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ORIGINAL ARTICLE

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Role of lipophilicity in determining cellular uptake and antitumour activity of gold phosphine complexes

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Abstract *Purpose*: The lipophilic cation $[Au(I)(dppe)_2]^+$ [where dppe is 1,2-bis(diphenylphosphino)ethane] has previously demonstrated potent in vitro antitumour activity. We wished to determine the physicochemical basis for the cellular uptake of this drug, as well as of analogues including the 1:2 adducts of Au(I) with 1,2-bis(di-n-pyridylphosphino)ethane (dnpype; n = 2, 3 and 4), and to compare in vitro and in vivo antitumour activity. *Methods and results*: Logarithmic IC_{50} values for the CH-1 cell line bore a parabolic dependence on drug lipophilicity, as measured either by high-performance liquid chromatography or by *n*-octanol-water partition. Cellular uptake of drug, as measured by inductively coupled plasma mass spectrometry, varied by over three orders of magnitude over the series. Logarithmic uptake had a parabolic dependence on drug lipophilicity but a linear relationship to logarithmic IC_{50} values. Free drug concentrations were determined under the culture conditions and logarithmic free drug IC_{50} values and uptake rates were linearly related to lipophilicity. Uptake of drug in vivo in tissue from murine colon 38 tumours was approximately proportional to the dose administered. Host toxicity varied according to lipophilicity with the most selective compound having an intermediate value. This compound was also the most active of those tested in vivo, giving a growth delay of 9 days following daily intraperitoneal dosing (10 days) at 4 μmol kg^{-1} day^{-1}. It was also significantly more active than another lipophilic cation, MKT-077. *Conclusions*: Alteration of lipophilicity of aromatic cationic antitumour drugs greatly affects cellular uptake and binding to plasma proteins. Changes in lipophilicity also affect host toxicity, and optimal lipophilicity may be a critical factor in the design of analogues with high antitumour activity.

Key words Lipophilic cation · Mitochondria · Hydrophobicity · Cellular uptake · Protein binding

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Introduction

The use of aromatic cations (also known as lipophilic cations) as anticancer drugs has had a long history. Strongly positively charged terephthalanilide derivatives were developed for clinical trials over 30 years ago, but were abandoned because of toxicity [5]. Bisquaternary derivatives of terephthalanilides, as well as a large number of related bisquaternary salts, were developed in an approach to controlling drug lipophilicity by appropriate substitution at the quaternary centre [2]. Many of these compounds showed excellent activity against the transplantable L1210 leukaemia in mice and activity was demonstrated to be a parabolic function of drug lipophilicity [17, 19]. One compound, ''quinolinium dibromide'', was selected for advanced preclinical evaluation but toxicity prevented further development [27]. A fluorescent member of this series was shown to localise in mitochondria of tumour cells [25].

Studies with other aromatic cations such as rhodamine 123 [23], dequalinium [38] and pyronine Y [18] suggested that a common feature of these compounds is to concentrate in mitochondria. Other investigations led to the identification of active antitumour compounds such as ditercalinium [30], AA-1 [36] and MKT-077 [24], the latter having been advanced to phase I clinical trial. All appear to have antimitochondrial effects [26, 30].

The metal-based aromatic cation $[Au(I)(dppe)_2]^+$ (where dppe is 1,2-bis(diphenylphosphino)ethane) was found to have potent in vitro antitumour activity as well as activity against several tumour models in mice [8]. There was some evidence for an antimitochondrial mode of action [20, 22] and preclinical development of $[Au(dppe)_2]^+$ was abandoned after the identification of severe hepatotoxicity in dogs [31] attributed to alterations in mitochondrial function [21, 35]. A series of derivatives of $[Au(dppe)_2]^+$ have been prepared with a variety of different bidentate pyridyl phosphine ligands, providing a series of structurally similar compounds (shown in Fig. 1) in which the hydrophilic character covers a very large range [11]. This has provided an opportunity to investigate the relationship of drug lipophilicity to activity.

