observations). However, a compound with intermediate hepatotoxicity and lipophilicity (SMIV) has shown some *in vivo* activity [19]. So, the optimal logk_w value for minimising hepatotoxicity and maximising their therapeutic selectivity remains unclear, but compounds with intermediate values appear most promising so far.

In summary, this study of the *in vitro* antitumour and hepatotoxicity profiles of a series of Au(I) and Ag(I) bidendate pyridyl phosphine complexes demonstrated activity against cisplatin-resistant human cancer cells and showed that *in vitro* cytotoxic potencies depend strongly upon their lipophilicity.

5. Abbreviations

41M-cisR cisplatin resistant sub-line of the 41M ovarian cancer cell-line
BCA bicinchoninic acid
CH1-cisR cisplatin resistant sub-line of the CH1 ovarian cancer cell-line
DMEM Dulbecco's modified Eagle's medium
d*n*pype 1,2-bis(di-*n*-pyridylphosphino)ethane)
dppe 1,2-bis(diphenylphosphino)ethane
ICP-MS inductively coupled plasma mass spectrometry
LDH lactate dehydrogenase
SRB sulforhodamine B

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