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Review

Targeting the mitochondrial cell death pathway with gold compounds☆

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This article is dedicated to Professor Peter J. Sadler, FRS, on the occasion of his 60th birthday.

Contents

Abstract

There is much recent evidence showing that mitochondria play a critical role in the regulation of apoptosis (programmed cell death), making them an attractive target for the design of new anticancer drugs. A variety of different strategies targeting mitochondria for cancer therapy have been described in recent literature. Several different classes of gold-based compounds (in both Au(I) and Au(III) oxidation states) have attracted interest as potential antitumour agents and there is evidence that many act by mechanisms involving mitochondrial cell death pathways. Two distinct classes of Au(I) phosphine complexes display antitumour properties, these having either linear two-coordinate, or tetrahedral four-coordinate geometries. Both classes appear to target mitochondria, but different mechanisms are likely to be involved, related to their differing propensity to undergo ligand exchange reactions with biological ligands. The anti-arthritic Au(I) phosphine drug, auranofin, has been shown to induce apoptosis *via* selective inhibition of the mitochondrial isoform of thioredoxin reductase, an enzyme which has emerged as a potential new drug target. Gold(I) compounds are among the most potent known inhibitors of thioredoxin reductase, attributable to binding of Au(I) to the redox-active selenocysteine residue. On the other hand $[Au(dppe)_2]^+$, and related tetrahedral Au(I) phosphine complexes, do not undergo ligand exchange reactions as readily as two-coordinate linear Au(I) complexes. Their antitumour activity may stem from the lipophilic, cationic properties, as for other delocalised lipophilic cations that accumulate in mitochondria.

Abbreviations: ANT, adenine nucleotide translocator; auranofin, 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*-triethylphosphine gold(1); bipy^{dmb}, 6-(1,1-dimethylbenzyl)-2,2′-bipyridine; $\Delta\psi_m$, mitochondrial membrane potential; dien, 2,2′-diethylenediamine; DLC, delocalised lipophilic cation; dppe, 1,2-bis(diphenylphosphino)ethane; dpmaa, 2,3-bis(diphenylphosphino)maleic acid; HEPES (buffer), *N*-(2-hydroxoethyl)piperazine-*N*′-2-ethanesulfonic acid; MOMP, mitochondrial outer membrane permeabilization; MPT, mitochondrial permeability transition; NHC, *N*-heterocyclic carbene; PBR, peripheral benzodiazepin receptor; py^{dmb}, 2-(1,1-dimethylbenzene)pyridine; PT pore, permeability transition pore; ROS, reactive oxygen species; Trx, thioredoxin; TxrR, thioredoxin reductase; VDAC, voltage dependent anion channel

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Examples from our own recent work on different types of Au(I) phosphine and *N*-heterocyclic carbene compounds under investigation as potential mitochondrial targeting antitumour agents are reported here. We also review recent related literature on auranofin and a variety of Au(III) antitumour compounds which either inhibit mitochondrial thioredoxin reductase, or induce apoptosis by other mitochondrial cell death pathways.

Keywords: Cancer; Mitochondria; Thioredoxin reductase; Gold; Phosphine; *N*-Heterocyclic carbene

1. Introduction

Mitochondria play a key role in the regulation of apoptosis (cell death) and in diseases characterised by abnormal apoptotic responses such as cancer. As a result there has been considerable recent interest in targeting mitochondrial cell death pathways in the development of new chemotherapeutic agents. There is potential to overcome the two overriding problems in cancer chemotherapy—the common occurrence of drug-resistant tumour cells and the lack of selectivity of cancer drugs in differentiating between cancerous cells and normal cells.

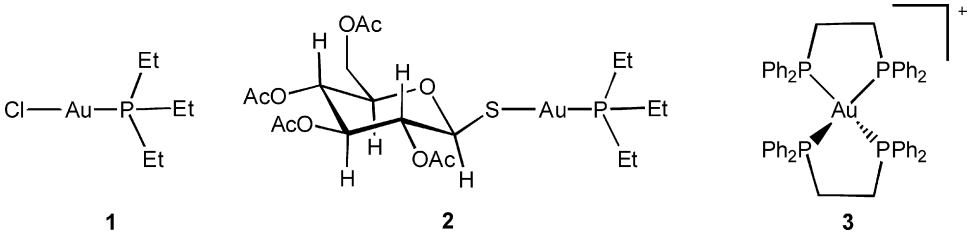
We have a long interest in the development of Au(I) phosphine antitumour agents [1] and research stemming from the early 1980s [2] points to two distinct classes of Au(I) phosphine antitumour agents in which the mode of action may depend on targeting mitochondrial function These are neutral linear two-coordinate Au(I) complexes such as Et_3PAuCl (**1**; Scheme 1) and the tetraacetylthioglucose gold(I) phosphine complex, auranofin (**2**) and cationic tetrahedral bis-chelated Au(I) phosphine complexes related to $[Au(dppe)_2]^+$ (**3**).

The possible role of mitochondria in the mechanisms of cytotoxicity and antitumour activity of these complexes was addressed in a 2002 review article in this journal [3]. Since then important new evidence has shown that auranofin induces apoptosis by a mechanism involving inhibition of the mitochondrial enzyme thioredoxin reductase (TrxR), which has recently emerged as a new target for drug development. The mechanism most likely depends on binding of Au(I) to the selenocysteine residue. As bis-chelated Au(I) complexes such as $[Au(dppe)_2]^+$ do not undergo ligand exchange reactions as readily as two-coordinate linear Au(I) complexes, the mode of action is likely to be different from that of auranofin; antitumour activity may stem from the lipophilic, cationic properties, as for other delocalised lipophilic cations that accumulate in mitochondria.

This article is based primarily on our own recent work on different types of Au(I) phosphine and *N*-heterocyclic carbene compounds under investigation as potential mitochondrial targeting antitumour agents. We also review recent related literature on auranofin and a variety of other Au(I) and Au(III) antitumour compounds which either inhibit mitochondrial thioredoxin reductase, or induce apoptosis by other mitochondrial cell death pathways.

1.1. Mitochondria as a target for chemotherapy

Apoptosis is a form of cell death characterised by the activation of caspases (cysteine aspartyl-specific proteases) that cleave multiple targets in the cell. The control of caspase activation represents a major decision point for determining whether a cell will continue to live or die by apoptosis. Mitochondria have long been known to be the cells main energy producers, responsible for over 80% of ATP production in normal cells; however, more recently research has shown that these organelles also pay a central role in the control of apoptosis. It is beyond the scope of this review to discuss this very active research area in detail, but for recent reviews see Refs. [4–12]. In vertebrate cells the mitochondrial (intrinsic) pathway is the major form of apoptosis, and the critical event responsible for caspase activation is mitochondrial outer membrane permeabilization (MOMP) referred to as the "point of no return" of cell death [6]. Numerous pro-apoptotic signal-transducing molecules and pathological stimuli converge on mitochondria to induce MOMP, which leads to the release of cytochrome *c* and other pro-apoptotic proteins from the intermembrane space. Caspase activation is then triggered *via* the formation of an Apaf-1 apoptosome complex, which recruits and activates caspase 9 and the cascade of apoptotic events leads to the disintegration of the cell without the release of harmful cell contents to the surrounding tissue [6,13]. MOMP also leads to the release of caspase-independent death effectors and loss of essential mitochondrial functions, so the event is lethal even in the absence of caspase activation. The mechanisms responsible for MOMP during apoptosis remain highly controversial but recent literature [6,13] describes two distinct classes of mechanism and each may function under different circumstances (see Fig. 1). One involves only the outer membrane and is mediated by members of the Bcl-2 family of apoptosis-regulating proteins acting directly on the outer membrane. The other mechanism involves the induction of a mitochondrial permeability transi-



Scheme 1. Examples of Au(I) phosphine antitumour complexes.