Materials and methods

Materials

The gold complexes were synthesised in the laboratory of Dr. Berners-Price according to published procedures: $[Au(dppe)_2]Cl$ (SMI) [6], $[Au(d2pype)_2]_2Cl_2$ (SMIV), $[Au(d3pype)_2]Cl$ (SMVIII) and $[Au(d4pype)_2]Cl$ (SMII) [12], $[Au(2pyppe)_2]Cl$ (SMIII) and $[Au_2(d3pype)_2]Cl_2$ (SMIX) [13]. MKT-077 was supplied by Dr. L.B. Chen (Dana-Farber Cancer Institute, Boston, Mass., USA). Other materials were obtained from Sigma Chemicals (St Louis, Mo., USA) or Life Technologies (Auckland, New Zealand) unless indicated. Drug stock solutions were made up at 300 μM before each experiment. SMII, SMVIII, SMIX and MKT-077 were dissolved in 0.9% (w/v) NaCl (Baxter Healthcare, Auckland, NZ). SMIV and SMIII were dissolved in 20 μl ethanol (Scharlau Chemicals, Barcelona, Spain) and made to volume with phenol red-free Dulbecco's modified Eagle medium. SMI was dissolved in 50 μl ethanol and added to phenol red-free Dulbecco's modified Eagle medium containing 1% (v/v) fetal calf serum. Final concentrations of ethanol under culture conditions were $< 0.3\%$ (v/v).

Measurement of lipophilicity

The lipophilicity of the series of compounds was determined using an HPLC technique for measuring drug log k_w [15]. The HPLC system consisted of a Waters model 510 pump, a Beckman Ultrasphere C18 column (4.6 × 150 mm, 5 μm) and a mobile phase comprising 50 mM phosphate buffer (pH 7.4) and varying concentrations of methanol pumped at 1 ml/min. Compounds were dissolved in methanol at 500 μM and injected onto the column (50 μl). UV absorbance was used for detection. Lipophilicity was determined by measuring the capacity factor (capacity factor = (retention volume − void volume)/(void volume) for each compound when the (actual or projected) mobile phase composition was 100% aqueous. Log k_w values were the averages of three determinations.

Drug lipophilicity was also determined by measuring log P using a shake-flask method [1]. Aqueous sodium chloride (0.9% w/v) and organic (*n*-octanol) phases were saturated for 1 week. Drugs were dissolved at a final concentration of 300 μM in the aqueous phase. An equal volume of saturated *n*-octanol was added and the solutions mixed for 30 min (45 rpm). Samples were centrifuged (1,500 *g*, 10 min) and the drug content of the organic and aqueous phases was determined by UV absorbance (280 nm). Log P was defined as the logarithmic ratio of drug concentrations in the organic and aqueous phases.

Culture techniques

The CH-1 human ovarian carcinoma cell line was obtained from Dr. Lloyd Kelland (Institute of Cancer Research, Surrey, UK). Cells were cultured as monolayers in Dulbecco's modified Eagle medium supplemented with fetal calf serum (10% v/v) and penicillin-streptomycin-glutamine (1% v/v) in an atmosphere of 5% CO_2/95% air at 37 °C. Cells were passaged every 3–7 days using trypsin (0.5% w/v) and disodium EDTA (0.2% w/v) in phosphate-buffered saline.

Growth inhibition assay

To obtain cells in the exponential phase of growth, cells were plated in 96-well plates (1,000 cells in 100 μl culture medium per plate) and incubated for 24 h in an atmosphere of 5% CO_2/95% air at 37 °C. Drug stock solutions were diluted in culture medium and added (100 μl per well) in triplicate to achieve exposure concentrations ranging from 0.005 to 100 μM. After incubation under the same conditions for 96 h, the drug-containing medium was poured off and the cells were fixed using 0.2 ml cold trichloroacetic acid (10% w/v). Plates were washed and cells stained with sulphorhodamine B (0.4% w/v) in 1% acetic acid (Scharlau Chemicals). After washing with acetic acid (1% v/v), the sulphorhodamine B dye was solubilised with TRIS-base (10 mM). Absorbance of stained wells was read at 564 nm. Growth inhibition was quanti-

