Dalton Transactions

PAPER

View Article Online
View Journal | View Issue

CrossMark click for updates

Cite this: *Dalton Trans.*, 2014, **43**, 17054

Gold(I) thiolates containing amino acid moieties. Cytotoxicity and structure–activity relationship studies

Alejandro Gutiérrez,[a] Lucia Gracia-Fleta,[b] Isabel Marzo,[b] Carlos Cativiela,[c] Antonio Laguna[a] and M. Concepción Gimeno*[a]

Received 28th July 2014,
Accepted 17th September 2014

DOI: 10.1039/c4dt02299h

www.rsc.org/dalton

Several gold(I) complexes containing a thiolate ligand functionalised with several amino acid or peptide moieties of the type [Au(SPyCOR)(PPh_2R')] (where R = OH, amino acid or dipeptide and R' = Ph or Py) were prepared. These thiolate gold complexes bearing biological molecules possess potential use as antitumor agents. Cytotoxicity assays in different tumour cell lines such as A549 (lung carcinoma), Jurkat (T-cell leukaemia) and MiaPaca2 (pancreatic carcinoma) revealed that the complexes exhibit good antiproliferative activity, with IC_{50} values in the low micromolar range. Several structural modifications such as in the type of phosphine, number of metal atoms and amino acid (type, stereochemistry and functionalisation) were carried out in order to establish the structure–activity relationship in this family of complexes, which has led to the design of new and more potent cytotoxic complexes. Observations of different cellular events after addition of the complexes indicated the possible mechanism of action or the biological targets of this type of new gold(I) drug.

Introduction

Metal-based drugs are an important class of compounds in medicinal chemistry employed clinically for the treatment of different diseases.[1] The most representative example is cisplatin,[2] a Pt complex used successfully in testicular, ovarian and other types of cancer. Although today this anticancer drug remains one of the most effective chemotherapeutic agents in clinical use, some drawbacks such as the lack of activity against other types of cancer, development of resistance and undesired toxic side-effects make necessary the design of new compounds that overcome these limitations and possess a good pharmaceutical profile, as potency or selectivity. The use of different metal compounds with different biological properties and targets has emerged as an alternative strategy.

Gold compounds have been employed since the last century for the treatment of rheumatoid arthritis and in the past few decades some gold(I) and gold(III) complexes with promising biological activities such as anticancer, antimicrobial, fungicidal, anti-HIV, or in the treatment of asthma or parasitic diseases, among others, have been prepared.[3] In addition to the excellent antiproliferative activities displayed by some of these gold(I) and gold(III) compounds, they have attracted a lot of attention because it seems that these species exert their therapeutic effect by a different mechanism, and over different targets in comparison with Pt drugs.

For this purpose, different approaches have been employed in order to prepare new gold complexes with antitumor activities, including the synthesis of (a) auranofin analogues (a gold(I) phosphine complex with a thioglucose ligand),[4] (b) gold(I) phosphine or diphosphine complexes,[5] (c) gold(I)-carbene derivatives,[6] and (d) gold(III) compounds (Fig. 1).[7]

Taking into account all these facts and in view of the importance of gold(I) thiolate complexes, we believe it would be interesting to prepare these gold-thiolate derivatives bearing in the skeleton different key biological molecules such as amino acids and peptides. Although gold thiolate derivatives containing carbohydrates or DNA bases are well known, only a few examples containing cysteine or glutathione species have been reported.[8]

In order to introduce the amino acid or peptide moieties in the structure we chose the nicotinic acid thiolate, a biological active molecule.[9] Starting from the complex [Au(SPyCOOH)(PR_3)], functionalisation of the carboxylic group with amino acid methyl esters or oligopeptides was carried out. The latter is an important class of biomolecules that constitute proteins

[a]*Departamento de Química Inorgánica, Instituto Síntesis Química y Catálisis Homogénea (ISQCH), CSIC-Universidad de Zaragoza, E-50009 Zaragoza, Spain. E-mail: gimeno@unizar.es*
[b]*Departamento de Química Orgánica, Instituto Síntesis Química y Catálisis Homogénea (ISQCH), CSIC-Universidad de Zaragoza, E-50009 Zaragoza, Spain*
[c]*Departamento de Bioquímica y Biología Celular, Universidad de Zaragoza, E-50009 Zaragoza, Spain*