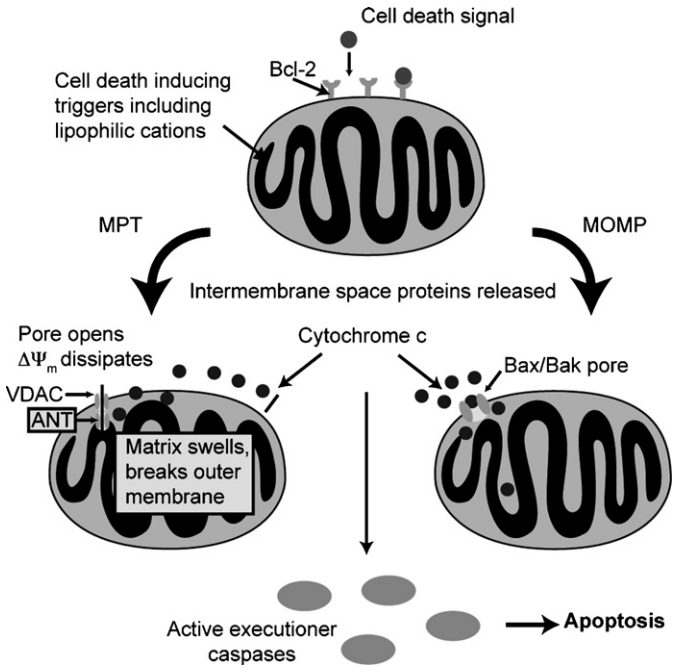


Fig. 1. A cartoon depicting apoptosis induction *via* the mitochondrial pathway, illustrating the two proposed mechanisms that lead to release of cytochrome *c* and other pro-apoptotic proteins from the inter-membrane space, triggering the activation of caspases (see Fig. 1 in Ref. [13] for a more detailed pictorial representation).

tion (MPT) *via* the opening of a pore in the inner membrane known as the permeability transition (PT) pore. Until recently this mechanism was generally regarded as the prime mechanism responsible for the permeabilization of the mitochondrial outer membrane and even though some recent literature suggests MPT is associated mainly with necrosis, signalling crosstalk between the MPT and Bcl-2 family proteins nevertheless provides evidence for an important role for the MPT in apoptosis [4,5]. Formation of the PT pore allows water and molecules up to ~1.5 kDa to pass through, causing swelling of the matrix which is sufficient to break the outer membrane to produce MOMP. The exact composition of the PT pore is controversial and a popular hypothetical model is that it forms by the apposition of trans-membrane proteins from the inner and outer mitochondrial membranes at contact sites between the two membranes [14]. Most models postulate roles for the adenine nucleotide translocator (ANT) in the inner membrane and the voltage dependent anion channel (VDAC) on the outer membrane. Other putative pore constituents are cyclophilin D, mitochondrial hexokinase and creatine kinase, Bcl-2 family proteins and the peripheral benzodiazepin receptor (PBR) [15,16].

These recent developments in understanding the central place of mitochondria as a regulator of cell death has prompted the idea of targeting mitochondria in new approaches to cancer chemotherapy. The importance and rapid growth of this area can be judged by many recent review articles [13,16–29] which include journal special issues [30,31]. A major impetus for this research is to develop strategies to overcome the common problem of resistance to cancer chemotherapy. Most commonly used anticancer drugs indirectly exploit mitochondria in their cytotoxic action by inducing apoptosis through signalling pathways that involve MOMP [28]. DNA damaging agents lead to activation of proapoptotic members of the Bcl-2 superfamily (cell-intrinsic pathway), while other anticancer agents act *via* cell surface death receptors and activation of apoptosis through a p53-independent signalling mechanism (cell-extrinsic pathway). A hallmark of cancer cells is their intrinsic resistance to the induction of MOMP when compared to normal cells, for example as a consequence of the overexpression of antiapoptotic proteins from the Bcl-2 family, mutations of proapoptotic Bcl-2 antagonists and p53, or overexpression of apoptosis-inhibitory PT pore components, such as hexokinase II, cyclophilin D and the PBR [28,32]. Chemotherapeutic agents that can induce MOMP by a direct action on mitochondria are attractive as they induce cell death irrespective of the upstream control mechanisms and have the potential to overcome these resistance mechanisms. A further impetus for this research is that many differences have been identified between mitochondria in normal and malignant cells and there is potential to exploit these mitochondrial alterations in cancer to produce new drugs with the potential to selectively target mitochondria in tumour cells. The design of selective anticancer drugs is an important goal to overcome the major toxicities associated with conventional (non-selective) anticancer treatments. A variety of different strategies targeting mitochondria for cancer therapy have been described in recent literature and summarized in several recent review articles (see for example Refs. [16,22,25,27–29]). The main approaches involve the modulation of the action of Bcl-2 family members, or the use of specific agents that induce MPT by targeting various components of the PT pore. The PT proteins PBR, creatine kinase, hexokinase and particularly ANT, have all been considered as targets for therapeutic intervention [27]. The list of agents shown to induce cell death by acting directly on mitochondrial membranes is now extensive [28] and includes a number of conventional therapeutics (e.g. etoposide [33,34] and paclitaxel [35]) as well as arsenite (As_2O_3) the treatment of choice for acute promyelomonocytic leukaemia [36,37]. The redox state of mitochondrial thiols is critical in the control of MPT [29] and agents that promote oxidative stress can be used to trigger MPT. For example, arsenite depletes glutathione and thiol-crosslinking agents (e.g. the trivalent arsenical peptide GSAO [16,38]) oxidise regulatory vicinal thiols in ANT [28].

One approach to the selective targeting of mitochondria in cancer cells exploits the long known phenomenon that carcinoma cells have an elevated mitochondrial membrane potential ($\Delta\psi_m$) compared to normal human epithelial cells [39]. Recent developments in mitochondrial research have lead to renewed interest [40] in the diverse class of compounds described as delocalised lipophilic cations (DLCs). These have a long history as potential antitumour drugs. Chen and coworkers studied more than 200 epithelial-derived cell lines/strains and found that carcinoma cells consistently had a higher level of uptake and retention of Rhodamine-123 (Rh-123) than normal human epithelial cells [41,42], which was attributed to the abnormally high $\Delta\psi_m$ characteristic of the carcinoma cells. Over the past 20 years several structurally diverse lipophilic cations have demonstrated strong activity in tumour models, e.g. Rh-123 [43], dequalinium

[44], AA1 [45], bis-quaternary ammonium heterocycles [46] and triarylalkylphosphonium salts [47]. Until recently their development was hindered by severe host toxicity, but one example (MKT-077) [48,49] reached Phase II clinical trials before being withdrawn due to renal toxicity. Another example of this class, F16, has shown promise as a mitochondriotoxic small molecule that selectively inhibits tumour cell growth [50]. All compounds of this class share a common mechanism for mitochondrial accumulation [51] but their mechanisms of mitochondrial toxicity are quite varied and not well understood [40]. Two recent predictive models have addressed the key factors that determine selective accumulation of DLCs in tumour cell mitochondria considering lipophilic–hydrophilic balance as well as the acid–base dissociation constant and the electric charge [52,53].

1.2. Thioredoxin reductase—a new drug target

Thioredoxin reductases are a class of homodimeric selenoenzymes that catalyse the NADPH-dependent reduction of thioredoxins, a family of ubiquitous disulfide reductases responsible for maintaining proteins in their reduced state (Fig. 2A). Thioredoxin reductases belong to the flavoprotein family of pyridine nucleotide–disulfide reductases that includes glutathione reductase. Members of the family are homodimeric proteins in which each monomer includes an FAD prosthetic group, an NADPH binding site and an active site containing a redox-active disulfide. Thioredoxin reductases have a broad substrate specificity attributable to a second redox-active site, a C-terminal -Cys-SeCys- (where SeCys is selenocysteine), that is not found in glutathione reductase. The thioredoxin reductase/thioredoxin system is present in most organisms, and mitochondria have a separate thioredoxin reductase/thioredoxin system. Thioredoxins serve as electron donors for a range of enzymes, including ribonucleotide reductase, which plays a critical role in DNA synthesis. In addition, thioredoxins are vital in the regulation of redox signalling pathways *via* thiol redox control and a growing number of transcription factors have been identified that require thioredoxin reduction for DNA binding. For several recent reviews on this active research field see Refs. [54–57].