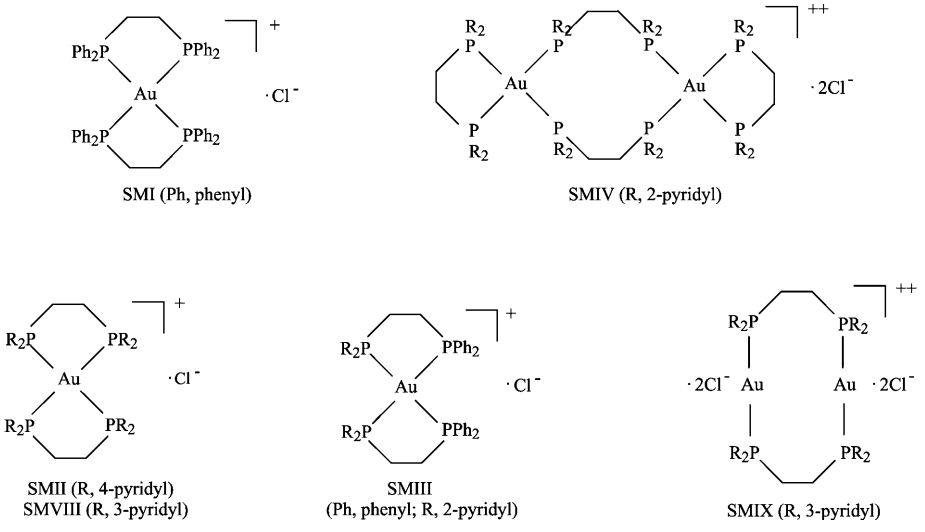

Fig. 1 Structures of gold phosphine complexes. SMIV has a dimeric structure $[Au(d2pype)_2]_2Cl_2$ in the solid state but in solution dissociates to give a solvent-dependent equilibrium mixture of monomeric and dimeric forms [12]. At the low concentrations used in the biological studies the complex will exist largely in the monomeric form [7, 12]. SMIX dissociates in aqueous solution to give an equilibrium mixture of $[Au_2(d3pype)_2]Cl_2$ (SMIX) and $[Au(d3pype)_2]Cl$ (SMVIII) [13]

tated by IC_{50} values, which were defined as drug concentrations reducing absorbance by 50% (average of three to nine determinations).

Measurement of drug uptake

Six-well plates were seeded with 5×10^4 cells in 3 ml culture medium per well. Cultures were incubated at 37 °C in an atmosphere of 5% CO_2/95% air until 70–80% confluent. The culture medium was then removed and replaced with drug containing medium at six concentrations (0–200 μM). After exposure to drug for 0.25, 0.5, 1, 2 or 4 h, the medium was removed and the cell monolayer washed four times with ice-cold phosphate-buffered saline. Milli-Q water (500 μl) was then added and the cell monolayer was scraped off the culture dish. A small sample (50 μl) was set aside for measuring protein content using the bicinchoninic acid reagent assays (BCA and BCA protein assay reagent; instructions 23230 and 233225, Pierce Chemicals, Rockford, Ill., USA). Samples (300 μl) were digested in 70% HNO_3 (500 μl) at 70 °C for 2 h then diluted 1:10 in water for inductively coupled plasma mass spectrometry (ICP-MS) analysis. Gold analysis was undertaken using a Hewlett-Packard HP4500 ICP-MS 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 of detection was 0.05 ng/ml. Other ICP-MS operating conditions were similar to those previously reported [33]. Data were fitted to one phase exponential equation $y = y_{max}(1 - e^{-kx})$, where y_{max} is the steady-state concentration of drug and k is the rate constant. The initial rate of drug accumulation was defined as $k \cdot y_{max}$. The dependence of initial drug accumulation rate on dose was analysed by linear regression ($n = 6$–15) and expressed as pmol mg^{-1} cellular protein h^{-1} μM^{-1} drug.

Measurement of free drug concentration

Drug solutions (0.1–100 μM) were made up in culture medium containing fetal calf serum (10% v/v) and left to sit in six-well plates at 37 °C for 1 h. Solutions (2.5 ml) were ultrafiltered using Centrisart tubes (cat. number 13239, MW cut-off 10,000; Sartorius, Goettingen, Germany) at 2,000 *g* for 15 min at room temperature. Gold content of ultrafiltrates (500 μl) was determined by ICP-MS. Binding of drugs to the ultrafiltration tubes and sample introduction equipment was minimised by adding a small amount of fetal calf serum (50 μl) to the ultrafiltrate side of the tubes before ultrafiltration, and cleaning the ICP-MS tubing between samples with 1% SDS (w/v). Free drug concentration (free fraction) was determined using linear regression analysis of added versus measured gold concentration ($n = 5$).

Measurement of antitumour activity in mice

Colon 38 tumours were grown subcutaneously following implantation of 1-mm^3 fragments under anaesthesia (pentobarbitone 90 mg/kg). When the tumour diameters were 4 ± 1 mm, groups of five mice were injected intraperitoneally (daily for 10 days) with drugs dissolved in saline. Tumour diameters were measured with callipers and volumes calculated as 0.52 a^2b, where a and b are the minor and major tumour axes. Tumour growth delays were measured at a time when the mean logarithmic tumour volumes had increased fivefold from their initial volume.