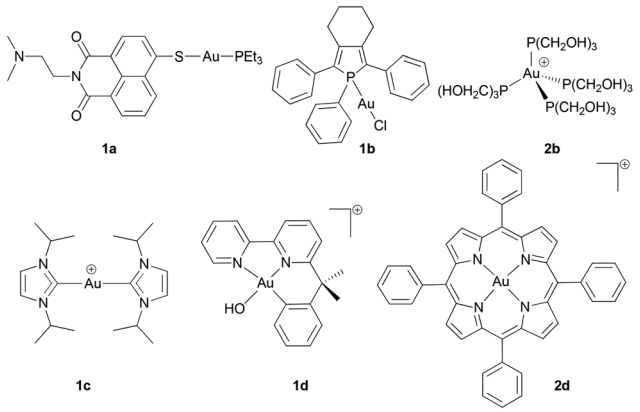


Fig. 1 Examples of gold(I) and gold(III) complexes of types (a)–(d) with anticancer activity.

and possess other different roles in the organism.[10] We think that the introduction of amino acids and peptides in our complexes might decrease the undesired toxic side-effects (they are biocompatible ligands) and they could serve as good carriers to deliver the gold atom to the biological target.[11] The major problem for cancer chemotherapeutics is crossing the cell membranes, and a strategy to overcome this is to make use of the peptide-based delivery systems, which can transport small peptides and peptide-like drugs to the cells. These peptide transporters are over-expressed in some types of tumours, these could be targeted by the gold-peptide compounds providing them with more internalization and subsequent selectivity.[12]

Several studies on the biological activity of gold compounds have revealed that the cytotoxicity is due mainly to the gold(I) atom, and the ligands bonded to gold are important as carriers to reach the biological target.[3] Phosphine ligands are important in the resulting potency of the final complex, and the thiolate ligand could participate in ligand-exchange reactions with other biomolecules in the organism.[13] Taking this into account, we have rationally designed these complexes considering these ideas and introducing more biologically compatible fragments in the molecule with the aim of facilitating the gold atom to easily reach its biological target.

In a previous study, we reported the synthesis of several gold(I) phosphine complexes with nicotinic acid thiolate functionalised with amino acids.[14] Three families of complexes (ester, acid and amide) were prepared. Here we report on the antiproliferative activity studies, tested by the MTT viability assay in different tumour human cell lines, which have shown that the gold(I) complexes prepared possess good antiproliferative activity in the low micromolar range. Following these results, we have introduced different structural modifications in these families of complexes, such as (1) the type of phosphine, (2) the charge and number of gold atoms per molecule, and (3) the number, type, stereochemistry or functionalisation of the coupled amino acid, in order to evaluate changes in the biological activity and thus can establish a structure–activity relationship. This has led to the synthesis of the most active complex of these families.

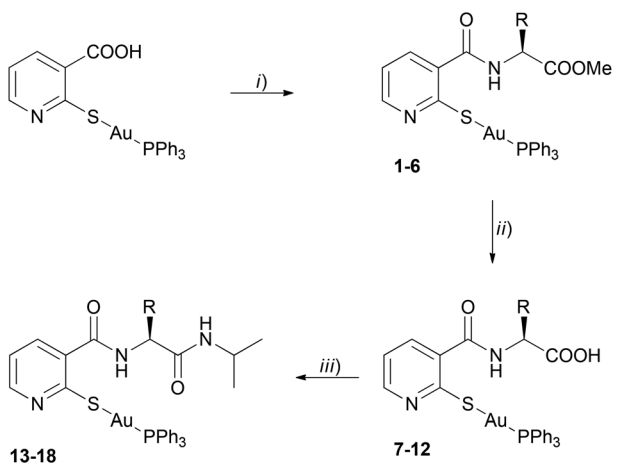
Further studies have been carried out over a selection of these complexes in order to know the mechanism of action. In the last few years, some selenium containing enzymes like TrX or GR, located at mitochondria, have been postulated as possible targets of gold(I) complexes, which seem to induce cell death *via* apoptosis by the mitochondrial (intrinsic) pathway.[5a,7d,15] To confirm this, several studies on ROS production, loss of mitochondrial membrane potential, cell and nuclei morphology and TrX inhibition have been performed.

Results and discussion

Synthesis and spectroscopic characterization

We have previously reported the preparation of several gold(I) complexes (**1–6**) obtained by coupling different amino esters (glycine, alanine, valine, phenylalanine, methionine or proline) with the free carboxylic acid of the complex [Au(SPyCOOH)(PPh_3)] (see Scheme 1).[14] This compound was prepared by Nomiya *et al.* some years ago, who reported its antimicrobial activity.[16] The basic hydrolysis of the ester complexes allowed access to the corresponding acid species (**7–12**). Finally, coupling of isopropylamine to these compounds afforded the corresponding amide derivatives (**13–18**).