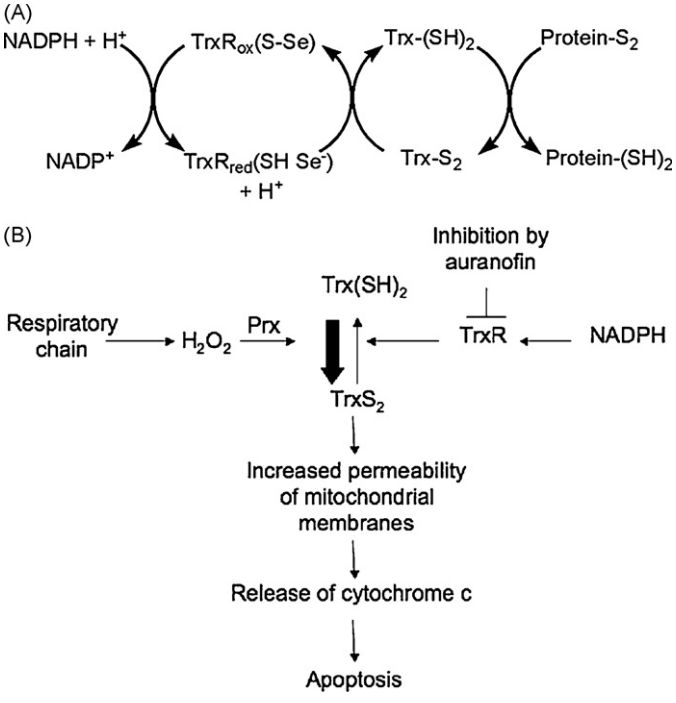


Fig. 2. (A) The thioredoxin reductase/thioredoxin system and (B) a scheme depicting the mechanism of auranofin alteration of the thiol redox balance in mitochondria leading to apoptosis, as proposed by Bindoli et al. [72]; Prx, peroxiredoxin (thioredoxin peroxidase); TrxR, thioredoxin reductase; $Trx(SH)_2$, reduced thioredoxin; $Trx(S)_2$, oxidised thioredoxin; reproduced from Ref. [72], with permission of Taylor & Francis Ltd.

The thioredoxin reductase/thioredoxin system is also believed to play a pathophysiologic role in several chronic diseases such as certain cancers, rheumatoid arthritis and Sjögren's syndrome [54]. The up-regulation of thioredoxins in malignant diseases is well established, with the growth-promoting effects of thioredoxin outweighing the beneficial anti-oxidant properties [58]. Elevated expression of thioredoxin is associated with increased proliferation of tumour cells, inhibition of apoptosis, aggressive tumour growth and decreased patient survival. In addition, high concentrations of thioredoxin reductase can support tumour cell drug resistance [58].

Due to the pathophysiological role of thioredoxin reductase, this enzyme has emerged as a potential new drug target [54,59,60]. Importantly, Au(I) containing drugs, which have been used for the treatment of rheumatoid arthritis (e.g. aurothioglucose and auranofin) are potent inhibitors of thioredoxin reductase [54]. Auranofin ($K_i = 4$ nM) [61] is particularly potent and Au(I) complexes are amongst the most effective and selective inhibitors of mammalian thioredoxin reductases found to date [54,62]. The interaction of Au(I) with other selenoenzymes (e.g. glutathione peroxidase and deiodinase I) is well known [63,64] and inhibition of thioredoxin reductase is attributable to binding of Au(I) to the C-terminal redox active -Cys-SeCys- centre [61,62]. Notably, the structurally and mechanistically closely related but selenium-free enzyme, glutathione reductase is inhibited by Au(I) compounds at 1000-fold higher concentrations [54]. Studies relating to the inhibition of mitochondrial thioredoxin reductase by Au(I) and Au(III) compounds are discussed below in Sections 2.1 and 4.

2. Au(I) phosphine compounds

2.1. Auranofin and related compounds

Auranofin (trade name Ridaura, **2**; Scheme 1) is an orally administered anti-arthritic gold drug. In common with many other linear two-coordinate Au(I) phosphine complexes, auranofin has been shown to inhibit the growth of cultured tumour cells *in vitro* [2,65,66]. Extensive early mechanistic studies provide evidence that both auranofin and Et_3PAuCl affect mitochondrial function (reviewed in Ref. [3]). These studies can now be reinterpreted to be consistent with an antitumour mechanism involving the induction of apoptosis rather than growth arrest of cycling cells [67]. An interesting development, reported by Bindoli and coworkers [68,69], is that auranofin, at submicromolar concentrations, induces MPT observed as mitochondrial

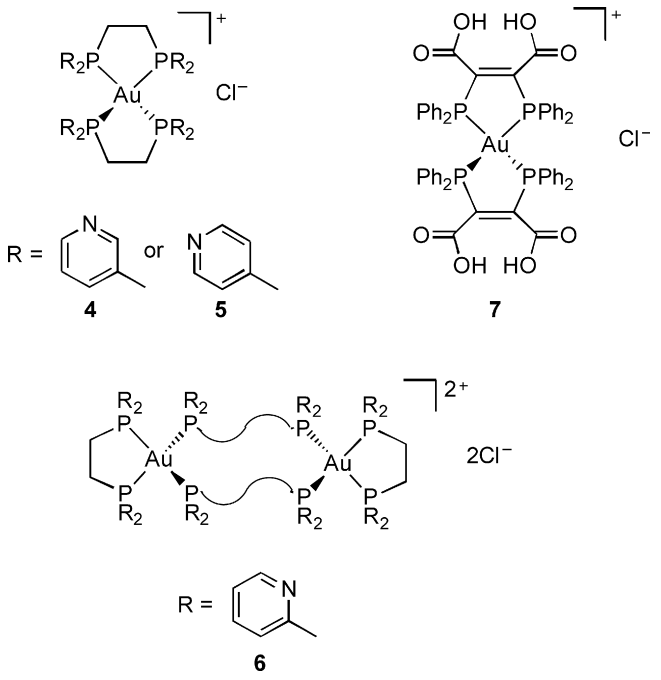
swelling and loss of membrane potential. Both events are completely inhibited by cyclosporin A, the specific inhibitor of MPT. The permeability changes were shown to occur at concentrations associated with the selective inhibition of mitochondrial thioredoxin reductase, and with few effects on the mitochondrial electron transport chain or glutathione reductase. The redox state of mitochondrial thiols is a key factor in the control of MPT and the thioredoxin reductase/thioredoxin system present in mitochondria may play a critical role [70]. Et_3PAuCl and aurothiomalate were also found to be potent inhibitors of mitochondrial thioredoxin reductase in the submicromolar range, but less potent than auranofin (Table 1) [71]. Recently it has been demonstrated that auranofin prevents the removal of hydrogen peroxide formed by the mitochondrial respiratory chain [72,73]. This is because hydrogen peroxide oxidises reducing equivalents such as thioredoxin that cannot be reduced by thioredoxin reductase when it is inhibited by auranofin. This then leads to an increase in hydrogen peroxide within mitochondria. In addition, the inhibition of thioredoxin reductase by auranofin means that thioredoxin oxidised by proteins, such as peroxiredoxin, cannot be reduced. Oxidised thioredoxin can act on several different targets leading to an opening of the PT pore or increasing the permeability of the outer membrane, thereby causing the release of apoptotic factors. This proposed mechanism of induction of apoptosis by auranofin, due to alteration of the thiol redox balance in mitochondria, is illustrated in Fig. 2B [72].

2.2. Tetrahedral Au(I) diphosphine complexes

The antitumour activity of the Au(I) phosphine complex $[Au(dppe)_2]^+$ (**3**) was first reported two decades ago [1]. The complex is a lipophilic cation and early studies provided some evidence for a mode of action involving effects on mitochondrial function [74], as well as the occurrence of DNA strand breaks and DNA-protein cross-links in tumour cells [1]. $[Au(dppe)_2]^+$ was selected for pre-clinical trials, but was abandoned when severe hepatotoxicity was observed in dogs. Structure activity relationships were evaluated for a wide range of diphosphine ligands and their metal complexes [2,75]. For complexes of the type $[Au(R_2P(CH_2)_nPR'_2)_2]X$ highest activity was found where R = R′ = phenyl and $n = 2$, 3 or *cis*-CH=CH. In general, activity was reduced, or lost altogether when the phenyl substituents on the phosphine were replaced by other substituents, but retained

Table 1
Inhibition of mitochondrial thioredoxin reductase by some Au(I) and Au(III) compounds (from Refs. [71,110])

Compound	IC_{50} (μM)
Au(I) compounds	
Aurothiomalate (myocrisin)	0.280
Et_3PAuCl	0.065
Auranofin	0.020
Au(III) compounds	
$[Au(dien)Cl]Cl_2$ (**14**)	0.42
$[Au(py^{dmb}\text{-}H)(OAc)_2]$ (**15**)	1.42
$[Au(bipy^{dmb}\text{-}H)(OH)]PF_6$ (**16**)	0.28
$[Au(bipy^{dmb}\text{-}H)(2,6\text{-xylidine-H})]PF_6$ (**17**)	0.21



Scheme 2. Hydrophilic analogs of $[Au(dppe)_2]^+$ (**3**).

when Au(I) is substituted by Ag(I) or Cu(I). Dppe complexes of other metals (e.g. Pt(II) and Pd(II)) were found to be less active than the phosphine alone.