Blood and tumours were collected from mice, under terminal halothane anaesthesia, 5–320 min after treatment ($n = 6$–11). Blood was centrifuged (1,500 *g* for 5 min) to prepare plasma. Plasma (100 μl) was diluted in 2.4 ml lysis buffer [ammonium EDTA (0.1% w/v), Triton X-100 (0.1% w/v); Serva, Heidelberg, Germany] in ammonium hydroxide (2.5% w/v; BDH Chemicals, Poole, UK) before analysis by ICP-MS. Tumours were washed in Milli-Q water, blotted dry and placed in preweighed 15-ml screw top tubes. Tumours were prepared for ICP-MS analysis as previously described [33]. They were left to stand overnight in 1 ml 70% nitric acid at room temperature. The following day tumours were digested at 70 °C for 2 h. Solubilized tissues were made to volume in 10-ml volumetric flasks using Milli-Q water. ICP-MS analysis was undertaken as described above. The local Animal Ethics Committee approved the work.

Results

Lipophilicity and inhibition of growth

Drug lipophilicity was measured by HPLC and *n*-octanol-water partition (Table 1). The two lipophilicity parameters (log k_w and log P) were linearly related ($r = 0.98$). The compounds had an extensive range of lipophilic character across the series. Growth inhibition was determined by growing CH-1 cells for 4 days in the presence of each drug. The IC_{50} values (Table 1) showed a second order (parabolic) dependence on drug lipophilicity (Fig. 2A; $r = 0.99$), with an initial fall and then a rise in IC_{50} values with increasing lipophilicity.

In vitro uptake

To determine whether the dependence of inhibition of growth on lipophilicity was related to drug uptake, cell-associated drug was measured as a function of time. For the more lipophilic drugs, uptake was rapid and complete by 2 h, while for the hydrophilic drugs uptake was linear over the time scale tested (4 h). Uptake determinations later than 4 h were hampered by cell loss due to

Table 1 Physicochemical and in vitro data of compounds tabulated in order of increasing lipophilicity

Compound	Log k_w[a]	Log P	IC_{50}[a] (μM)	Drug uptake[a,b]	Percentage free fraction[a]
SMI	5.42 ± 0.18	1.41	0.082 ± 0.013	498 ± 52	0.005 ± 0.005
SMIII	4.44 ± 0.10	1.14	0.017 ± 0.001	956 ± 249	0.16 ± 0.025
SMIV	2.93 ± 0.07	−0.92	0.20 ± 0.023	67.9 ± 6.3	44 ± 6.5
SMIX	2.53 ± 0.02	–	0.60 ± 0.17		18.7 ± 1.4
SMVIII	2.38 ± 0.06	−1.46	0.93 ± 0.07	2.23 ± 0.34	56 ± 0.2
SMII	2.25 ± 0.02	−1.77	3.06 ± 0.27	0.49 ± 0.07	59 ± 0.5
MKT-077		−1.6[c]	1.10 ± 0.16		

[a] Mean ± SEM (n = 3–15)
[b] pmol mg^{-1} cellular protein h^{-1} μM^{-1} drug
[c] Published data [37]

