

Synthesis and Antitumour Activity of Gold(I) and Silver(I) Complexes of Hydrazine-Bridged Diphosphine Ligands

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ABSTRACT

A known synthetic route was used to prepare two known hydrazine-bridged phosphine ligands and four new ligands with variable groups on the hydrazine bridge (methyl and ethyl), as well as positions on the aryl phosphine groups (phenyl, methoxyphenyl, dimethylaminophenyl). A range of gold(I) and silver(I) complexes were synthesized utilizing these phosphine ligands. Both the phosphine-bridged dimetal and cationic bis(diphosphine) metal complexes were isolated. An interesting phenomenon of the spontaneous oxidation of gold(I) to gold(III) (and reduction of gold(III) to gold(I)) upon complexation with ((*N,N*-dimethyl)-4-aminophenyl)dialkylhydrazine ligands is described. Thirteen of the synthesized complexes were subjected to anticancer activity screening against HeLa, Jurkat, A2780, cisplatin-resistant A2780, CoLo 320 DM and MCF7. Most of the complexes were found to inhibit the cancerous cells at low μM concentrations and in some cases nM concentrations. Two of the complexes were tested for their ability to reduce the mitochondrial membrane potential of PBMC cells as a possible mechanism of action of anticancer activity.

KEYWORDS

Gold(I), silver(I), hydrazine, diphosphine, antitumour, anticancer, mitochondrial membrane potential.

1. Introduction

The use of inorganic compounds as cancer treatment became well established with the FDA approval of cisplatin in 1978.¹ Cisplatin, carboplatin and oxaliplatin are widely used in clinical settings today. Platinum, however, is not the only transition metal that infers antitumour potential, as was seen when auranofin (a gold-based anti-arthritis compound) was shown to have *in vitro* antitumour activity.² Since then, many other gold compounds have been identified as potential anticancer agents.^{3–7} Of all the gold compounds tested as anticancer agents, gold phosphine compounds stand out as some of the most active compounds.⁸

Studies done by Berners-Price *et al.*^{9–11} led to the development of the bischelated gold(I) phosphine antitumour compound $[\text{Au}(\text{dppe})_2]\text{Cl}$ (dppe = bis(diphenylphosphino)ethane), whose active entity was found to be the cationic ion $[\text{Au}(\text{dppe})_2]^+$. Whereas auranofin exhibited only modest antitumour activity in one animal tumour model, $[\text{Au}(\text{dppe})_2]^+$ exhibited significant antitumour activity in a range of tumour models in mice. It was selected for pre-clinical trials, but was abandoned after the identification of severe hepatotoxicity in dogs.^{9,10}

Further studies done by Berners-Price and co-workers¹² found that the antitumour activity of $[\text{Au}(\text{dppe})_2]\text{Cl}$ and related complexes showed a dependence on the lipophilic/hydrophilic balance. Specifically, a compound of intermediate lipophilicity showed significant antitumour activity, less dose-limiting toxicity and higher gold concentration in plasma and tumours compared with the more lipophilic or hydrophilic analogues.⁸

Thus, the high toxicity of $[\text{Au}(\text{dppe})_2]^+$ was attributable to the high lipophilicity of the cation which resulted in non-selective uptake into mitochondria in all cells. Delocalized lipophilic cations (e.g. Rhodamine 123) have a long history as potential

antitumour drugs and have been shown to preferentially concentrate within tumour cell mitochondria in response to elevated mitochondrial membrane potentials, which are characteristic features of carcinoma cells.¹³ Clinical development of compounds of this type has been hindered by severe toxicity, but several classes of delocalized lipophilic cations have demonstrated a relationship between antitumour selectivity and lipophilicity.^{9,10}

One way of changing the lipophilicity balance in a compound is the introduction of heteroatoms. Substitution of the carbon bridge of dppe with heteroatom analogues allows for the design of new ligand systems with altered lipophilic properties. The discovery of bis(dihalophosphino)amines (Fig. 1) has led to extensive research into the transition metal chemistry of this class of ligands.^{14–16} By 1994 more than 300 published papers described the rich coordination chemistry of $\text{R}_2\text{PN}(\text{R}')\text{PR}_2$ -type ligands.¹⁷

Recently, interest in the corresponding hydrazine-bridged diphosphines (Fig. 1), such as bis(dihalo, diaryl or dialkylphosphino)dialkylhydrazine, have been piqued as easier and more effective synthetic pathways were pioneered. These synthetic pathways were first investigated by Reddy and Katti^{18–20} and soon afterwards, explored further by others.²¹

The hydrazine-bridged diphosphines possess the same two atom spacing as dppe between the phosphorous atoms which makes them ideal chelators of transition metals. The added functionality of the hydrazine also changes the lipophilic properties, which in turn makes them good candidates to research as potential antitumour agents.

Here we describe the synthesis and characterization of gold(I) and silver(I) complexes of various bis(diarylphosphino)-1,2-dimethylhydrazine and bis(diarylphosphino)-1,2-diethylhydrazine, as well as their *in vitro* antitumour activity

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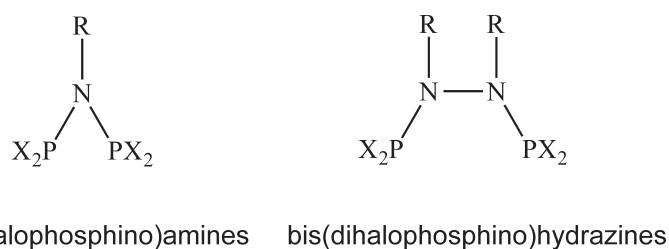


Figure 1 Nitrogen-bridged diphosphines.

against HeLa, Jurkat, A2780, cisplatin-resistant A2780, CoLo 320 DM and MCF7.

2. Results and Discussion

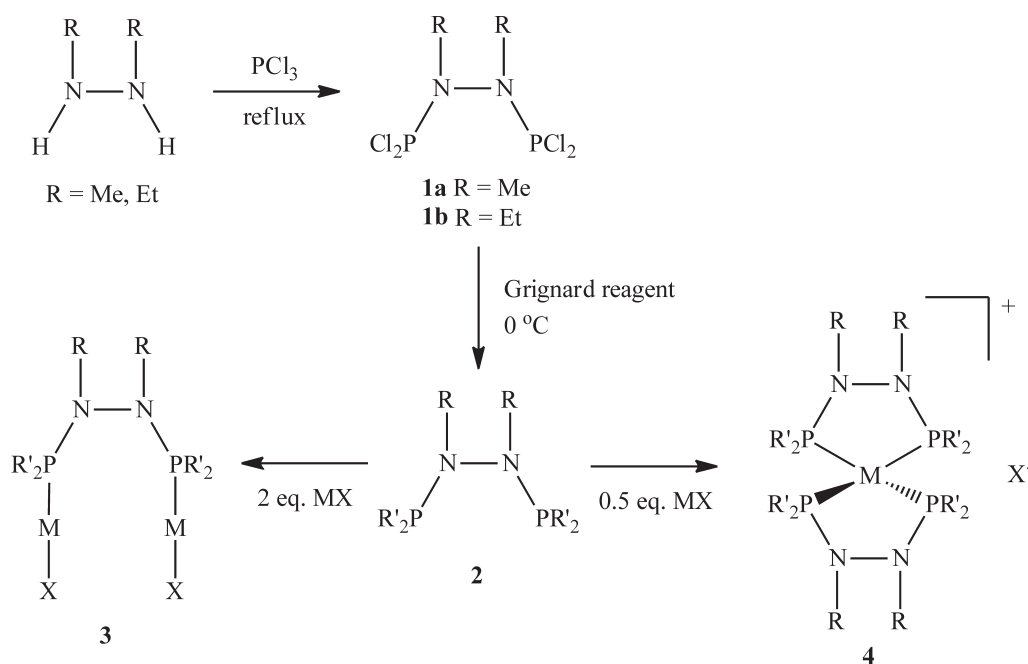
2.1. Synthesis

Chlorophosphines are a well known starting material for the synthesis of phosphine ligands and previously reported methods were used to synthesize two hydrazine-bridged chlorophosphines namely bis(dichlorophosphino)-1,2-dimethyl- and -diethylhydrazine (**1**, Scheme 1).^{18,19,21} The corresponding six bis(diarylphosphino)-1,2-dialkylhydrazines (**2**) (where alkyl = methyl or ethyl and aryl = phenyl, 4-methoxyphenyl or *N,N*-dimethyl-4-aminophenyl) were further synthesized utilizing the appropriate Grignard reagent as described for bis((diphenyl)phosphino)-1,2-dimethyl- and -diethylhydrazine