In more recent work (a collaboration with Mark J. McKeage) we have investigated a series of Au(I) complexes of bidentate pyridylphosphine ligands, related to the parent compound $[Au(dppe)_2]^+$ [76,77]. Importantly, we showed that the selectivity of these complexes for cancer cells over normal cells can be 'tuned' by adjusting their lipophilic/hydrophilic balance through ligand design. The position of the N-atom in the pyridyl ring influences the interaction with solvent providing a series of compounds which are structurally similar to $[Au(dppe)_2]^+$, and in which the hydrophilic character covers a very large range. The 3- and 4-pyridyl complexes (**4** and **5**, Scheme 2), exist as simple monomeric cations in the solid state and in solution and have a much higher water solubility than the 2-pyridyl complex (**6**) which crystallises in the solid state as a dimer [76,78].

An important consideration is that for these bis-chelated Au(I) phosphine complexes the high thiol reactivity characteristic of linear Au(I) complexes is considerably reduced. $[Au(dppe)_2]^+$ and the pyridyl phosphine analogs have been shown to be stable in the presence of thiols and to remain intact in human blood plasma [1,77]. The structural integrity of the compounds therefore allowed the relationship between biological activity and lipophilicity to be explored [77]. Cytotoxicity studies carried out against a panel of human ovarian carcinoma cell lines *in vitro* showed a general increase in potency (and decrease in selectivity) with increasing lipophilicity of the compound [76,77]. When correction for drug binding to serum proteins was made, linear correlations were observed for both cellular uptake and growth inhibition (IC_{50}) of CH-1 cells and the lipophilicity of the Au(I) phosphine complex [77]. Antitumour activity *in vivo* of $[Au(dppe)_2]Cl$ and the pyridylphosphine analogs also showed

a dependence on lipophilicity in subcutaneous colon 38 tumours [77]. The most lipophilic and hydrophilic compounds had no significant tumour growth delay, whereas the 2-pyridyl analog (intermediate lipophilicity) showed significant antitumour activity, less dose limiting toxicity and higher gold concentration in plasma and tumours compared with more lipophilic and more hydrophilic analogs (Fig. 3). Non-specific binding to proteins is likely to account for the high host toxicity of the highly lipophilic $[Au(dppe)_2]^+$, whereas the most hydrophilic compounds may be limited by high rates of excretion as a consequence of low protein binding [77]. Overall, these results are consistent with the trends derived from predictive models for the selective accumulation of DLCs in tumour cells based on hydrophilic/lipophilic character [52,53].

A different approach to reducing the lipophilicity of the parent $[Au(dppe)_2]^+$ complex is to retain the aromatic substituents, which appear to be important for antitumour activity [2] and introduce hydrophilic groups into the ethane bridge of dppe to increase water solubility. The bis-chelated 2:1 adduct of Au(I) with 2,3-bis(diphenylphosphino)maleic acid, $[Au(dpmaa)_2]Cl$ (**7**; Scheme 2), is highly water soluble and the crystal structure shows a well defined hydrogen bond network involving the carboxyl groups (Fig. 4) [79]. Interestingly, $[Au(dpmaa)_2]Cl$ exhibited no significant cytotoxicity when assessed against eight different cancer lines [79]. The complex was shown to be stable after prolonged incubation in cell culture media containing 5% fetal calf serum and the lack of cytotoxicity maybe a reflection of a low degree of cellular uptake. Under physiological conditions the carboxyl groups are likely to be deprotonated so the complex has an overall negative charge. These results lend support to the hypothesis that the antitumour activity of $[Au(dppe)_2]^+$ and related compounds depend on their accumulation in tumour cell mitochondria as a consequence of their lipophilic-cationic character. The mitochondrial matrix carries a net negative charge and anionic molecules do not accumulate [53].

Several lines of evidence from early work point to the involvement of mitochondria as a target in the antitumour activity of $[Au(dppe)_2]^+$ and these studies were discussed at length in the previous review by McKeage et al. [3]. Due to the central role of MOMP in apoptosis, most chemotherapeutic agents that induce apoptosis are likely to show signs of MOMP when added to intact cells. To prove that a given drug acts by direct effects on mitochondria requires demonstration of MOMP when added to purified mitochondria in a cell free-system [20]. Early

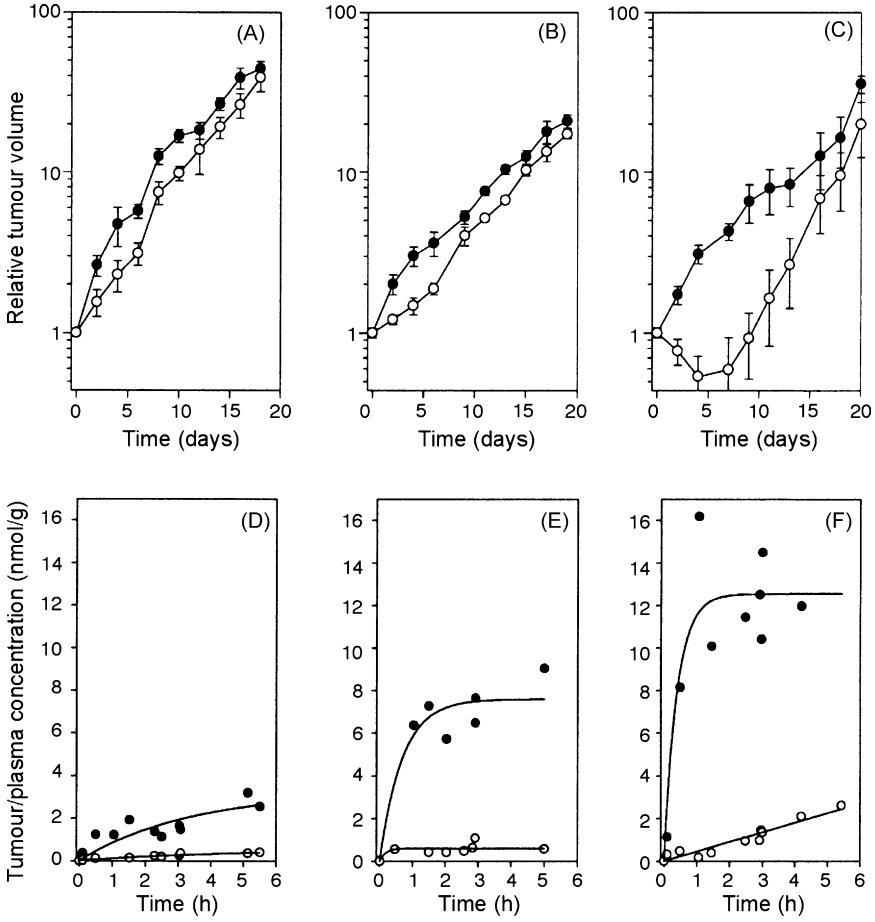


Fig. 3. Activity of $[Au(dppe)_2]Cl$ (A) and the pyridyl phosphine analogs **5** (R = 4-pyridyl) (B) and **6** (R = 2-pyridyl) (C) against murine subcutaneous colon 38 tumours. Tumour volumes (relative to volume at commencement of treatment) of control mice (●) and treated nice (◯). Panels D–F show the drug concentrations (as Au) of each complex shown in the panel above in plasma (◯) and colon 38 tumour tissue (●) as a function of time after drug administration. Reproduced from Ref. [77], with kind permission of Springer Science and Business Media.