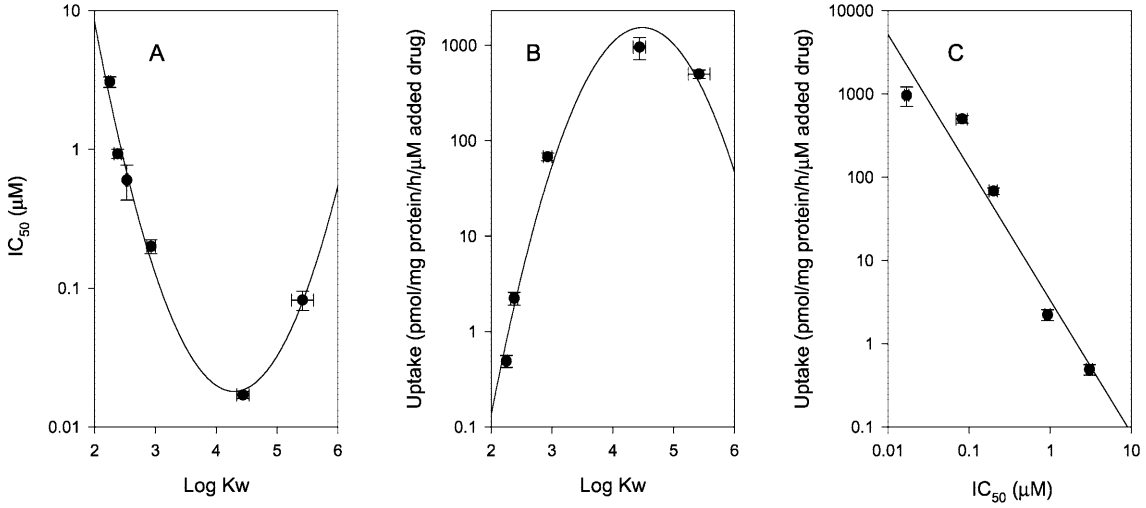

Fig. 2A–C Relationships between lipophilicity, uptake and growth inhibition of gold phosphine derivatives in CH-1 tumour cells in vitro. **A** Growth inhibition versus lipophilicity. **B** Uptake versus lipophilicity. **C** Growth inhibition versus uptake. *Vertical and horizontal lines* represent the SEM ($n = 3$–15). The *symbols* represent compounds with differing log k_w values: SMI (5.42); SMIII (4.44); SMIV (2.93); SMIX (2.53); SMVIII (2.38); SMII (2.25)

drug cytotoxicity. Uptake data for SMI and SMIV are shown in Fig. 3. The initial rate of uptake (Table 1) was found also to show a parabolic dependence on drug lipophilicity (Fig. 2B; $r = 0.99$). A linear correlation was obtained between logarithmic IC_{50} values and logarithmic uptake (Fig. 2C; $r = 0.97$). However, the slope of the regression (−1.59), indicated that halving the IC_{50} value was associated with a tripling of the uptake rate.

Correction for protein binding

The high affinity of the more lipophilic drugs with cells indicated that they were also likely to bind avidly to serum proteins present in the culture medium. Percentage free drug fractions were therefore determined in the presence of 10% fetal calf serum, and showed a very extensive range (Table 1). For SMI, the free drug concentration was within the error of determination. The logarithmic free fraction was linearly related to log k_w (Fig. 4A; $r = 0.98$). The data in Fig. 3 enabled the determination of free drug IC_{50} values and uptake rates. Both parameters, when expressed in logarithmic units, were linearly related to lipophilicity (Fig. 4B; $r = 0.99$ and Fig. 4C; $r = 0.99$). Moreover, the range of IC_{50} values was almost 10^6 and the range of uptake rates was 10^7.

Relationship of in vivo antitumour activity to lipophilicity

Three of the drugs (SMI, SMII and SMIV) were tested in vivo against the murine colon 38 adenocarcinoma. In a parallel experiment, plasma and tumour tissue were removed and analysed for gold content (Fig. 5). The aromatic cation MKT-077 was also tested for compari-

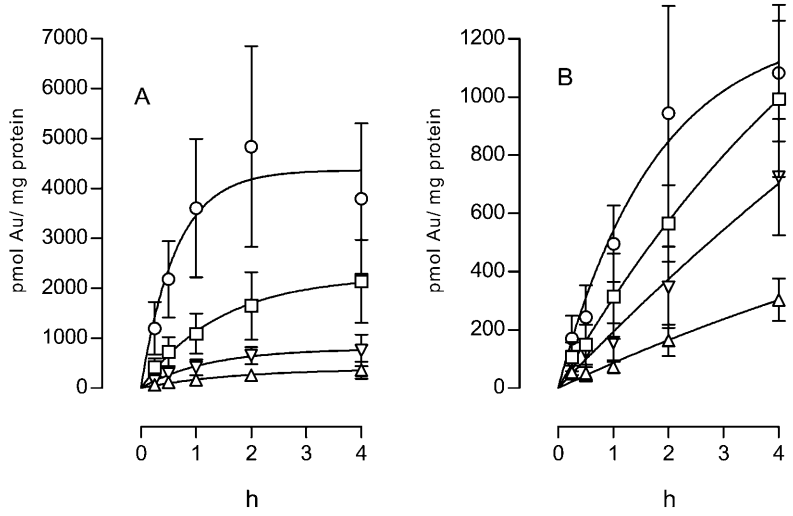

Fig. 3 Gold uptake in CH-1 cells exposed to SMI (**A**) or SMIV (**B**) at 1 (△), 2.5 (▽), 5 (□) and 10 μM (○). *Vertical lines* represent the SEM ($n = 3$)