in the literature.^{18,19} Oxidation of the ligands were minimized through the increased speed of the brine workup leading to overall better yields and purer products. The ligands were isolated as sticky yellow liquids, except in the case of **2b**, where it was possible to crystallize the ligand successfully.²²

The phosphine ligands (**2**) were found to oxidize and decompose over time, with faster oxidation and decomposition observed when dissolved. This phenomenon was seen to more readily apply to the methyl hydrazine ligands as compared to the ethyl substituted hydrazine ligands. This trend was further amplified upon complexation of the ligands to gold and silver.

The ligands were used to complex both gold(I), (THT)AuCl, and silver(I), AgNO₃, metals in a 1:2 and 2:1 ratio. This gave rise to phosphine-bridged dimetal complexes (**3**) as well as discreet cationic bis(diphosphine) metal complexes (**4**) (Scheme 1). A small excess of ligand to metal ratio was found to minimize the



Ligands (2)		Phosphine-bridged (3)		Bis(diphosphine) (4)		
R	R'	M = Au X = Cl	M = Ag X = NO ₃	M = Au X = Cl	M = Ag X = NO ₃	
Me	Ph	2a *	3a #	3g	4a	4g
Et	Ph	2b *,#	3b #	3h #	4b	4h
Me	PhOMe	2c	3c #	3i	4c	4i
Et	PhOMe	2d	3d #	3j	4d	4j
Me	PhNMe ₂	2e	3e	3k	4e	4k
Et	PhNMe ₂	2f	3f	3l	4f	4l

* Known compounds. # previously described by the authors

Scheme 1
Synthesis of gold(I) and silver(I) complexes.

Table 1 ^{31}P NMR shifts for the dimethyl- and diethylhydrazine ligands and the corresponding gold(I) and silver(I) complexes.

R	R'	Ligands	
Me		1a	160.9
Et		1b	156.3
Me	Ph	2a	63.3
Et	Ph	2b	63.4
Me	PhOMe	2c	61.4
Et	PhOMe	2d	61.0
Me	PhNMe ₂	2e	61.5
Et	PhNMe ₂	2f	60.4

Phosphine-bridged (3)					
R	R'	Gold(I)		Silver(I)	
Me	Ph	3a	87.1	3g	Unstable
Et	Ph	3b	87.6	3h	77.1, d, $^1J_{\text{P,Ag}} = 782.9$
Me	PhOMe	3c	85.1	3i	Unstable
Et	PhOMe	3d	84.7	3j	74.2, d, $^1J_{\text{P,Ag}} = 834.2$
Me	PhNMe ₂	3e	Unstable	3k	Unstable
Et	PhNMe ₂	3f	83.5	3l	74.5, d, $^1J_{\text{P,Ag}} = 819.6$

Bis(diphosphine) (4)					
R	R'	Gold(I)		Silver(I)	
Me	Ph	4a*	83.9	4g*	68.4 dd, $^1J_{\text{P,Ag}} = 245.7; ^1J_{\text{P,Ag}} = 283.5$
Et	Ph	4b	86.5	4h	69.1, dd, $^1J_{\text{P,Ag}} = 248.1; ^1J_{\text{P,Ag}} = 285.3$
Me	PhOMe	4c	81.9	4i	66.2 dd, $^1J_{\text{P,Ag}} = 246.9; ^1J_{\text{P,Ag}} = 284.9$
Et	PhOMe	4d	83.9	4j	67.5 dd, $^1J_{\text{P,Ag}} = 249.9; ^1J_{\text{P,Ag}} = 285.3$
Me	PhNMe ₂	4e	Unstable	4k*	66.6 dd, $^1J_{\text{P,Ag}} = 246.4; ^1J_{\text{P,Ag}} = 284.2$
Et	PhNMe ₂	4f	Mixture	4l*	67.6, dd, $^1J_{\text{P,Ag}} = 248.3; ^1J_{\text{P,Ag}} = 286.1$

NMR analysis was performed in CDCl_3 , * *d*-DMSO

reduction of the metals to either red gold or black silver precipitates or metal mirrors.

Phosphine-bridged silver complexes were especially prone to reduction of the metal and regularly formed silver mirrors or metal precipitations. The crystal structure showing the intricate network of molecules of one of these complexes seems to give a clue to the inherent instability of these complexes.²³

The instability of the ligands, and to a lesser degree the complexes, made complete characterization impossible in some cases. Notably for elemental analysis or where long NMR times were necessary (even if homogeneous crystals were analyzed).

2.2. NMR-Spectroscopy Studies

Analysis of the ^{31}P NMR spectra of the various complexes showed a general downfield shift of about 25 ppm from the ligand precursors for both gold complex types, bridged and bischelated (Table 1). Taking into account the deshielding effect of the electron withdrawing gold cation on the phosphorous atoms, this is not unusual and is also observed for gold dppe complexes. In the analogous gold dppe complexes, the bridged complex, $\text{ClAu}(\text{dppe})\text{AuCl}$, is observed at 27.1 ppm and the bischelated complex, $[\text{Au}(\text{dppe})_2]^+$, is observed at 20.7 ppm, while the dppe ligand is observed at -22 ppm.²⁴ Generally the

synthesized bridged gold(I) complexes also showed a marginal downfield shift from the bischelated complexes.

Silver(I) complexes showed similar trends to those observed with gold(I) complexes with respect to their ^{31}P NMR shifts. The downfield shifts of the silver complexes as compared to the free ligands are not as pronounced as with gold, with a shift of only about 10 ppm for the bridged complex *vs* almost 25 ppm for the gold analogues. A more noticeable difference can be seen between the bridged and bischelates, when compared to the similar effect observed for gold.

A characteristic feature of these complexes was the interaction of the spin active isotopes ^{107}Ag and ^{109}Ag with ^{31}P resulting in a doublet for the bridged complex and a doublet of doublets for the bischelates. Studies by Berners-Price *et al.*^{25,26} have shown a similar trend as evident by ^{31}P NMR signals for $(\text{AcO})\text{Ag}(\text{dppe})\text{Ag}(\text{OAc})$ at 3.3 ppm and $[\text{Ag}(\text{dppe})_2]^+$ at 4.4 ppm *vs* the free ligand signal at -12.3 ppm. Berners-Price *et al.*²⁶ have found that the broad signals in $(\text{AcO})\text{Ag}(\text{dppe})\text{Ag}(\text{OAc})$ resolved into a complex multiplet upon cooling to 221 K, indicating the three combinations of ^{107}Ag and ^{109}Ag in the bridged species. Coupling constants of $^1J(^{109}\text{Ag}-^{31}\text{P}) = 757$ Hz, $^1J(^{107}\text{Ag}-^{31}\text{P}) = 657$ Hz and various smaller couplings were found for $(\text{AcO})\text{Ag}(\text{dppe})\text{Ag}(\text{OAc})$. The bischelated silver(I) complex,

Table 2 IC₅₀ (μM) of cells following treatment with experimental compounds (mean of three triplicate experiments ± standard error of measurement).