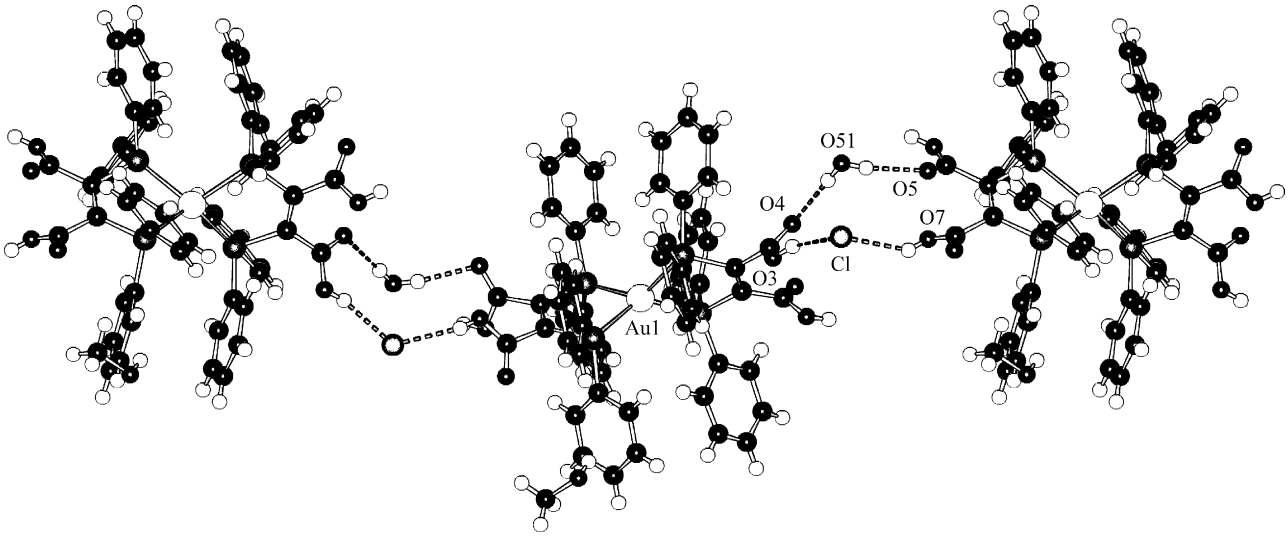


Fig. 4. H-bonding network linking $[Au(dpmaa)_2]^+$ entities in the complex $[Au(dpmaa)_2]Cl{\cdot}H_2O{\cdot}CH_3OH$. Reproduced from Ref. [79], with permission from Elsevier.

studies by Hoke et al. [80,81] on isolated hepatocytes showed that $[Au(dppe)_2]^+$ stimulated O_2 consumption and caused ATP depletion. A disruption in the electrochemical potential in isolated mitochondria treated with $[Au(dppe)_2]^+$ caused secondary calcium efflux. In the context of recent research into mitochondrial function these observations are consistent with a mechanism involving the mitochondrial permeability transition pore complex. In recent work (in collaboration with David A. Day) we have demonstrated that $[Au(dppe)_2]^+$ causes dose-dependent permeabilization of mitochondrial membranes [82]. At low concentrations (e.g. 10 μM), $[Au(dppe)_2]^+$ uncouples mitochondrial respiration and induces cyclosporin-A sensitive swelling of the mitochondria in the presence of low concentrations of Ca^{2+}, suggesting opening of the mitochondrial permeability transition pore complex. However, higher doses (>30 μM) cause severe inhibition of respiration and rapid permeabilization of mitochondrial membranes, which is insensitive to cyclosporin A and Ca^{2+} [82]. It is possible that the cytotoxic mechanism of $[Au(dppe)_2]^+$ involves induction of apoptosis in cancer cells by MPT, but the effects are overridden by the high lipophilicity which results in the rapid unselective permeabilization of mitochondria membranes in all cells. We have conducted preliminary studies on the hydrophilic pyridylphospline analogs which show that cyclosporin-A sensitive swelling of the rat liver mitochondria occurs at higher concentrations than for $[Au(dppe)_2]^+$. As shown in Fig. 5, swelling induced by 20 μM concentrations of the 2- and 3-pyridyl analogs is completely inhibited by 1 μM CSA, whereas for $[Au(dppe)_2]^+$ the sensitivity to CsA is lowered at this concentration, indicating the onset of non-specific membrane permeabilization. Overall, these results suggest that by fine-tuning the hydrophilic–lipophilic balance in the optimal range, it may be possible to identify compounds of this class able to target the mitochondria of tumour cells with great specificity. With this aim in mind we have synthesized Au(I) adducts of the 3-carbon bridge analog of the 2-pyridyl ligand (1,3-bis(di-2-pyridylphosphino)propane, d2pypp). The *in vitro* cytotoxic activity of $[Au(d2pypp)_2]Cl$ has been assessed in human normal and cancer breast cells and selective toxicity to the cancer cells was found [83]. Studies probing the mechanism of antitumour activity of the pyridylphosphine complex are in progress.

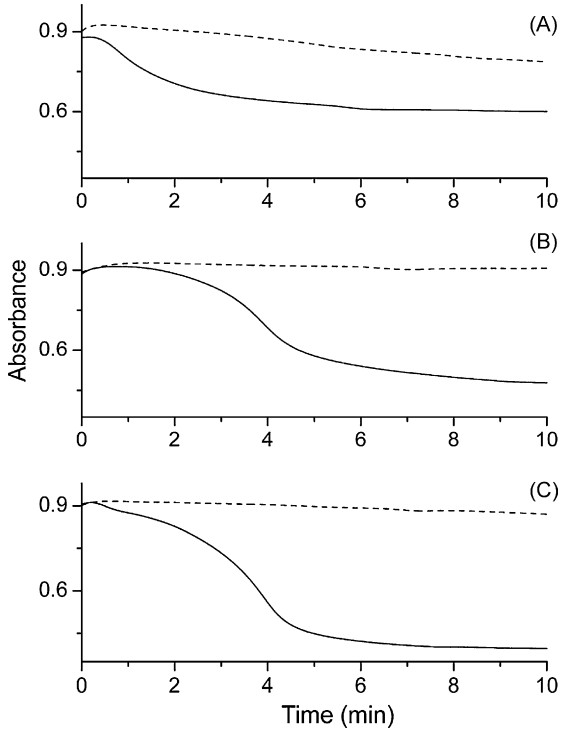


Fig. 5. Induction of mitochondrial swelling by $[Au(dppe)_2]Cl$ (**1**) (A) and the pyridyl phosphine analogs, **6** (R = 2-pyridyl) (B) and **4** (R = 3-pyridyl) (C) in the presence (dashed line) and absence (solid line) of CsA (1 μM). The assays were started by the addition of rat liver mitochondria (final concentration 0.25 mg mitochondrial protein/mL) to a buffer consisting of mannitol (195 mM), sucrose (65 mM), KCl (10 mM), HEPES (10 mM), KH_2PO_4 (2 mM), $MgCl_2$ (1 mM) succinate (5 mM) and $CaCl_2$ (25 μM) at pH 7.2 and 25 °C. Stock solutions of compounds in methanol (∼1 mM) were added to give final complex concentrations of 20 μM.