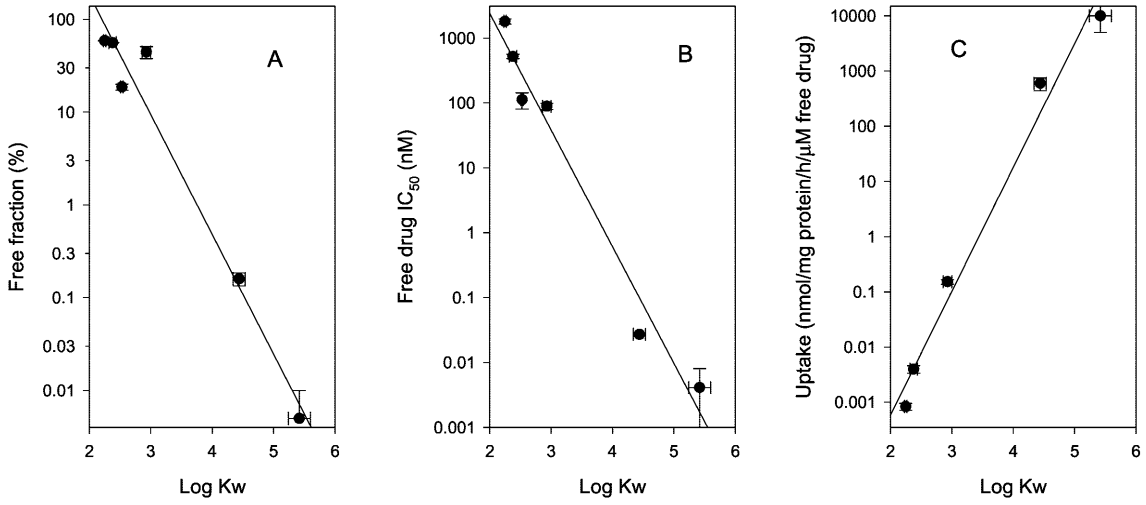

Fig. 4A–C Relationship between lipophilicity, free drug fraction, free drug uptake and free drug growth inhibition of gold phosphine derivatives in CH-1 tumour cells in vitro. **A** Free fraction versus lipophilicity. **B** Free drug IC_{50} versus lipophilicity. **C** Free drug uptake versus lipophilicity. *Vertical and horizontal lines* represent the SEM ($n = 3$–15)

son. Drug was administered daily for 10 days, commencing when the tumour diameters were between 3 and 4 mm. SMIV was found to have significant activity in this system, inducing a 9-day growth delay (Fig. 5; Table 2). SMIV was also found to have higher gold concentrations in tumours and plasma than the other two compounds.

Discussion

The homologous series of gold-containing phosphine derivatives utilised in this study provides an excellent opportunity to relate lipophilicity to anticancer activity. The hydrophilic nature of these compounds can be varied over a very large range without losing aromatic character (Fig. 1) by replacement of the phenyl substituents on the diphosphine linkages with pyridyl ligands [10, 11, 12]. Recently improved methods allowed the synthesis of 3- and 4-pyridyl phosphines in good yield [14]. The degree of hydrophilicity depends critically on the presence of the pyridyl ligand and on the position of the N atom. Replacing phenyl ligands with 4-pyridyl groups has previously been shown to introduce major changes in the interaction of the compounds with solvent, without altering the nature of the AuP_4 core or the overall topology of the cation [11].

An important consideration for biological evaluation of metal-based compounds is whether they are stable in solution and especially under the testing conditions. Gold compounds, in particular, often react readily with thiols and cytotoxicity can be reduced by reaction with -SH groups in serum and cell culture media. For bis-chelated Au(I) phosphine complexes the high thiol reactivity is considerably reduced. $[Au(dppe)_2]Cl$ (SMI) has been shown to be stable in the presence of thiols and to remain intact in human plasma [8, 9]. Similarly, the pyridylphosphine compounds SMIV, SMII and SMVIII have been shown to remain intact after prolonged incubation in either blood plasma or cell culture media [7, 13]. The maintenance of the structural integrity of these metal-based compounds under the testing conditions therefore makes it a valid exercise to investigate the relationship between the drug lipophilicity and biological activity.