Compound	HeLa	Jurkat	Resting PBMC	Stimulated PBMC	Tumour specificity
ref	0.75 ± 0.27	0.03 ± 0.05	0.33 ± 0.32	0.13 ± 0.10	0.6
3b	0.86 ± 0.15	0.74 ± 0.43	4.3 ± 0.6	0.94 ± 0.90	3.3
3c	1.0 ± 0.3	1.0 ± 0.0	7.3 ± 0.1	5.7 ± 1.3	6.4
3d	5.5 ± 2.5	0.4 ± 1.4	7.5 ± 4.0	13 ± 0	3.4
3f	39 ± 9	18 ± 3	32 ± 1	25 ± 2	1.0
3j	4.6 ± 0.3	2.5 ± 1.5	1.5 ± 0.6	1.1 ± 0.3	0.4
4a	1.3 ± 0.6	0.44 ± 0.31	1.4 ± 0.2	0.84 ± 0.27	1.3
4b	0.7 ± 0.7	0.31 ± 0.18	0.78 ± 0.26	0.65 ± 0.13	1.4
4c	2.5 ± 0.5	1.9 ± 0.1	3.2 ± 0.7	4.5 ± 1.0	1.8
4d	0.56 ± 0.05	1.8 ± 0.3	3.2 ± 0.26	2.9 ± 0.1	2.6
4g	1.1 ± 0.2	0.65 ± 0.23	3.7 ± 0.6	3.1 ± 0.6	3.9
4i	0.71 ± 0.60	0.38 ± 0.16	2.0 ± 0.1	6.5 ± 0.3	7.8
4j	0.91 ± 0.47	1.1 ± 0.4	4.2 ± 0.6	3.4 ± 0.8	3.7
4l	1.7 ± 0.5	3.1 ± 0.6	10 ± 1	4.9 ± 1.0	3.1

[Ag(dppe)₂]⁺, showed well-resolved couplings with coupling constants of ¹J(¹⁰⁹Ag-³¹P) = 266 Hz and ¹J(¹⁰⁷Ag-³¹P) = 231 Hz. With the addition of excess ligand, a slight peak broadening was observed which is indicative of relatively slow exchange on the NMR time scale (>400 s⁻¹) of free and bound dppe ligand.

The difference in ³¹P NMR shifts between the synthesized gold and silver hydrazine complexes is also observed for the silver and gold complexes of dppe, with ³¹P signals of [Au(dppe)₂]⁺ at 20.7 ppm and [Ag(dppe)₂]⁺ at 4.4 ppm compared to the dppe free ligand ³¹P signal found at -12.3 ppm.

The synthesis of the complexes [(((CH₃)₂N-4-C₆H₄)₂PN(CH₃)N(CH₃)P((C₆H₄-4-N(CH₃)₂)₂)₂ Au)⁺Cl⁻ (**4k**) and [(((CH₃)₂N-4-C₆H₄)₂PN(C₂H₅)N(C₂H₅)P((C₆H₄-4-N(CH₃)₂)₂)₂ Au)⁺Cl⁻ (**4l**) was accompanied by an unexpected phenomenon. Initially the reaction mixture of the complexes in THF after the addition of gold(I) precursor showed the characteristic yellow colour associated with complexes of this type, but upon stirring for prolonged periods the solution darkened and turned red. The same colour change was observed when the solvent was removed *in vacuo* to afford a yellow solid. This solid changed from yellow to red in the absence of solvent while standing under an argon atmosphere. When doing the same reaction in DCM, the colour change was observed to be markedly faster. Due to the breakdown of the methylhydrazine ligand upon complexation this effect could not be studied in this complex, but the ethylhydrazine ligand showed greater stability and ³¹P NMR studies showed that the colour change was accompanied by a shift in the phosphorous signal from 85.9 ppm to 96.5 ppm. These two peak signals seemed to reach an equilibrium when the signal intensities had reached a ratio of 1:2. This equilibrium ratio was reached after about 15 min in DCM and 40 min in THF and then remained constant for several days. The 96.5 ppm peak was ascribed to a gold(III) species and as a control, a gold(III) source, HAuCl₄, was added to the ethylhydrazine ligand and a red solution ensued. The same equilibrium ratio was once again observed in the NMR spectrum, indicating a definitive equilibrium between the gold(I) and gold(III) species of this complex.

2.3. Cytotoxicity Assays

A range of thirteen gold(I) and silver(I) compounds that could be isolated and suitably characterized was subjected to biological screening (Table 2). [Au(dppe)₂]⁺Cl⁻ (**ref**) was used as a reference compound. The range of compounds was initially screened against three cell lines, namely HeLa, Jurkat and PBMC. The HeLa cell line is a well known and studied cancerous cell line

and often used for activity screening. The cancerous Jurkat cell line was used to determine the toxicity of the compounds to cancerous human T-cells, while normal PBMC, harvested from healthy human blood donors, were used to determine the toxicity of compounds to normal human T-cells.

IC₅₀ values, described as the amount of compound delivered to cause 50 % mortality to viable cells, were determined by performing the standard MTT assay after incubation with the compounds for the allocated amount of days.

A summary of the IC₅₀ values obtained from the cytotoxicity profiles is given in Table 2, reported as the mean value of three experimental results and given in μM concentrations of administered compound.

From the results obtained from the screening, tumour specificity of the compounds was calculated (Table 2). Specificity was calculated as follows: the sum of the IC₅₀ values from normal cells (resting and stimulated PBMC in this case) divided by the sum of the IC₅₀ values of the cancerous cells (HeLa and Jurkat cells in this case).

From the IC₅₀ values of the bridged gold compounds (**3**) a trend can be seen with complexes being more active as follows: phenyl > methoxyphenyl >> dimethylaminophenyl (Table 2). Furthermore, the methoxyphenyl substituted diphosphine containing complexes also show good specificity. The same trend can be seen to some extent in the bischelated gold complexes (**4**), with methylhydrazine complexes being more active than the ethylhydrazine complexes. The bischelated silver complexes (**4g–4l**), on the other hand, showed good specificity all around, but no clear trend could be observed.

When one compares the bridged to the bischelated gold compounds directly (i.e. **3b** vs. **4b**; **3c** vs. **4c**; and **3d** vs. **4d**), the only general trend that can be seen is an increase in selectivity in the case of the bridged gold over the bischelated gold complexes. From the one example available for the silver complexes (i.e. **3j** vs. **4j**), the reverse seems to be true.

Why there would be this reversal of properties between gold and silver complexes is unclear at present, but one possibility is the inherent reactivity of silver with halide salts in biological media to form silver-halide precipitations. As the bischelated silver complexes are expected to be more stable in the presence of halides, they have a better chance of reaching the target site intact.⁹

Based on the specificity displayed by the compounds, five potential inhibitors (and the reference complex) were chosen for further evaluation against other cancerous cell lines. The

Table 3 IC₅₀ (μM) of cells following treatment with experimental compounds (mean of three experiments ± standard error of measurement).