In the present work, we report the biological activity of the previously described complexes **1–18** and, in addition, we have introduced different structural modifications in these families of complexes with the purpose of obtaining new derivatives with better activity, in order to establish some structure–activity relationships (see Fig. 2).



Scheme 1 Preparation of complexes **1–18** of the ester, acid and amide families. (i) HCl·H-AA-OMe, PyBOP, DIPEA, MeCN, (ii) LiOH, MeOH and (iii) $^{i}PrNH_2$, PyBOP, DIPEA, MeCN.

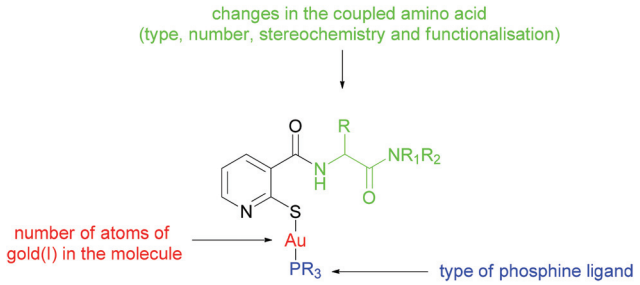


Fig. 2 Selected modifications introduced in our complexes.

Synthesis of the complexes with a different type of phosphine. The complex [Au(SPyCOOH)(PPh_2Py)] (**19**) can be readily prepared by reaction of [AuCl(PPh_2Py)] with 2-mercaptoniconitic acid in acetone in the presence of K_2CO_3, yielding the pure product in high yield. This complex presents a carboxylic moiety on the thiolate ligand that can be used for coupling amino acid esters such as L-alanine methyl ester, to give the derivative [Au(SPyCOAlaOMe)(PPh_2Py)] (**20**) that was obtained in the pure form by chromatography (see Scheme 2). Complexes **19** and **20** are analogous to complexes [Au(SPyCOOH)(PPh_3)] and **2** reported previously,[14] except for the type of phosphine ligand bonded to the gold atom, for which the 2-diphenylphosphino pyridine ligand is employed instead of triphenylphosphine. Both phosphines exhibit similar electronic and steric properties, although PPh_2Py possesses a pyridine ring, which could further bind to other metals, interact with other biomolecules or alter the lipophilicity of the complex.

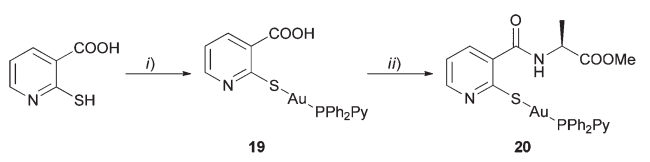
Complexes **19** and **20**, as all the complexes reported in this work, were fully characterized according to the usual techniques (^{1}H, ^{31}P{^{1}H}, ^{13}C{^{1}H}-NMR and IR spectroscopy, MS spectrometry and elemental analysis). The ^{1}H and ^{13}C{^{1}H} NMR spectra show the expected resonances from all the protons and carbon atoms that are essentially the same observed for [Au(SPyCOOH)(PPh_3)] and **2**, and will not be discussed further here. The only exception are the signals arising at the pyridine of the phosphine ligand, for which a correct assignment could be done by carrying out 2D-NMR experiments (^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC), since these resonances and those of the pyridine belonging to the thiolate unit appear at similar chemical shift. The ^{31}P{^{1}H} NMR spectra present a unique resonance for both complexes corresponding to the phosphorus of the 2-diphenylphosphino pyridine ligand, in both cases the chemical shift is observed at 35.9 ppm, a value similar to those observed for other thiolate gold(I) phosphine complexes (35–37 ppm). The IR spectra present, among others, absorptions for the free carboxylic acid at around 3400–3049 (br, s), which is no longer observed for complex **20**, and for the latter the bands belonging to the new amide bond at 3280 (br, w) and 1637 (s), and the ester at 1736 (s) appear. The mass spectra (ESI+) present for both compounds the protonated molecular peak $[M + H]^+$ (**19**) or with a sodium cation $[M + Na]^+$ (**20**), together with the association fragment $[M + AuPPh_2Py]^+$ observed for **20**.