3. Au(I) *N*-heterocyclic carbene complexes

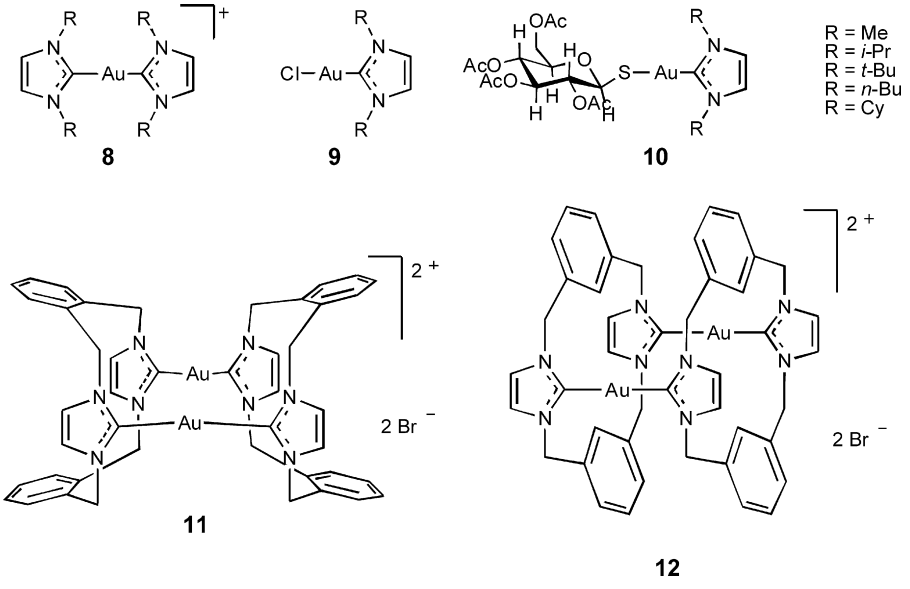
Metal complexes of *N*-heterocyclic carbene ligands (NHCs) are of great current interest for a range of applications. NHCs are a class of electron donating ligand, which form strong σ bonds to metal ions in both high and low oxidation states [84–86]. Some similarities between NHC and phosphine ligands have been noted and NHCs have been used in place of phosphines to prepare catalysts, perhaps most notably for the second generation Grubbs catalyst [85,87]. Our long-standing interest in the antitumour properties of Au(I) phosphine complexes, and the similarities between NHCs and phosphines, led to our investigation of Au–NHC complexes as potential new antitumour agents that target mitochondrial function (a collaboration with Murray V. Baker). Au–NHC chemistry has been recently reviewed [88].

Our previous studies on tetrahedral Au(I) complexes of bidentate phosphine ligands have yielded promising results, however difficulties encountered in the preparation of the phosphine ligands have limited the approach. Metal complexes of NHCs are often prepared from easily synthesized imidazolium salts, offering the possibility of the generation of a large library of metal complexes from which compounds with optimal biological properties can be selected.

A series of mononuclear Au(I)–NHC complexes of the type $[(R_2Im)_2Au]^+$ (**8**; Scheme 3) have been prepared [89] in which the alkyl substituents on the NHC ligands were varied in a manner to allow the generation of complexes with an essentially unchanged structural core, but differing hydrophilic/lipophilic properties. The log *P* values for these compounds vary across the series within the range −1.09 to 1.73 and increase in the expected manner: R = methyl (Me) < *iso*-propyl (*i*-Pr) < *tert*-butyl (*t*-Bu) < *n*-butyl (*n*-Bu) < cyclohexyl (Cy). The propensity of five of the compounds to induce mitochondrial membrane permeabilization was assessed by a standard assay that measures increases in mitochondrial volume (swelling) based on a decrease in the apparent absorbance (540 nm) of a mitochondrial suspension in the presence of the compound. As shown in Fig. 6, at concentrations between 1 and 10 μM the compounds induced dose-dependent, Ca^{2+}-sensitive, mitochondrial swelling at rates that increased with the lipophilicities of the complexes, with the most lipophilic compounds inducing the most rapid onset of swelling. The swelling was completely inhibited by cyclosporin A, demonstrating that the swelling caused by these compounds results from the induction of MPT, rather than a non-specific mechanism [89]. As described elsewhere in this review, the biological effects displayed by a variety of Au(I) and Au(III) compounds are consistent with their inhibition of the selenoenzyme, thioredoxin reductase. It is reasonable to suspect that the activity displayed by the Au(I)–NHC complexes studied here may also be due to the inhibition of this enzyme. We have used NMR spectroscopy to evaluate the reactivity of two cationic mono-nuclear gold carbene complexes (**8**, R = Me, *i*-Pr) with selenocysteine (SeCys) and cysteine (Cys) as a model of their potential reactivity towards the SeCys residue of thioredoxin reductase. These studies showed that the Au(I)–NHC complexes were significantly more reactive towards SeCys than Cys [90].

A series of neutral Au(I) *N*-heterocyclic carbene complexes of the general form (NHC)AuX (X = Cl and 2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl-1-thiolato; **9** and **10**; Scheme 3) have also been prepared as NHC-based analogues of Et_3PAuCl (**1**) and auranofin (**2**) [91]. In a similar manner to the cationic complexes described previously, the alkyl substituents of the NHC ligands were varied to allow modulation of the lipophilic properties of the resultant complexes. Studies investigating the affects of these complexes on mitochondrial function are in progress. Other researchers have also been developing Au(I)–NHC complexes bearing biologically compatible groups [92].

A family of dinuclear Au(I) carbene complexes have been synthesized from various imidazolium-linked cyclophanes and related acyclic bis(imidazolium) salts (e.g. **11** and **12**; Scheme 3) [93]. Seven of the dinuclear gold(I) complexes of both cyclophane and acyclic bidentate carbene ligands were evaluated for



Scheme 3. Examples of mono- and di-nuclear Au(I) *N*-heterocyclic carbene complexes.

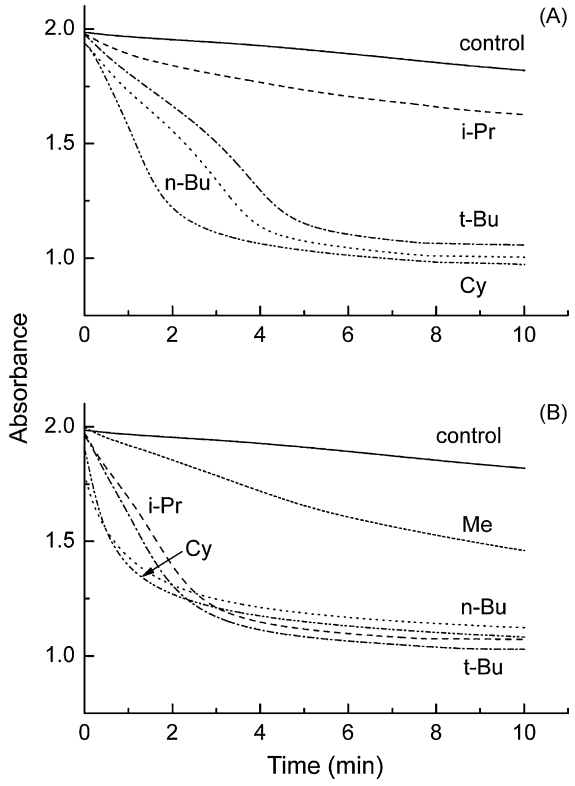
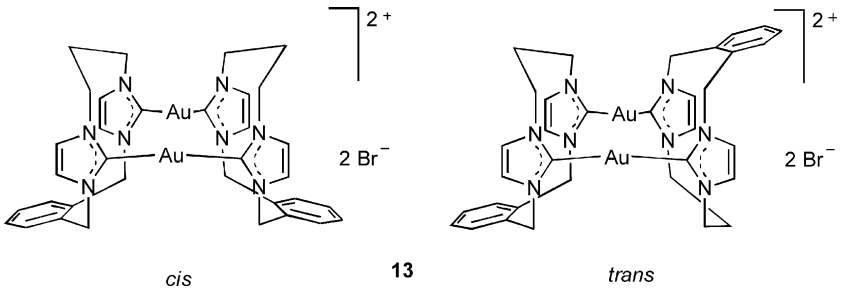


Fig. 6. Induction of mitochondrial swelling by a series of mononuclear Au(I)–NHC complexes of the type $[(R_2Im)_2Au]^+$ (**8**, R = Me, *i*-Pr, *t*-Bu, *n*-Bu and Cy; Scheme 3), as monitored by the decrease in the apparent optical density (at 540 nm) of a suspension of rat liver mitochondria at 25 °C. The complex concentrations are (A) 1 μM and (B) 10 μM; in the control no addition was made. In all cases (except R = *n*-Bu, 10 μM) swelling was blocked by the addition of cyclosporin A (1 μM) (results not shown) and did not differ significantly from the control. Reproduced from Ref. [89], by permission of The Royal Society of Chemistry.

their ability to induce MPT in isolated mitochondria [94]. Six of the compounds, at concentrations of 10 μM, induced Ca^{2+} sensitive MPT as evidenced by mitochondrial swelling. In the absence of added Ca^{2+} the compounds were either inactive or their activity was significantly reduced. The mitochondrial swelling was completely blocked by the addition of cyclosporin A. The rates and levels of uptake of Au into mitochondria for these compounds were estimated using inductively coupled plasma optical emission spectroscopy (ICP-OES). Significant differences were found in the levels at which the different compounds accumulated in the mitochondria, however the levels of Au uptake did not correlate strongly with the rate at which they induced mitochondrial swelling.