Compound	A2780	A2780cis	CoLo 320 DM	MCF-7	MCF-12A	Tumour specificity
ref	0.02 ± 0.07	0.23 ± 0.14	0.46 ± 0.04	0.20 ± 0.09	0.44 ± 0.17	1.1
3c	0.28 ± 0.41	1.9 ± 0.3	1.3 ± 0.0	0.79 ± 0.36	1.6 ± 0.3	4.3
3d	0.92 ± 0.19	2.0 ± 0.6	0.33 ± 0.80	1.1 ± 0.1	0.76 ± 0.22	5.1
4g	0.34 ± 0.11	0.96 ± 0.57	0.24 ± 0.81	0.63 ± 0.42	0.88 ± 0.37	2.8
4i	0.03 ± 0.19	5.5 ± 0.6	0.51 ± 0.25	0.96 ± 0.07	13 ± 0	5.8
4l	0.50 ± 0.08	2.0 ± 0.7	3.0 ± 0.8	0.58 ± 0.30	4.5 ± 0.9	3.6

compounds displaying some of the highest specificity included: two bridged gold compounds (**3c** and **3d**) and three bischelated silver compounds (**4g**, **4i** and **4l**) (Table 2).

It can already be noted that compounds **3c** and **4i** show a ten-fold higher specificity compared to the reference [Au(dppe)₂]Cl (**ref**). Both these complexes have the same ligand system, i.e. (CH₃O-4-C₆H₄)₂PN(CH₃)N(CH₃)P(C₆H₄-4-OCH₃)₂, but different metal centres, the bridged complex (**3c**) being a gold complex and the bischelated complex (**4i**) being a silver complex.

The five selected compounds were further tested on A2780 (human ovarian cancer), A2780cis (human ovarian cancer – cisplatin resistant), Colo 320 DM (human colon cancer), MCF-7 (human breast cancer) and MCF-12A (immortalized human non-cancerous breast cells) cell lines. A summary of IC₅₀ values obtained, is given in Table 3.

From the further assays done on these five compounds a broader specificity profile was determined (Table 3). The broader specificity was calculated by taking the sum of the IC₅₀ values from normal cells (MCF-12A, resting and stimulated PBMC) divided by the sum of the IC₅₀ values of the cancerous cells (HeLa, Jurkat, A2780, A2780cis, CoLo 320 DM and MCF-7).

All five compounds showed low IC₅₀ values for the three cancer cell lines A2780, CoLo 320 DM and MCF-7, but higher IC₅₀ values were observed for the cisplatin-resistant A2780cis cell line. Only compounds **4i** and **4l**, both bischelated silver complexes, showed good selectivity for the cancerous MCF-7 cell line over the non-cancerous MCF-12A cell line (derived from normal breast epithelial cells²⁷).

The two compounds with the highest specificities (**3d** and **4i**)

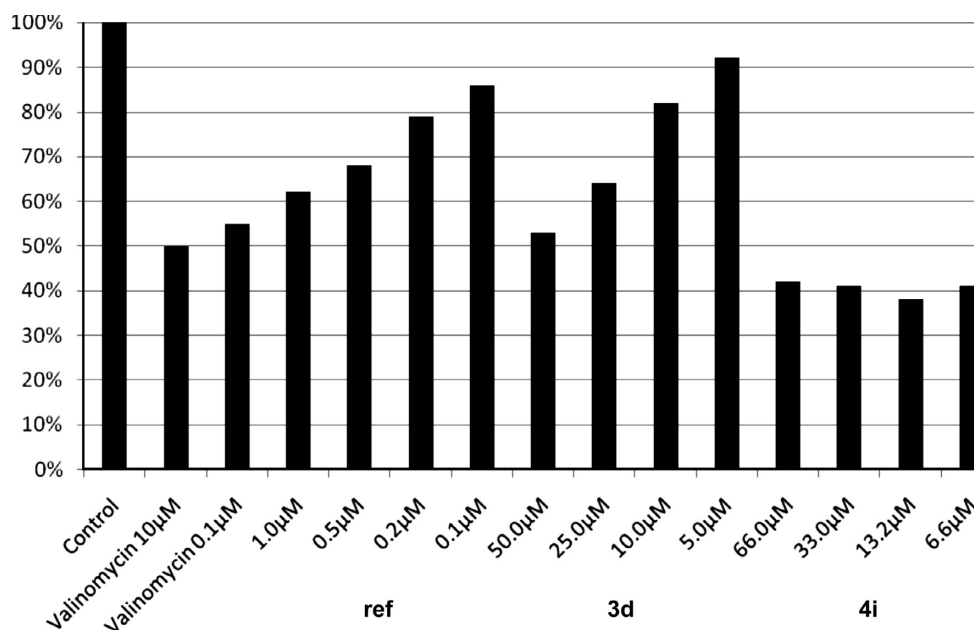
showed a five times higher specificity compared to the reference (**ref**). However, the overall specificities of these five compounds are not ideal.

2.4. Mitochondrial Membrane Potential Assay

The understanding of the role of mitochondria in apoptosis is still developing, but it is clear that mitochondria are critically involved in deciding whether a cell undergoes apoptosis.²⁸ By activation of caspases, cells irreversibly initiate apoptosis.²⁹ As the mitochondrial membrane potential is far larger than that of others areas of the cells, lipophilic cations accumulate selectively within mitochondria, where they can easily pass through the hydrophobic barrier of the lipid bilayer.³⁰

Mitochondria are thus seen as cell-based targets for discrete lipophilic cations and the depolarization of the mitochondrial membrane potential would indicate that the complexes under investigation cause cell death *via* a mitochondrial mode of action. A mitochondrial membrane potential assay (using the fluorescent probe JC-1 after a 24 hour incubation of the cells) is used to determine whether the application of a test compound lowers or increases the mitochondria membrane potential. There is a direct correlation between the fluorescent absorption of JC-1 and the membrane potential.

From the extended *in vitro* evaluations the two compounds with the highest SI values, a bridged gold complex (**3d**) and a bischelated silver complex (**4i**), along with the reference compound (**ref**) was used in mitochondrial membrane potential assay. Results of the assay on PBMC are displayed in Fig. 2. Valinomycin is used as positive control of the method as this is known to reduce the membrane potential of mitochondria.

**Figure 2** Effect of test compound at various concentrations on the depolarization of the mitochondrial membrane potential.

Concentrations of test agent used were taken at 1×, 2×, 5× and 10× the IC₅₀ value previously obtained from cytotoxicity studies. Percentage of normal membrane activity is given as an easy comparison.

A clear trend is evident from the PBMC mitochondria membrane potential assay (Fig. 2). Complex **3d** compares to the [Au(dppe)₂]Cl reference compound (**ref**) in that a decrease of the membrane potential is correlated to an increase in the concentration of the compound. Complex **4i** on the other hand showed a huge decrease in membrane potential independent of the concentration of the administered complex. This reduction (approx. 60 %) of membrane potential was more than that observed for valinomycin (approx. 50 %). The concentration dependence of compounds **ref** and **3d** implies a reversible interaction with the membrane while the concentration independence of compound **4i** may imply a less labile or permanent interaction and disruption.

3. Conclusion

This paper reports the successful preparation of two previously described hydrazine-bridged diphosphine ligands (**2a–b**) and four new hydrazine-bridged diphosphine ligands (**2c–f**). The synthesis of both gold(I) and silver(I) bridged and bischelated complexes of these six ligands are reported. The curious gold(III) complex of **4f** was obtained, making it unique as a gold(III) tetrakisphosphine complex. All of the synthesized complexes compared favourably to analogues reported in literature.