Synthesis of a dinuclear gold derivative. The reaction between the oxonium salt, $[O(AuPPh_3)_3]BF_4$, and 2-mercapto nicotinic acid yielded the desired dinuclear gold(I) complex $[Au_2(SPyCOOH)(PPh_3)_2]BF_4$ (**21**). In this reaction, stoichiometric amounts of the oxonium salt must be used (2/3 equiv.) to avoid mixtures due to the very reactive $[AuPPh_3]^+$ fragment that could bind to other donor atoms of the molecules such as N or O (Scheme 3).

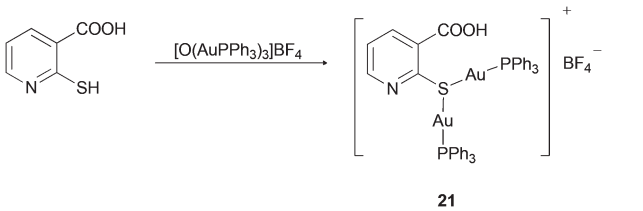
In complex **21** two gold(I) atoms are bonded to the sulfur centre, and it presents great interest because on the one hand, we could expect an increase in the cytotoxicity of the final complex due to the introduction of another gold(I) atom in the molecule, and on the other hand, this compound is charged and presents high lipophilicity that could help the complex to cross the cell membranes and/or accumulate in the mitochondria.

The ^{1}H NMR spectrum shows all the resonances expected for the molecule, in a similar way to that observed for the compound [Au(SPyCOOH)(PPh_3)], except for the aromatic protons of the triphenylphosphine that integrate for 30 instead of 15 H. The protons of the pyridine rings appear slightly downfield in relation to the resonances observed for the mononuclear derivative. The ^{31}P{^{1}H} NMR spectrum presents a unique resonance at 33.3 ppm for the equivalent gold(I) phosphine fragments, about 3–4 ppm more shielded than the mononuclear complex, a feature observed for similar dinuclear gold(I) complexes. In the ^{19}F{^{1}H} NMR spectrum a signal at −153.9 ppm is observed and corresponds to the counter anion tetrafluoroborate. The ^{13}C{^{1}H} NMR and IR spectra are essentially the same as the mononuclear analogue, while the mass spectra (ESI+) presents the molecular peak $[M]^+$.

Change in the coupled amino acid. In addition to the different types of L-amino acids employed previously (Gly, Ala, Val, Phe, Met and Pro) in the preparation of complexes **1–18**, we have made several modifications in the coupling process, such as in the amino acid (lysine), in the use of a dipeptide



Scheme 2 Synthesis of the complexes **19** and **20** with a different phosphine coordinated to Au(I). (i) [AuCl(PPh_2Py)], K_2CO_3, acetone, (ii) HCl·H-Ala-OMe, PyBOP, DIPEA, MeCN.



Scheme 3 Synthesis of the dinuclear complex **21**.

(H-Gly-Pro-OMe), in the functionalisation of the amino acid with a tertiary amide, or by a change in the stereochemistry using D-enantiomers (Ala, Phe), as summarised in Scheme 4.

The amino ester derivatives [Au(SPyCOLys(Boc)OMe)(PPh_3)] (**22**) and [Au(SPyCOGlyProOMe)(PPh_3)] (**24**) can be readily prepared by the coupling of the lysine ester or a dipeptide [HGly-ProOMe] (previously synthesized in solution by standard peptide chemistry), in a similar way to that reported for the synthesis of compounds **1–6**. The desired complexes were obtained in good yields and in a pure form after chromatographic purification, confirming the generality of this procedure for the incorporation of thiolate gold fragments into more complex molecules. Boc group deprotection in the lysine side chain afforded the complex [Au(SPyCOLys(H)OMe)(PPh_3)] (**23**), with the free ε-amino group in the side chain. This complex may be interesting under physiological conditions because it is a water-soluble species with a basic group in the molecule. Coupling of diethylamine through the free carboxylic acid of complex **2** gave the compound [Au(SPyCOAla-NEt_2)(PPh_3)] (**25**), in which we have a tertiary amide instead of the secondary amide in the analogue **14**. Furthermore, we carried out a change in the stereochemistry of the coupled amino ester. The use of enantiomerically pure D alanine or phenylalanine amino esters yielded complexes [Au(SPyCO(D)-AlaOMe)(PPh_3)] (**26**) or [Au(SPyCO(D)PheOMe)(PPh_3)] (**27**) that are the corresponding enantiomers of previously described **2** or **4**. Finally, hydrolysis of complex **26** gave the amino acid derivative [Au(SPyCO(D)AlaOH)(PPh_3)] (**28**), corresponding to D-alanine, which is the analogue of **8**.