Structural analysis of the dinuclear complexes showed that the cyclophane ligand framework provides fine control over the intra-molecular distance between the gold atoms [93]. For example, compound **11** (Scheme 3) supports a short Au···Au interaction of 3.0485(3) Å, while compound **12** shows a significantly longer Au···Au distance of 3.7917(4) Å. Short Au···Au distances of less than 3.6 Å are generally considered to indicate the presence of an attractive (aurophilic) interaction and compounds displaying these short Au···Au are often luminescent. This relationship is well demonstrated by these molecules with compound **11** being luminescent (both in solution and the solid state) while compound **12** is not. The luminescence associated with attractive Au···Au (aurophilic) interactions has been of great interest and complexes possessing such properties offer potential as luminescent display devices and sensors [95–98]. Our interest in targeting specific cellular organelles (mitochondria) led us to consider the possibility of exploiting the native luminescence of **11** to determine its intracellular distribution using fluorescence microscopy; however, its luminescence profile (λ_{ex} 260 nm, λ_{em} 400 nm) is unsuitable for such studies, as the high-energy excitation would lead to interference from other cellular components. Previous reports have shown that the excitation and emission energies associated with short Au···Au interactions are, in some cases, red-shifted as a result of a contraction of the Au···Au distance [99–102]. With this in mind, in collaboration with the Baker group, we synthesised the new dinuclear Au(I) complex **13** (Scheme 4) [103]. As the ligand is unsymmetrical about the imidazolium units *cis* and *trans* isomers of **13** were obtained. Aqueous solutions of each isomer display identical electronic absorption and emission properties. Two emission bands are seen with relative intensities that depend on the excitation wavelength used (λ_{ex} 313 nm, λ_{em} 396 nm; λ_{ex} 355 nm, λ_{em} 496 nm), and each of the emission bands show distinct excitation spectra. Importantly, the lower energy excitation and emission bands are of suitable wavelengths for cellular distribution studies allowing us to study the uptake and distribution of *cis*-**13** in single living cancer cells using fluorescence confocal microscopy [103]. An important consideration in determining the feasibility of this approach is establishing whether the Au(I) complex is likely to retain its dinuclear (luminescent) structure under biological conditions, as it might be expected that the structure would be degraded by facile ligand exchange reactions with biological thiols. Remarkably, *cis*-**13** showed



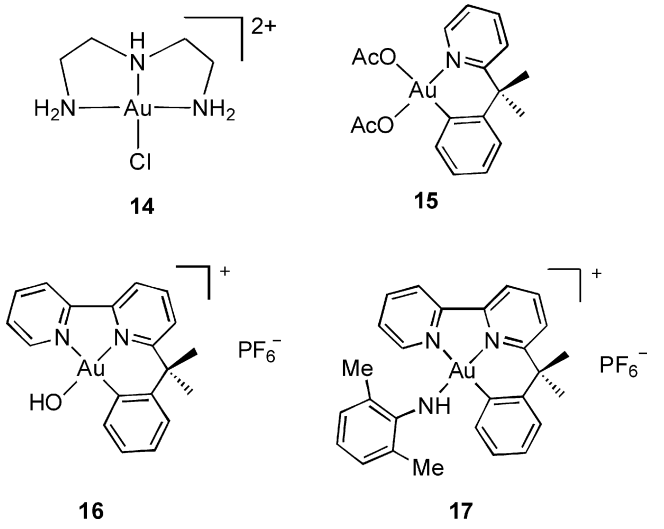
cis **13** *trans*

Scheme 4. Luminescent dinuclear Au(I) *N*-heterocyclic carbene complex with short Au···Au distance used for cellular distribution studies.

no reaction with reduced glutathione after prolonged incubation under physiological conditions (pH 7, 37 °C). The mouse macrophage cancer cell line RAW264.7 was chosen for this initial study because their adherent properties make these cells ideal for investigation by confocal microscopy. By the use of co-localization studies with a lysosomal dye (LysoTracker red) and a mitochondrial dye (MtoTracker green) we observed localization of *cis*-**13** within lysosomes rather than mitochondria in the RAW264.7 cells. Notably, *cis*-**13** exhibits only modest cytotoxicity in this cell-line, but is more potent in other cell lines such as human promyelocytic leukaemia cell line, HL 60 [103]. Fluorescence microscopy studies of both zinc bis(thiosemicarbazone) complexes [104] and a dinuclear platinum complex labelled with a fluorescent tag [105,106] have shown sequestration of the complexes in lysosomes. These latter studies have demonstrated also that intracellular distribution is strongly dependent on the cell type. Further studies are in progress using other cell lines to investigate whether a correlation exists between cytotoxicity and localization to mitochondria.

4. Au(III) compounds

In early work Au(III) complexes were investigated for antitumour activity based on the rationale that as they are isolectronic and isostructural with square planar Pt(II) complexes, they may form similar DNA adducts. A difficulty is that most Au(III) complexes are rapidly reduced to Au(I) under physiological conditions but some Au(III) complexes have been identified which have significant antitumour properties and in which the Au(III) oxidation state is stabilized by appropriate choice of ligands (see [107] for a recent review of this topic). Messori and co-workers have investigated the antitumour activity of Au(III) complexes which are stabilized by simple polyamine ligands (e.g. $[Au(en)_2]Cl_3$ and $[Au(dien)Cl]Cl_2$ [108]) and also ligands based on the 2,2-bipyidyl motif such as the cyclometallated complex $[Au(bipy^{dmb}\text{-}H)(OH)]PF_6$ ($bipy^{dmb}$ = 6-(1,1-dimethylbenzyl)-2,2′-bipyridine) [109]. Some examples are shown in Scheme 5.

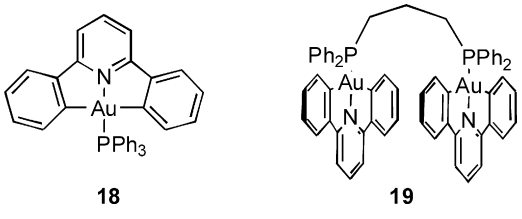


Scheme 5. Examples of Au(III) antitumour complexes shown to inhibit mitochondrial thioredoxin reductase.

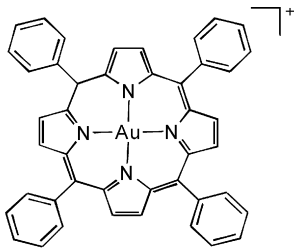
It has been shown that interactions of these Au(III) complexes with DNA are relatively weak, indicating that DNA is not the primary target [107]. Several of these Au(III) complexes have been investigated for their effects on mitochondrial function and their ability to inhibit mitochondrial thioredoxin reductase [71]. All of the tested compounds induced MPT in isolated mitochondria, whilst having little or no effect on the mitochondrial respiratory chain. In addition these Au(III) complexes were shown to be potent inhibitors of mitochondrial thioredoxin reductase (see Table 1). A recent study has investigated the mechanism of cytotoxicity of three of these organogold(III) compounds (**15**, **16** and **17**; Scheme 5) [110] in ovarian A2780 human cancer cells. They were shown to promote apoptosis to a greater extent than cisplatin, while causing only modest cell cycle modifications; it is proposed that mitochondrial pathways are directly involved in the apoptotic process *via* the selective inhibition of thioredoxin reductase. A variety of other Au(III) compounds have been shown recently to be potent inhibitors of mammalian cytosolic thioredoxin [111,112], but in these cases there is no evidence to show whether the Au(III) oxidation state is stable under the testing conditions.