Toxicity studies were carried out on thirteen metal complexes of silver(I) and gold(I) and related to results of the known antitumour agent [Au(dppe)₂]Cl (**ref**). The most active complexes were found to be bridged gold and bischelated silver complexes. Five complexes were tested against various other cell lines to obtain a better toxicology profile. The bridged gold complexes ((CH₃O-4-C₆H₄)₂PN(C₂H₅)N(C₂H₅)P(C₆H₄-4-OCH₃)₂)₂Au₂Cl₂(**3d**) and the bischelated silver complex [((CH₃O-4-C₆H₄)₂PN(CH₃)N(CH₃)P(C₆H₄-4-OCH₃)₂)₂Ag]⁺NO₃⁻ (**4i**) were seen to exhibit the best specificities and subsequently were used in mitochondria membrane potential assay. While both the reference compound (**ref**) and **3d** showed concentration dependence in their lowering of membrane potential, **4i** showed a non-concentration dependent lowering of membrane potential. The reason for this dramatic reduction is still unclear. Further investigation of this family of compounds is under way at the National Institute of Health's (NIH, U.S.A.) Developmental Therapeutic Program (DTP).

4. Experimental

4.1. Synthetic Reagents and General Procedures

All manipulations were carried out under argon, using standard Schlenk techniques. Solvents were distilled from drying agents and degassed. NMR spectra were recorded in CDCl₃ and *d*-DMSO at ambient probe temperature using the following Bruker instruments: AVANCE 300 (¹H 300.13; ³¹P 121.5; ¹³C 75.5 MHz), AVANCE DRX 400 (¹H 400.13; ³¹P 161.9; ¹³C 100.6 MHz) and referenced internally to residual solvent resonances (chemical shift data in δ). ¹³C NMR and ³¹P NMR spectra were all proton-decoupled. FAB-MS spectra were collected using a VG70-SEQ instrument in positive ion mode. Elemental analysis data was determined by either the University of Cape Town, Cape Town, South Africa (Thermo Flash EA1112 CHNS-O elemental analyzer) or the Institute for Soil, Climate and Water, Pretoria, South Africa (Carlo Erba NA1500 Nitrogen/Carbon/ Sulphur Analyzer which has

been modified to allow CHN analysis).

The following abbreviations are used throughout the experimental section: bs = broad singlet, d = doublet, dd = doublet of doublet, m = multiplet, s = singlet. Coupling constants (*J*) are given in Hz. (THT)AuCl (THT = tetrahydrothiophene) starting material was synthesized according to literature procedures.³¹

4.2. Ligand Synthesis

4.2.1. Bis(dichlorophosphino)-1,2-dimethylhydrazine (**1a**)

1a was synthesized according to slightly modified literature methods.^{18,19} A final purification step was included, where dry diethylether was added to allow complete precipitation of all solids. The liquid phase was decanted and the diethylether removed *in vacuo* to afford the pure product as colourless liquid. ¹H NMR (CDCl₃, 300 MHz) δ_H 3.13 (t, CH₃, ³J(¹H-³¹P) = 3.7 Hz, 6H). ¹³C NMR (CDCl₃, 75 MHz) δ_C 33.4 (d, CH₃, ²J(¹³C-³¹P) = 1.8 Hz). ³¹P NMR (CDCl₃, 162 MHz) δ_P 160.9.

4.2.2. Bis(dichlorophosphino)-1,2-diethylhydrazine (**1b**)

1b was synthesized according to a modified literature method as for **1a**.¹⁸ ¹H NMR (CDCl₃, 300 MHz) δ_H 3.75 and 3.23 (m, CH₂CH₃, 4H), 1.27 (t, CH₂CH₃, ³J(¹H-¹H) = 7.1 Hz, 6H). ¹³C NMR (CDCl₃, 75 MHz) δ_C 42.2 (s, CH₂CH₃), 14.0 (s, CH₂CH₃). ³¹P NMR (CDCl₃, 162 MHz) δ_P 156.3.

4.2.3. Bis(diphenylphosphino)-1,2-diethylhydrazine (**2a**)

Ligand **2a** was synthesized in a manner similar to published methods.^{18,19} ¹H NMR (CDCl₃, 300 MHz) δ_H 7.39 (bs, Arom, 8H), 7.23 (bs, Arom, 12H), 2.66 (bs, CH₃, 6H). ³¹P NMR (CDCl₃, 162 MHz) δ_P 63.3. MS 429 (10 %, M + 1).

4.2.4. Bis(diphenylphosphino)-1,2-diethylhydrazine (**2b**)

2b was synthesized as described for **2a** and has been previously characterized.²²

4.2.5. Bis(di(4-methoxyphenyl)phosphino)-1,2-dimethylhydrazine (**2c**)

Ligand **2c** was synthesized in a manner similar to published methods.^{18,19} The corresponding (4-methoxyphenyl)magnesium bromide Grignard reagent and ligand were synthesized in tetrahydrofuran (THF). **2c** was isolated as a sticky yellow liquid in 74 % yield. ¹H NMR (CDCl₃, 300 MHz) δ_H 7.32 (bs, Arom, 8H), 6.81 (d, Arom, ³J(¹H-¹H) = 8.4 Hz, 8H), 3.73 (s, OCH₃, 12H), 2.60 (s, NCH₃, 6H). ¹³C NMR (CDCl₃, 75 MHz) δ_C 160.3 (s, Arom), 134.2 (m higher order, Arom), 131.1 (bs Arom), 114.2, (m, Arom), 55.5 (s, OCH₃), 38.1 (m, NCH₃). ³¹P NMR (CDCl₃, 121 MHz) δ_P 61.4.

4.2.6. Bis(di(4-methoxyphenyl)phosphino)-1,2-diethylhydrazine (**2d**)

2d was synthesized as described for **2c**. A sticky yellow liquid was obtained in 75 % yield. ¹H NMR (CDCl₃, 300 MHz) δ_H 7.44 (m, Arom, 4H), 7.26 (m, Arom, 4H), 6.80 (m, Arom, 8H), 3.71 (s, OCH₃, 12H), 2.99 (m, CH₂CH₃, 6H), 0.88 (t, CH₂CH₃, ³J(¹H-¹H) = 5.4 Hz, 4H). ³¹P NMR (CDCl₃, 121 MHz) δ_P 61.0.

4.2.7. Bis(di(*N,N*-dimethyl-4-aminophenyl)phosphino)-1,2-dimethylhydrazine (**2e**)

2e was synthesized as described for **2c**. A sticky yellow liquid was obtained in 82 % yield. ¹H NMR (CDCl₃, 400 MHz) δ_H 7.44 (bs, Arom, 8H), 6.79 (d, Arom, ³J(¹H-¹H) = 8.3 Hz, ³J(¹H-³¹P) = 17.5 Hz, 8H), 3.01 (s, N(CH₃)₂, 24H), 2.74 (bs, NCH₃, 6H). ³¹P NMR (CDCl₃, 162 MHz) δ_P 61.5.

4.2.8. Bis(di(*N,N*-dimethyl-4-aminophenyl)phosphino)-1,2-diethylhydrazine (**2f**)

2f was synthesized as described for **2c**. A sticky yellow liquid was obtained in 90 % yield. ¹H NMR (CDCl₃, 400 MHz) δ_H 7.52 (bs, Arom, 4H), 7.31 (bs, Arom, 4H), 6.70 (t, ³J(¹H-³¹P) = 6.0 Hz,

Arom, 8H), 2.96 (m, N(CH₃)₂, 24H), 3.1 (m, CH₂CH₃, 4H), 0.92 (t, ³J(¹H-¹H) = 7.0 Hz, CH₂CH₃, 6H). ¹³C NMR (CDCl₃, 75 MHz) δ_c 149.2 (m, Arom), 133.7 (m, Arom), 131.7 (m Arom), 111.0 (m, Arom), 46.9 (s, CH₂CH₃), 39.0 (m, N(CH₃)₂), 13.4 (s, CH₂CH₃). ³¹P NMR (CDCl₃, 162 MHz) δ_p 60.4.