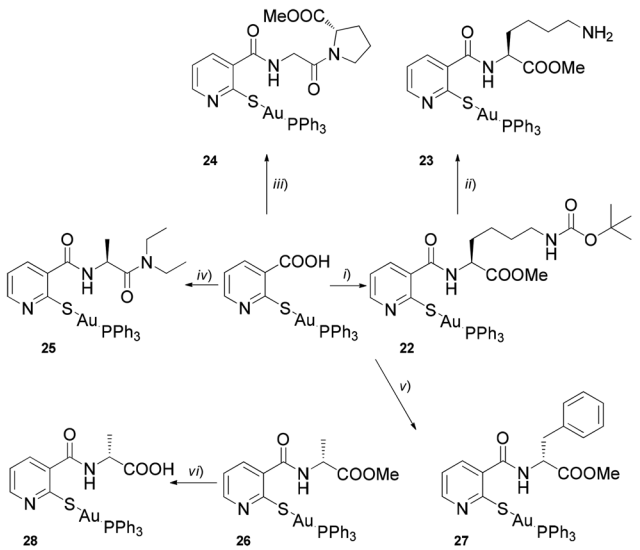
The different structural modifications introduced in the molecule could influence the activity and/or the mechanism of action of the resulting complexes, and might determine the interactions of the complexes with different biomolecules or receptors in the organism, and have influence on the cytotoxicity or biodistribution of the final complex.

All the complexes were prepared as discussed earlier, except in the case of complex **23**, where the removal of the Boc group was carried out employing standard conditions (TFA), carefully to avoid the loss of the integrity of the Au–S and Au–P bonds, as was observed when 3 M HCl/AcOEt was employed.

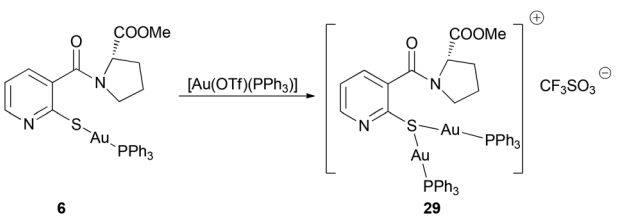
The ^1H and $^{13}C\{^1H\}$ NMR spectra show all the signals corresponding to the coupled amino esters, dipeptide or amine, similar to that observed in the spectra of complexes **1–18**, and are in agreement with the proposed formulation. The $^{31}P\{^1H\}$ NMR spectra show in all cases a singlet in the range of 35–37 ppm, which are also in agreement with a phosphorus atom *trans* to a sulfur centre. In the mass spectra (ESI+) the molecular peaks, as the protonated species $[M + H]^+$, appear in all the cases. In addition the species generated in the experiment $[M + AuPPh_3]^+$ were observed. The D-enantiomers (**26–28**) show exactly the same physical properties as their L-enantiomers (**2**, **4** and **8**). To confirm the stereochemical configuration of the chiral centre (Cα amino acid), the measurement of the optical rotation angle was carried out, obtaining approximately opposite values to that observed for the L-analogues.

Synthesis of the hybrid complex of 6 and 21. As we will comment in the next section, complexes **6** and **21** were found to be the most cytotoxic against different tumor cell lines. These derivatives contain firstly proline as the coupled amino ester (**6**), and secondly two gold atoms bonded to the thiolate unit in **21**. According to the values obtained and the structure–activity relationship observed, we decided to prepare the hybrid complex between these two candidates. The reaction between complex **6** and [Au(OTf)(PPh_3)], generated *in situ*, afforded the desired compound [Au_2(SPyCOProOMe)(PPh_3)$_2$]-OTf (**29**), conjugating the most important characteristics of both complexes mentioned above (Scheme 5).

This complex presents the following SAR: functionalization as esters, containing a rigid and conformationally restricted amino acid and more than one gold(I) atom per molecule. The ^1H NMR spectrum of complex **29** appears as a mixture of rotamers (ratio 1 : 0.8), showing similar signals to those observed for **6**, with the exception of the protons of the pyridine (the *ortho* and *para* protons appear slightly downfield while the *meta* protons appear upfield), and the aromatic protons of PPh_3 that integrate for 30 H instead of 15 H (Fig. 3). The ratio between both rotamers can be calculated in the integral of the resonance for CαH.