Che and coworkers have recently described a series of interesting cyclometallated Au(III) compounds $[Au_m(C\hat{}N\hat{}C)_mL]^{n+}$ (m = 1–3; n = 0–3; HC^N^CH = 2,6-diphenylpyridine) which contain a variety of N-donor or other auxiliary ligands (L). These complexes exhibit potent cytotoxicity against a panel of cancer cell lines including a cisplatin resistant variant [113] and the Au(III) oxidation state has been shown to be very stable under physiological conditions. The complexes appear to act by a variety of different mechanisms dependent on the nature of the auxiliary ligand, which influences the DNA-binding affinity. In the context of this review, of particular interest are a series of complexes where the $[Au(C\hat{}N\hat{}C)L]^+$ unit is ligated to a variety of mono- and bi-dentate phosphine ligands (e.g. $[Au(C\hat{}N\hat{}C)(PPh_3)]^+$ (**18**) and $[Au_2(C\hat{}N\hat{}C)_2(\mu\text{-dppp})]^{2+}$ (**19**); Scheme 6). These complexes show a cytotoxicity at least 10-fold higher than other Au(III) analogues and react only weakly with DNA. Notably, these complexes are lipophilic cations and have been shown to be stable in solution, so mitochondria are a possible target.

A further series of interesting Au(III) antitumour complexes recently reported by Che and co-workers are a series of Au(III) porphyrins that exhibit potent *in vitro* and *in vivo* anticancer properties toward hepatocellular carcinoma and nasopharyngeal carcinoma [114–118]; the prototypical compound (called 1a)



Scheme 6. Examples of cyclometallated Au(III) phosphine compounds with antitumour activity.

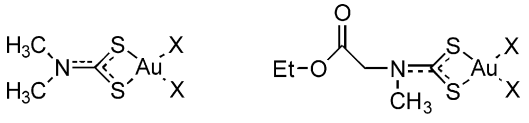


20

Scheme 7. Structure of Au(III) porphyrin 1a.

which has been studied most extensively is shown in Scheme 7 (**20**). The mode of action has been probed in great detail by a range of methods including functional proteomic approaches and it has been demonstrated that mitochondria are the major cellular target [115]. Au(III) porphyrin 1a induces apoptosis though both caspase-dependent and caspase-independent mitochondrial pathways. It is proposed that the complex causes depletion of $\Delta\psi_m$ via the generation of ROS and the altered cellular oxidative state regulates MOMP.

Finally, mention needs to be made of the antitumour Au(III) dithiocarbamate complexes that have been recently reported in the literature by Fregona and co-workers [119–121]. The complexes [Au(DMDT)X_2] (**21**) and [Au(ESDT)X_2] (**22**) (where DMDT = *N*,*N*-dimethyldithiocarbamate and ESDT = ethylsarcosinedithiocarbamate; X = Cl, Br; Scheme 8) are more cytotoxic *in vitro* than cisplatin including in human tumour cells lines intrinsically resistant to cisplatin. The complexes undergo rapid hydrolysis in solution but remain in the Au(III) oxidation state; they do interact with DNA but this is not considered to be the primary biological target. A very recent report [121] indicates that the mechanism involves inhibition of the proteasome. A representative compound of this series, [Au(DMDT)Br_2] was shown to potently inhibit the activity of a purified rabbit 20S proteasome and 26S proteasome in intact MDA-MB-231 human breast cancer cells, resulting in the accumulation of ubiquinated proteins and the proteasome target protein p27 and induction of apoptosis. Inhibition of the Proteasome activity and accumulation of p27 were found also in MDA-MB-231 xenografts in nude mice treated with the compound, resulting in significant inhibition of tumour growth. While the mechanism of proteasome inhibition is not known, one suggestion is that [Au(DMDT)Br_2] might stimulate production of ROS, which then oxidise and inactivate the Proteasome. It would be interesting to investigate possible effects of these compounds in stimulating ROS production within mitochondria.



X = Cl or Br

21 **22**

Scheme 8. Examples of Au(III) dithiocarbamate antitumour complexes.

5. Concluding remarks

Gold-based drugs have been used for decades for the treatment of rheumatoid arthritis and there has been a significant resurgence of interest in these compounds stemming mainly from the fact that Au(I) compounds are among the most potent known inhibitors of thioredoxin reductase, attributable to binding of Au(I) to the redox-active selenocysteine residue. The antitumour properties of a variety of Au(I) phosphine compounds was discovered in the 1980s and the emergence of thioredoxin reductase as a significant new drug target has renewed interest in understanding their mechanisms of action. Of particular interest is the antiarthritic Au(I) phosphine drug, auranofin, which has been shown recently to open mitochondrial pathways to apoptosis through selective inhibition of mitochondrial thioredoxin reductase. Two distinct classes of Au(I) phosphine complexes display antitumour properties, these having either linear two-coordinate or bis-chelated, tetrahedral four-coordinate geometries. Both appear to target mitochondria, but different mechanisms are likely to be involved related to their differing propensity to undergo ligand exchange reactions with biological ligands. All known active analogs of $[Au(dppe)_2]^+$ are lipophilic cations (albeit with different hydrophilic character and charge), which are stable in the presence of thiols, and with aromatic substituents reducing the likelihood of oxidative side reactions in comparison to alkyl-substituted phosphines. We have shown that by modulating the hydrophilic–lipophilic balance in $[Au(dppe)_2]^+$ analogs, it is possible to achieve a degree of selectivity for tumour cells over normal cells. This behaviour is consistent with that of other classes of delocalised lipophilic cations, which exhibit antitumour activity and selectively accumulate in tumour cell mitochondria as a consequence of the elevated $\Delta\psi_m$ that is a common feature of tumour cells. Previous structure activity relationship studies for Au(I) complexes suggested that phosphine ligands are an essential requirement for antitumour activity; however, our recent studies have now shown that Au(I) complexes with *N*-heterocyclic carbene ligands, designed to mimic the two classes of Au(I) phosphine compounds, induce similar effects in isolated mitochondria. It is reasonable to propose therefore that the main role of the ligand (phosphine or NHC) is in controlling the chemical reactivity of the Au(I) centre and influencing the overall properties of the complex (lipophilicity, charge, ligand exchange behaviour) which are the important determinants of the biological activity. The results obtained to date show that Au(I)–NHC complexes are readily synthesized, with structural features which impart desirable biological properties that are readily tuned by alteration of the substituents. Studies are in progress to investigate whether the Au(I)–NHC compounds exhibit similar antitumour properties to the Au(I) phosphine compounds.

Until recently investigations of gold-based antitumour drugs were largely restricted to Au(I) compounds as the Au(III) oxidation state is generally too strongly oxidising for use in biological media. However, over the past few years, a range of Au(III) compounds have emerged which display significant antitumour properties, and in which the Au(III) oxidation state is stabilised by appropriate choice of ligands. Importantly, these new com-

pounds are active *via* mechanisms that differ from those of the Pt(II) antitumour agents such as cisplatin. Mitochondria may be the major target for at least some of these Au(III) compounds; several have been shown to be potent inhibitors of mitochondrial thioredoxin reductase and a Au(III) porphyrin complex (a lipophilic cation) has been shown to induce apoptosis though both caspase-dependent and caspase-independent mitochondrial pathways.

Overall, these studies highlight the potential for the design of a wide range of gold and other metal-based drugs able to target the mitochondrial cell-death pathway by different mechanisms. The current interest in thioredoxin reductase as a new drug target has stimulated interest in investigating a range of metal-based compounds as thioredoxin reductase inhibitors. For example, thioredoxin reductase has recently been identified as a target for motexafin gadolinium, a Gd(III) complex of a porphyrin-like synthetic macrocycle, that is currently in phase III clinical trials for the treatment of brain metastasis of lung cancers [122]. A note of caution is that the development of new metal-based drugs requires a good understanding of the fundamental coordination chemistry that will occur under biological conditions, to ensure that the key balance between stability and appropriate reactivity in biofluids and cells is achieved. Understanding the ligand-exchange reactions of gold-based drugs under physiological conditions is particularly challenging and this topic has been addressed in a recent review article by Isab [123]. For example, we showed previously by ^{31}P NMR experiments that the bridged digold complex ClAu(dppe)AuCl, which contains linear two-coordinate Au(I), is essentially a prodrug for the lipophilic, cationic, tetrahedral Au(I) complex $[Au(dppe)_2]^+$, with conversion induced by thiols and plasma proteins [124].

Finally, in recent work we have demonstrated the feasibility of probing the intracellular distribution of dinuclear Au(I) compounds, and their possible localization to mitochondria, by exploiting the inherent luminescence that stems from the aurophilic interaction.

Note added in proof

It has been reported [125] that the mixed phosphine gold complex chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) inhibits melanoma cell growth by inducing mitochondria-mediated apoptosis.

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