4.3. Phosphine Complex Synthesis

4.3.1. Bis(diphenylphosphino)-1,2-dimethylhydrazine di(gold(I) chloride) (3a)

The synthesis and characterization of **3a** has been described previously.³²

4.3.2. Bis(diphenylphosphino)-1,2-diethylhydrazine di(gold(I) chloride) (3b)

The synthesis and characterization of **3b** has been described previously.³³

4.3.3. Bis(di(4-methoxyphenyl)phosphino)-1,2-dimethylhydrazine di(gold(I) chloride) (3c)

The synthesis and characterization of **3c** has been described previously.³⁴

4.3.4. Bis(di(4-methoxyphenyl)phosphino)-1,2-diethylhydrazine di(gold(I) chloride) (3d)

The synthesis and characterization of **3d** has been described previously.²³

4.3.5. Bis(di(N,N-dimethyl-4-aminophenyl)phosphino)-1,2-diethylhydrazine di(gold chloride) (3f)

3f was synthesized using **2f** as described for **3a** (182 mg, 0.34 mmol) and (THT)AuCl (200 mg, 0.68 mmol). The purple-blue reaction mixture yielded a green powder on drying *in vacuo* (86 % yield). ¹H NMR (*d*-DMSO, 400 MHz) δ_H 7.61 (m, Arom, 8H), 6.87 (d, ³J(¹H-¹H) = 7.6 Hz, Arom, 4H), 6.72 (d, ³J(¹H-¹H) = 7.6 Hz, Arom, 4H), 3.16 (bs, CH₂CH₃, 4H), 3.01 and 2.96 (s, N(CH₃)₂, 24H), 0.85 (t, ³J(¹H-¹H) = 6.8 Hz, CH₂CH₃, 6H). ³¹P NMR: (*d*-DMSO, 162 MHz) δ_p 83.5. MS 1057 (25 %, M-Cl). MP 170–172 °C.

4.3.6. Bis(diphenylphosphino)-1,2-dimethylhydrazine di(silver(I) nitrate) (3g)

The complex could be observed utilizing ³¹P NMR, but could not be isolated due to the instability of the complex. ³¹P NMR: (*d*-DMSO, 121 MHz) δ_p 75.28 (d, 1(^{107/109}Ag-³¹P) 764.2 Hz).

4.3.7. Bis(diphenylphosphino)-1,2-diethylhydrazine di(silver(I) nitrate) (3h)

The synthesis and characterization of **3h** has been described previously.³⁵

4.3.8. Bis(di(4-methoxyphenyl)phosphino)-1,2-diethylhydrazine di(silver(I) nitrate) (3j)

3j was synthesized using **2d** as described for **3h** (167 mg, 0.29 mmol) and AgNO₃ (100 mg, 0.59 mmol). The suspension turned colourless. The solvent was removed *in vacuo* to afford the product as a white solid (70 % yield). ¹H NMR: (CDCl₃, 400 MHz) δ_H 7.54 (m, Arom, 8H), 7.00 (m, Arom, 8H), 3.85 (s, OCH₃, 12H), 3.41 and 3.18 (bs, CH₂CH₃, 4H), 1.02 (bs, CH₂CH₃, 6H). ³¹P NMR: (CDCl₃, 162 MHz) δ_p 74.2 (d, ¹J(^{107/109}Ag-³¹P) 834.2 Hz). MS: 826 (12 %, M-NO₃). MP: 91–92 °C.

4.3.9. Bis(di(N,N-dimethyl-4-aminophenyl)phosphino) diethylhydrazine di(silver(I) nitrate) (3l)

3l was synthesized using **2f** as described for **3h** (182 mg, 0.29 mmol) and AgNO₃ (100 mg, 0.59 mmol). The reaction mixture was seen to be a purple-blue solution, which on drying *in vacuo* yielded a green solid (74 % yield). ¹H NMR: (*d*-DMSO, 400 MHz) δ_H 7.43 (m, Arom), 6.78 (m, Arom), 3.11 (m, CH₂CH₃, 4H), 2.97 and 2.99 (s, N(CH₃)₂, 24H), 0.66 (t, ³J(¹H-¹H) = 6.8 Hz,

CH₂CH₃, 6H). ³¹P NMR: (*d*-DMSO, 162 MHz) δ_p 74.5 (d, ¹J(^{107/109}Ag-³¹P) 819.6 Hz). MS: 951 (10 %, M-CH₃, 10 %), 751 (100 %, L + Ag + CH₃/N). EA: Calc: (Ag₂P₂N₈O₆C₃₆H₅₀) C 44.64 %, H 5.20 %, N 11.57 % Found: C 44.14 %, H 5.23 %, N 10.60 %. MP: 118–120 °C.

4.3.10. Bis(bis(diphenylphosphino)-1,2-dimethylhydrazine)gold(I) chloride (4a)

(THT)AuCl (100 mg, 0.34 mmol) was suspended in THF (5 mL). To the stirred suspension were added two equivalents of **2a** (291 mg, 0.68 mmol) in DCM (2 mL). The gold precursor dissolved and the solution turned yellow. The solvent was *in vacuo* removed to afford the product as a yellow solid (86 % yield). ¹H NMR: (CDCl₃, 300 MHz) δ_H 7.33 (t, Arom, ³J(¹H-¹H) = 7.6 Hz, 8H), 7.21 (t, Arom, coupling not resolved, 16H), 7.12 (t, Arom, (¹H-¹H) = 7.6 Hz, 16H), 2.69 (t, coupling not resolved, NCH₃, 12H). ³¹P NMR: (CDCl₃, 121 MHz) δ_p 83.9. MS: 1053 (100 %, M-Cl). MP: 84–86 °C.

4.3.11. Bis(bis(diphenylphosphino)-1,2-diethylhydrazine)gold(I) chloride (4b)

4b was synthesized using **2b** as described for **4a** (310 mg, 0.68 mmol) and (THT)AuCl (100 mg, 0.34 mmol). The product was obtained as a yellow solid (92 % yield). ¹H NMR: (*d*-DMSO, 300 MHz) δ_H 7.84 (m, Arom, 16H), 7.36 (m, Arom, 24H), 2.94 (m, CH₂CH₃, 8H), 0.96 (t, CH₂CH₃, ³J(¹H-¹H) = 6.9 Hz, 12H). ¹³C NMR: (*d*-DMSO, 100.6 MHz) δ_c 132.5 (m, Arom), 131.9 (m, Arom), 130.5 (m, Arom), 128.7 (m, Arom), 46.3 (d, CH₂CH₃, ²J(¹³C-³¹P) = 22.5 Hz), 14.1 (m, CH₂CH₃). ³¹P NMR: (*d*-DMSO, 121 MHz) δ_p 86.5. MS: 1109 (75 %, M-Cl). MP: 109–110 °C.