Scheme 4 Synthesis of the complexes **22–28**. (i) HCl·H-Lys(Boc)-OMe, PyBOP, DIPEA, MeCN, (ii) TFA–DCM (1 : 1 v/v), (iii) HCl·H-Gly-Pro-OMe, PyBOP, DIPEA, MeCN, (iv) HCl·H-Gly-OMe, PyBOP, DIPEA; LiOH, KHSO4; $HNEt_2$, PyBOP, DIPEA; MeCN, (v) HCl-H-(D)-Aa-OMe, PyBOP, DIPEA, MeCN, (vi) LiOH, $KHSO_4$.



Scheme 5 Synthesis of complex **29**.

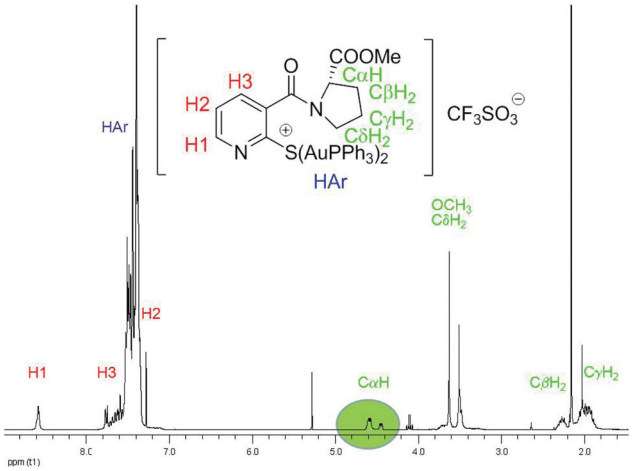


Fig. 3 ^{1}H NMR spectrum of complex **29**, showing the presence of rotamers.

In the $^{13}C\{^{1}H\}$ NMR spectrum the complex also appears as a mixture of rotamers, and the resonances do not present any significant variation in relation to **6** and **21** (Fig. 4). The $^{31}P\{^{1}H\}$ NMR spectrum shows one resonance at 33.0 ppm, with a chemical shift similar to **22**, and consequently the two $[AuPPh_3]^+$ fragments are equivalents. The $^{19}F\{^{1}H\}$ NMR spectrum shows one resonance at −78.0 ppm corresponding to the triflate counter anion. The mass spectrum (ESI+) presents the molecular peak $[M]^+$.

Biological evaluation

Cytotoxic activity. The cytotoxic activity of the previously synthesised complexes (**1–18**), and the new complexes prepared including all the structural modifications (**19–29**) were tested against three different human tumour cell lines: A549 (lung carcinoma), Jurkat (T-cell leukaemia) and MiaPaca2 (pancreatic carcinoma). The sensitivity of non-tumor R69 (lymphoid cell line) and 293 T (embryonic kidney fibroblasts) to some of the complexes was also evaluated. Compounds are soluble in DMSO and in the DMSO–water mixtures used in the tests, which contain a small amount of DMSO (concentration is always <0.5%). We did not observe any precipitation of the complexes while performing the tests. Their colourless D_6-DMSO solutions are very stable at room temperature, as shown in the $^{31}P\{^{1}H\}$ NMR spectra in which the signals remain the same for weeks.

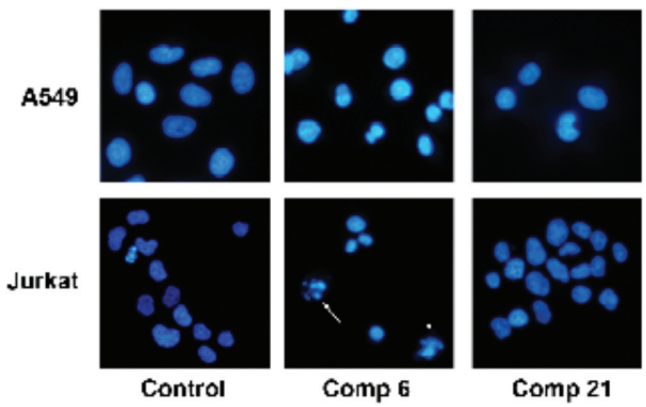


Fig. 4 Compounds **6** and **21** induce apoptosis and necrosis in Jurkat cells and non-apoptotical cell death in A549 cells. Nuclei were stained with Hoechst 333248 and cells were photographed under UV light. Arrows point to apoptotic nuclei showing condensed and fragmented chromatin.