We have studied the inter-relationships among drug activity, uptake and lipophilic–hydrophilic balance. ICP-MS [4] was used to determine tumour-associated gold as a measure of drug uptake. Growth inhibitory activity, determined in vitro using CH-1 cells, was found to have a parabolic dependence on lipophilicity (Fig. 2), with cytotoxic potency showing a 1,000-fold variation across the series with a maximum at log k_w values of 4–5. Other workers have also shown parabolic relationships between lipophilicity and antitumour properties of aromatic cations [17, 19, 29]. Logarithmic drug uptake into CH-1 cells showed an almost exactly complementary parabolic dependence on lipophilicity, except that there was a 10,000-fold variation across the series. Logarithmic cytotoxicity was thus a linear function of logarithmic uptake (Fig. 2).

Since lipophilicity determines the degree of protein binding as well as cellular uptake, it is necessary to examine the cytotoxicity and uptake in relationship to free drug concentrations. Correction for free drug concentrations converted the parabolic dependence of activity and uptake on lipophilicity to a linear one: almost all (99%) of the non-linearity of the relationships to drug lipophilicity was explained by changes in free drug concentration under the culture conditions. Extracellular drug binding under test conditions could contribute to variability in drug activity of other homologous series of aromatic cations with differing lipophilicity.

Antitumour activity in vivo also showed an apparent dependence on drug lipophilicity (Fig. 5). The most lipophilic compound (SMI) had a low maximum tolerated dose, low concentrations of tumour-associated gold