4.3.12. Bis(bis(di(4-methoxyphenyl)phosphino)diethylhydrazine) gold chloride (4c)

4c was synthesized using **2c** as described for **4a** (310 mg, 0.68 mmol) and (THT)AuCl (100 mg, 0.34 mmol). The product was obtained as a bright yellow solid (83 % yield). ¹H NMR: (CDCl₃, 300 MHz) δ_H 7.16 (d, ³J(¹H-¹H) = 7.6 Hz, Arom, 16H), 6.64 (d, ³J(¹H-¹H) = 8.5 Hz, Arom, 16H), 3.73 (s, OCH₃, 24H), 2.58 (bs, NCH₃, 12H). ¹³C NMR: (CDCl₃, 75 MHz) δ_c 161.9 (bs, Arom), 134.2 (m, Arom), 126.0 (m, Arom), 114.6 (s, Arom), 55.6 (bs, OCH₃), 37.4 (s, NCH₃). ³¹P NMR: (CDCl₃, 121 MHz) δ_p 81.9. MS: 1293 (85 %, M-Cl), 274 (54 %, MeNP(PhOMe)₂), 245 (98 %, P(PhOMe)₂), 138 (100 %, P(PhOMe)). EA: Calc: (AuClP₂O₈N₄C₆₀H₆₈) C 54.20 %, H 5.16 %, N 4.21 %. Found: C 51.10 %, H 4.96 %, N 3.72 %. MP: 96–97 °C

4.3.13. Bis(bis(di(4-methoxyphenyl)phosphino)diethylhydrazine) gold chloride (4d)

4d was synthesized using **2d** as described for **4a** (312 mg, 0.68 mmol) and (THT)AuCl (100 mg, 0.34 mmol). The product was obtained as a bright orange solid (90 % yield). ¹H NMR: (CDCl₃, 300 MHz) δ_H 7.73 (Arom, 4H), 6.95 (Arom, 4H), 3.84 and 3.86 (s, OCH₃, 6H), 3.46 (m, CH₂CH₃, 1H), 3.27 (m, CH₂CH₃, 1H), 0.79 (t, CH₃, 3H, J = 2.1 Hz) ³¹P NMR: (CDCl₃, 121 MHz) δ_p 83.9. MS: 1349 (25 %, M-Cl), 138 (100 %, P(PhOMe)). MP: 89–92 °C.

4.3.14. Bis(bis(di(N,N-dimethyl-4-aminophenyl)phosphino) diethylhydrazine) gold chloride (4f)

4f was synthesized using **2b** as described for **4a** (358 mg, 0.68 mmol) and (THT)AuCl (100 mg, 0.34 mmol). The yellow reaction mixture slowly turned red over time, giving a mixture of products. *Yellow compound*: ³¹P NMR: (CDCl₃, 161.98 MHz) δ_p 84.9. *Red compound* ³¹P NMR: (CDCl₃, 161.98 MHz) δ_p 96.5.

4.3.15. Bis(bis(diphenylphosphino)-1,2-dimethylhydrazine)silver(I) nitrate (4g)

Silver nitrate (50 mg, 0.29 mmol) was suspended in THF (5 mL). To the stirred suspension were added 2 equivalents of **2a**

(252 mg, 0.59 mmol) in DCM (2 mL). The silver nitrate dissolved and the suspension turned colourless. The solvent was removed *in vacuo* to afford the product as a white solid (76 % yield). ¹H NMR: (*d*-DMSO, 300 MHz) δ_H 7.51 (bs, Arom), 7.34 (bs, Arom), 2.73 (bs, CH₃). ³¹P NMR: (*d*-DMSO, 121 MHz) δ_P 68.42 (dd, ¹J(¹⁰⁷Ag-³¹P) = 245.7 Hz, ¹J(¹⁰⁹Ag-³¹P) = 283.5 Hz). MS: 963 (38 %, M-NO₃), 535 (58 %, Ligand + Ag), 214 (100 %, PPh). MP: 96–98 °C.

4.3.16. Bis(bis(diphenylphosphino)-1,2-diethylhydrazine)silver(I) nitrate (4h)

4h was synthesized using **2b** as described for **4g** (269 mg, 0.59 mmol) and AgNO₃ (50 mg, 0.29 mmol). The product was obtained as a white solid (84 % yield). ¹H NMR: (CDCl₃, 300 MHz) δ_H 7.39 (bs, Arom, 16H), 7.19 (bs, Arom, 24H), 2.94 (unresolved q, CH₂CH₃, 8H), 0.85 (t, ³J(¹H-¹H) = 6.0 Hz, CH₂CH₃, 12H). ¹³C NMR: (CDCl₃, 75 MHz) δ_C 133.5 (Arom), 132.8 (Arom), 131.5 (Arom), 128.9 (Arom), 48.9 (CH₂CH₃), 14.8 (CH₂CH₃). ³¹P NMR: (CDCl₃, 161.98 MHz) δ_P 69.06 (dd, ¹J(¹⁰⁷Ag-³¹P) = 248.1, ¹J(¹⁰⁹Ag-³¹P) = 285.3 Hz). MS: 1022 (45 %, M-NO₃), 563 (56 %, Ligand + Ag), 455 (47 %, Ligand). EA: Calc: (AgP₄N₅O₃C₅₂H₅₂) C 62.11 %, H 5.58 %, N 6.47 %. Found: C 60.08 %, H 5.54 %, N 5.85 %. MP: 127–130 °C.

4.3.17. Bis(bis(di(4-methoxyphenyl)phosphino)dimethylhydrazine)silver nitrate (4i)

4i was synthesized using **2c** as described for **4g** (252 mg, 0.59 mmol) and AgNO₃ (50 mg, 0.29 mmol). The product was obtained as light yellow solid (79 % yield). ¹H NMR: (CDCl₃, 400 MHz) δ_H 7.21 (d, coupling not resolved, Arom, 16H), 6.70 (d, ³J(¹H-¹H) = 8.4 Hz, Arom, 16H), 3.75 (OCH₃, 24H), 2.54 (NCH₃, 12H). ¹³C NMR: Due to sample breakdown ¹³C could not be done. ³¹P NMR: (CDCl₃, 162 MHz) δ_P 66.2 (dd, ¹J(¹⁰⁷Ag-³¹P) = 246.9 Hz, ¹J(¹⁰⁹Ag-³¹P) = 284.9 Hz). MS: 1206 (36 %, M-NO₃), 655 (34 %, L + Ag), 245 (100 %, P(PhOMe)₂). EA: Calc: (AgP₄N₅O₁₁C₆₀H₆₈) C 56.88 %, H 5.41 %, N 5.53 %. Found: C 56.78 %, H 5.51 %, N 4.84 %. MP: 90–93 °C.

4.3.18. Bis(bis(di(4-methoxyphenyl)phosphino)diethylhydrazine)silver nitrate (4j)

4j was synthesized using **2d** as described for **4g** (341 mg, 0.59 mmol) and AgNO₃ (50 mg, 0.29 mmol). The product was obtained as a light yellow solid (83 % yield). ¹H NMR: (CDCl₃, 400 MHz) δ_H 7.53 (d, Arom, ³J(¹H-¹H) = 8.8 Hz, 16H), 6.99 (d, Arom, ³J(¹H-¹H) = 8.8 Hz, 16H), 3.78 (OCH₃, 24H), 2.90 (bs, CH₂CH₃, 8H), 0.85 (bs, CH₂CH₃, 12H). ¹³C NMR: (CDCl₃, 100.6 MHz) δ_C 161.8 (m, Arom), 132.8 (m, Arom), 113.2 (m, Arom), 52.6 (s, OCH₃), 42.9 (m, CH₂CH₃), 13.2 (m, CH₂CH₃). ³¹P NMR: (CDCl₃, 162 MHz) δ_P 67.5 (dd, ¹J(¹⁰⁷Ag-³¹P) = 249.9, ¹J(¹⁰⁹Ag-³¹P) = 285.3 Hz). MS: 1261 (22 %, M-NO₃), 683 (20 %, L + Ag), 245 (100 %, P(PhOMe)₂). EA: Calc: (AgP₄N₅O₁₁C₆₄H₇₆) C 58.10 %, H 5.79 %, N 5.29 %. Found: C 58.27 %, H 5.96 %, N 4.88 %. MP: 76–78 °C.