Cells were exposed to different concentrations of each compound for a total of 24 h. Using the colorimetric MTT viability assay,[17] the IC_{50} values (final concentration <0.5% DMSO) were calculated from dose–response curves obtained by non-linear regression analysis. IC_{50} values are concentrations of drugs required to inhibit tumour cell proliferation by 50% compared to control cells treated with DMSO alone.

The IC_{50} values for the ester (**1–6**), acid (**7–12**) and amide (**13–18**) complexes are collected in Table 1. Cytotoxicity values of cisplatin, measured under the same conditions, are included too and used for comparison purposes. It should be noted that the values measured at 24 h can be considerably greater than other values reported in the studies in the bibliography at 48 h or 72 h. In order to evaluate the cytotoxicity of the new complexes prepared, the cytotoxicity of the precursor compound, [Au(SPyCOOH)(PPh_3)], was also determined.

As can be observed, all the complexes synthesized were active against all the different tumour cell lines at low concentrations (low micromolar range). The starting material [Au(SPyCOOH)(PPh_3)], with known antimicrobial activity, possesses also cytotoxic activity. Complexes **1–18** exhibit good antiproliferative activities, with IC_{50} values ranging from 7.4 to 30.5 μM in A549 cells, 2.4 to 7.7 μM in Jurkat cells, and 8.2 to 27.2 μM in MiaPaca2. The Jurkat cell line was the most sensitive to our compounds, while A549 or MiaPaca2 showed more resistance to the complexes. Importantly, the compounds showed some selectivity for leukaemia cells against non-tumour cells (Jurkat *vs.* R69) but this difference was not seen in the case of solid tumours.

The family of ester complexes (**1–6**) displayed better cytotoxicity values than the precursor complex in all the cell lines, except in the MiaPaca2 where both showed similar potency. Particularly, the proline derivative **6** was the most potent of this family of complexes. The acid complexes (**7–12**) were more active than the precursor complex only in the A549 cell line. The other cell lines were less active, except the proline **12**, alanine **8** and glycine **7** derivatives in each case. In comparison with the ester compounds, the acid complexes were less active. The amide complexes (**13–18**) were more active than the precursor only in the Jurkat cell line. The other cell lines were less active than the precursor complex and also than the ester and acid analogues.

These data indicate that the coupling of amino esters to the precursor gold-thiolate complex affords more cytotoxic compounds. The functionalisation as acids or amides decreases the activity of the complexes. Finally, the type of amino acid coupled has an influence on the antiproliferative activity of the complex; the best results have been achieved mainly with the

Table 1 IC_{50} (μM) of complexes **1–18** after 24 h of incubation

Compounds	A549	MiaPaca2	Jurkat	293T	R69
Cisplatin	105 ± 0.90	71 ± 0.80	7.4 ± 0.10	14.0 ± 0.20	65 ± 0.92
[Au(SPyCOOH)(PPh_3)]	15.5 ± 0.92	9.2 ± 0.28	4.6 ± 0.08	4.6 ± 0.13	16 ± 0.64
1	11.5 ± 0.55	9.7 ± 0.22	3.8 ± 0.07	4.2 ± 0.08	8.6 ± 0.33
2	13.7 ± 0.71	11.0 ± 0.20	4.0 ± 0.07	2.7 ± 0.07	2.2 ± 0.08
3	10.9 ± 0.40	10.2 ± 0.25	3.3 ± 0.05	10.7 ± 0.31	19.0 ± 0.60
4	8.9 ± 0.36	12.3 ± 0.37	4.0 ± 0.08	5.5 ± 0.16	14.0 ± 0.59
5	8.2 ± 0.41	12.8 ± 0.32	4.1 ± 0.06	3.7 ± 0.07	9.6 ± 0.36
6	7.4 ± 0.34	9.4 ± 0.19	2.4 ± 0.04	10.0 ± 0.19	4.0 ± 0.13
7	14.7 ± 0.88	8.2 ± 0.13	7.6 ± 0.11	35.2 ± 0.53	25.9 ± 1.04
8	7.7 ± 0.22	10.7 ± 0.16	3.7 ± 0.06	12.8 ± 0.27	6.1 ± 0.18
9	14.7 ± 0.20	12.3 ± 0.32	4.3 ± 0.08	11.3 ± 0.29	6.5 ± 0.25
10	15.9 ± 0.50	11.5 ± 0.29	6.7 ± 0.13	65.5 ± 1.12	33.1 ± 1.13
11	14.1 ± 0.55	14.5 ± 0.26	3.6 ± 0.06	49.3 ± 1.53	28.4 ± 1.16
12	14.3 ± 0.61	11.6 ± 0.20	7.5 ± 0.07	3.0 ± 0.08	4.0 ± 0.11
13	28.3 ± 1.02	27.2 ± 0.65	3.9 ± 0.06	14.4 ± 0.42	4.9 ± 0.16
14	19.1 ± 0.67	8.1 ± 0.23	3.9 ± 0.07	8.1 ± 0.28	1.4 ± 0.04
15	14.4 ± 0.60	12.5 ± 0.32	3.8 ± 0.06	7.6 ± 0.26	5.2 ± 0.15
16	18.8 ± 0.71	14.1 ± 0.29	3.7 ± 0.06	14.6 ± 0.57	6.8 ± 0.23
17	19.4 ± 0.62	15.2 ± 0.33	5.3 ± 0.11	13.6 ± 0.40	3.0 ± 0.09
18	30.5 ± 0.82	19.2 ± 0.36	7.7 ± 0.15	5.8 ± 0.16	4.0 ± 0.16