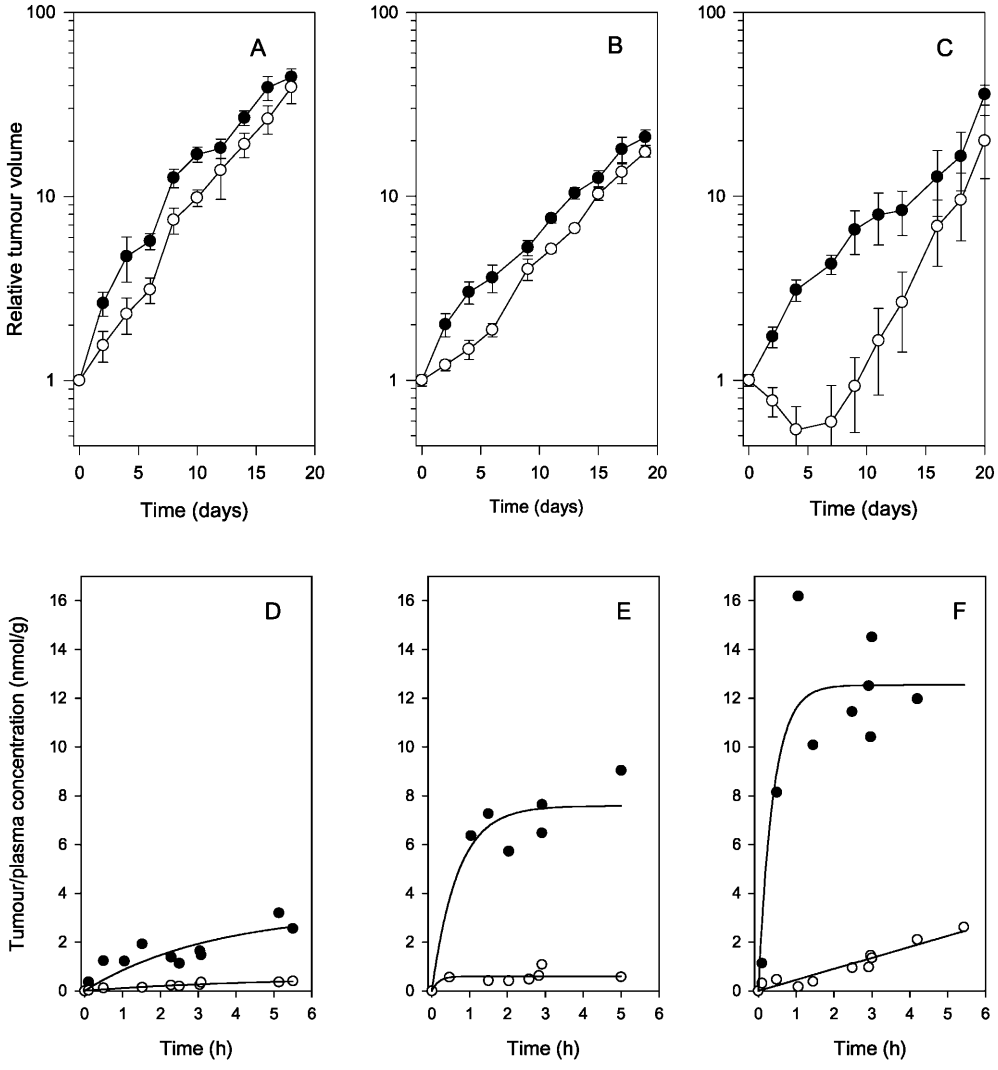

Fig. 5A–C Activity of SMI (**A**), SMII (**B**) and SMIV (**C**) against murine subcutaneous colon 38 tumours. Tumour volumes (relative to volume at commencement of treatment) of control mice (●) and treated mice (○). *Horizontal lines* represent the SEM ($n = 5$). **D–F** Drug concentrations (as Au) of SMI (**D**), SMII (**E**) and SMIV (**F**) in plasma (○) and colon 38 tumour tissue (●) as a function of time after drug administration

Table 2 In vivo data for mice with subcutaneous colon 38 tumours

Compound	Dose (Au) μmol kg^{-1} day^{-1}	Growth delay (days)[a]	Maximum plasma Au content[b] (μM)	Maximum tumour Au content[b] (nmol/g)
SMI	1.5	–	0.54 ± 0.16	3.3 ± 1.3
	2	3.5 ($P < 0.01$)		
	3	Toxic		
SMII	3	–	0.59 ± 0.09	7.6 ± 0.7
	6	2.5 ($P < 0.01$)		
	9	4 ($P < 0.01$)		
SMIV	8	9 ($P < 0.001$)	2.4 ± 0.1	13 ± 0.8[c]
	12	Toxic		
MKT-077	5	4 ($P < 0.01$)		

[a] Values in brackets denote significance of difference between control and treated groups when tumour volume of control group was four times that at the start of treatment ($n = 5$)
[b] Mean ± SEM ($n = 6$–11)
[c] Significantly higher than that for SMI or SMII ($P < 0.001$)

and no significant tumour growth delay. The most hydrophilic compound (SMII) also showed minimal growth delay. However, SMIV, which had intermediate lipophilicity, showed significant activity with a growth delay of 9 days (Fig. 5). Such activity compares with growth delays for etoposide, cyclophosphamide, doxorubicin and 5-fluorouracil of 1.5, 7, 8 and 14 days, respectively, under comparable conditions [3].

The determination of free drug fractions allows comparison of in vitro and in vivo activity. Assuming that binding is similar to serum proteins of different species, the free drug fractions in mouse plasma can be

calculated to be 0.0005%, 12.6% and 7.4% for SMI, SMII and SMIV, respectively. The maximal plasma concentrations achieved in vivo calculated from the data in Fig. 5 are 0.39, 0.59 and 2.4 μM, respectively, and from these the maximal free drug concentrations achieved are 0.0019, 75 and 180 nM, respectively. Comparison with the corresponding free drug IC_{50} values of 0.0041, 1,800 and 89 nM, respectively, (Table 2) indicates that only SMIV in vivo exceeds the IC_{50} value for CH-1 cells. Although the relationship between CH-1 and colon 38 drug sensitivity is not known because colon 38 cells do not grow as a cell line, the results are consistent with the hypothesis that SMIV is more active than SMI and SMII because host toxicity is not so limiting.

Non-specific binding to proteins or other macromolecules might explain the high host toxicity associated with very lipophilic aromatic cations such as SM1. Other workers have shown relationships between drug lipophilicity and the specificity of binding of aromatic cations to mitochondrial proteins [32]. In contrast, the most hydrophilic compounds may be limited by high rates of excretion as a consequence of low protein binding. Renal clearance has been shown to increase with drug hydrophilicity with other series of compounds [34]. The investigational drug MKT-077 is a comparatively hydrophilic ($\log Pc = -1.6$) aromatic cation [37] that has shown nephrotoxicity in the clinic [16, 28]. Pharmacological studies have shown low concentrations of MKT-077 in tumour tissue as compared to the renal cortex [37].

In conclusion, aromatic cations with lipophilicity intermediate to the clinical candidates SM1 and MKT-077 will be required to optimise the antitumour activity of this class of antitumour compound. Changes in drug lipophilicity appear to alter host toxicity associated with non-specific binding and renal drug elimination. Compounds that can be delivered at high doses are expected to prove more active because of the dependence of activity on tumour drug concentration in in vitro and in vivo models.

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