4.3.19. Bis(bis(di(N,N-dimethyl-4-aminophenyl)phosphino)dimethylhydrazine)silver nitrate (4k)

4k was synthesized using **2e** as described for **4g** (354 mg, 0.59 mmol) and AgNO₃ (50 mg, 0.29 mmol). ³¹P NMR: (*d*-DMSO, 161.98 MHz) δ_P 66.6 (dd, ¹J(¹⁰⁷Ag-³¹P) = 246.4, ¹J(¹⁰⁹Ag-³¹P) = 284.2 Hz).

4.3.20. Bis(bis(di(N,N-dimethyl-4-aminophenyl)phosphino)diethylhydrazine)silver nitrate (4l)

4l was synthesized using **2f** as described for **4g** (371 mg, 0.59 mmol) and AgNO₃ (50 mg, 0.29 mmol). The product was obtained as a light brown solid (76 % yield). ¹H NMR: (*d*-DMSO,

400 MHz) δ_H 7.6–6.4 (various multiplets, Arom), 2.87 (NCH₃, 48H), 2.67 (m, CH₂CH₃, 8H), 0.88 (bs, CH₂CH₃, 12H). ³¹P NMR: (*d*-DMSO, 162 MHz) δ_P 67.6 (dd, ¹J(¹⁰⁷Ag-³¹P) = 248.3, ¹J(¹⁰⁹Ag-³¹P) = 286.1 Hz). MS: 1365 (50 %, M-NO₃), 735 (74 %, L + Ag), 271 (100 %, P(PhNMe₂)₂). EA: Calc: (AgP₄N₁₅O₃C₇₂H₁₀₀) C 60.58 %, H 7.06 %, N 12.76 %. Found: C 59.25 %, H 6.86 %, N 12.24 %. MP: 125–127 °C.

4.4. Biological Assays

4.4.1. Biological Reagents and General Procedures

Roswell Park Memorial Institute medium (RPMI) Eagle's minimal Essential Medium (EMEM) and Dulbecco's minimal Essential Medium (DMEM) culture media were commercially obtained (Sigma-Aldrich) and supplemented with 1 % penicillin/streptomycin mixture (10 000 U penicillin/mL and 10 000 μg streptomycin mL⁻¹) (Adcock-Ingram) and 10 % heat inactivated foetal calf serum (FCS) (Adcock-Ingram). DMEM culture medium used for cultivating MCF-7 cell line was further supplemented with 2 % nonessential amino acids. Culture medium used for MCF-12A cell line was made up from a 1:1 mixture of DMEM and Ham's F12 medium and was supplemented with hydrocortisone (10 mg mL⁻¹), chloratoxin (1 mg mL⁻¹), insulin (20 mg mL⁻¹), 10 % FCS and epidermal growth factor (100 mg mL⁻¹). All culture medium was stored at 4 °C.

The cell cultures used are listed in Table 4.

4.4.2. Compound Preparation

Stock solutions (1 mM) of the compounds were prepared by dissolving the compound in dimethylsulfoxide (DMSO) (1 mL). The stock solutions were dispensed in 50 μL aliquots into Eppendorf tubes and stored at -20 °C. Dilutions of the stock solutions were made directly before they were administered to stabilized cells in 96 well plates. Eight serial dilutions of the compound solutions in question were prepared from the stock solution. Serial dilutions typically started at a final concentration in the well of 200 μM, but the starting concentration was adjusted to the appropriate range of activity of the compound. The final percentage DMSO solvent per well was less than 0.5 %.

4.4.3. IC₅₀ Toxicity Assay

This procedure applies to all cell lines with the exception of peripheral blood mononuclear cells (PBMC). Culture medium (80 μL) was dispensed into the 96 wells of the plate and pre-warmed. A cell suspension (100 μL) of the appropriate concentration (see Table 4) was dispensed into wells and the plate was left to incubate for one hour at 37 °C and 5 % CO₂. The compound solution (20 μL) or the culture medium (in the case of the control experiments) was dispensed into wells in triplicate and incubated for seven days. Cell viability was determined by MTT assay³⁶ utilizing a multiplate absorbance reader (BIO-TEK Instruments, ELx800 UV Universal microplate reader).

Similar procedures were followed for PBMC as described above with the following amendments. PBMC were isolated from a healthy human adult volunteer using Histopaque 1077 and 60 μL culture medium was dispensed into each well of the plate. Five minutes after the addition of complex solution, PHA (20 μL, BIOWEB) was added to stimulate lymphocytes in one set of experiments, while culture medium (20 μL) was added to the set of resting lymphocyte experiments. The plates were incubated for three days.

4.4.4. Determination of the Cell Membrane Potential of Mitochondria

A standard flow cytometric method with the cationic mitochondrial membrane stain, JC-1, was used for the study as

Table 4 Summary of the cell cultures used in biological studies. American Type Culture Collection (ATCC) codes are also provided.

Code	Description	ATCC code	Culture medium	Initial cell conc. (cells mL ⁻¹)
HeLa	Human adenocarcinoma of the cervix	CCL-2	EMEM	6.25 × 10 ³
CoLo 320 DM	Human colon cancer	CCL-220	RPMI	5 × 10 ³
Jurkat	Human T-cell cancer	NRBM 0062	RPMI	7.5 × 10 ³
A2780	Human ovarian cancer	EACC 93112519	RPMI	6.25 × 10 ³
A2780cis	Human ovarian cancer (cisplatin resistant)	EACC 93112519 exposed to cisplatin	RPMI	6.25 × 10 ³
MCF-7	Human breast cancer	HTB 22	DMEM	5 × 10 ³
MCF-12A	Modified human noncancerous breast cells	CRL 10782	DMEM	5 × 10 ³
PBMC	Peripheral blood mononuclear cells		RPMI	2 × 10 ⁶

described by Cassarizza *et al.*³⁷ PBMC (1.8 mL) at appropriate concentration (see Table 4) were incubated for one hour in flow cytometer tubes. To this was added the appropriate compound dilution (0.2 mL) and after five minutes, PHA (0.22 mL). All experiments were carried out in the same manner using a control sample (no addition of test compound) and multiples (1×, 2×, 5×, 10×) of the IC₅₀ value of the complex as determined in previous toxicological experiments. The cells were left to incubate for 24 hours. Two tubes that were incubated without complex were treated with 0.1 mM and 1 mM Valinomycin (known to decrease mitochondrial membrane potential), respectively and incubated for ten minutes. The tubes were centrifuged for five minutes at 1800 rpm. The obtained pellet was resuspended in medium (900 μl) and JC-1 [5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide] (100 μl, 37.5) was added. The tubes were left in the dark for twenty minutes and then centrifuged for five minutes at 1800 rpm. The isolated pellet was washed with PBS (3 mL). The obtained pellet was resuspended in PBS supplemented with 10 % FCS and analyzed using a flow cytometer (Beckman Coulter Cytomics FC 500 flow cytometer equipped with a 488 argon laser).

4.4.5. Analysis of Results

The results of the assays are expressed as the mean percentage (%) of the control ±SEM. The statistical program GRAPHPAD was used to determine the IC₅₀ concentration of the complex treated groups.

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