incorporation of the proline moiety, although glycine or alanine provided very active compounds too.

Compared with the reference drug cisplatin, our complexes (**1–18**) exhibit much better antiproliferative activities *in vitro* in all the cell lines.

The IC_{50} values of the modified derivatives (**19–29**) are listed in Table 2. The modified complexes were also active against the different human tumor cell lines at low concentrations (low micromolar range), with IC_{50} values measured after 24 h of incubation, ranging from 6.9 to 33.5 μM in A549 cells, 3.3 to 8.6 μM in Jurkat cells and 7.5 to 29.3 μM in MiaPaca2. The Jurkat cell line was more sensitive to our complexes than A549 or MiaPaca2. The values compared with cisplatin are considerably lower for our complexes in the toughest cell lines, A549 or MiaPaca2, and present similar values in the Jurkat cell line.

The complex **19** or **20** with different types of phosphine coordinated to gold(I) afforded slightly more cytotoxic complexes in the R69 (non-tumor) and Jurkat cell lines, respectively. In the other cell lines the complexes were slightly less active than their analogues with triphenylphosphine. Then, the effect of changing the triphenylphosphine for 2-diphenylphosphino pyridine was not significant, probably because both phosphines have similar electronic and steric properties.

The coordination of an additional $[AuPPh_3]^+$ fragment afforded a more potent complex. The dinuclear derivative **21** showed better antiproliferative activity than the precursor complex in all cell lines. This is in agreement with the fact that the cytotoxic activity of the complex is mainly due to the gold(I) atom and the thiolate ligand first, and later the phosphine ligands are lost in exchange reactions with other biomolecules in the organism.

The coupling of a different type of amino ester (lysine) afforded compound **22** that exhibited good antiproliferative activity, in the same range as the most potent compounds of the ester family. The removal of the protective group to yield a water-soluble complex and with a basic group in the side chain (**23**) decreased dramatically the potency of the compound. This could be explained because the free amino group is a powerful nucleophile that could react with other biomolecules in the organism, resulting in a shorter life-time of the complex, or may prevent the gold(I) to reach its target.

Table 2 IC_{50} (μM) of complexes **19–29** after 24 h of incubation

Compounds	A549	MiaPaca2	Jurkat	293T	R69
19	NT	18.2 ± 0.51	5.2 ± 0.07	14.0 ± 0.28	4.9 ± 0.18
20	15.7 ± 0.66	17.4 ± 0.48	3.8 ± 0.04	12.0 ± 0.19	3.2 ± 0.13
21	6.9 ± 0.21	7.5 ± 0.19	3.3 ± 0.04	7.8 ± 0.19	1.8 ± 0.05
22	8.3 ± 0.39	13.1 ± 0.26	3.4 ± 0.06	3.5 ± 0.11	2.8 ± 0.08
23	32.5 ± 1.24	29.3 ± 0.70	36.5 ± 0.77	>25	10.4 ± 0.35
24	18.7 ± 0.64	22.5 ± 0.67	8.6 ± 0.14	17.9 ± 0.45	15.4 ± 0.60
25	33.5 ± 1.31	>50	NT	8.3 ± 0.19	1.9 ± 0.05
26	16.5 ± 0.92	17.1 ± 0.39	4.2 ± 0.05	7.7 ± 0.24	3.0 ± 0.08
27	18.3 ± 0.75	15.1 ± 0.27	3.6 ± 0.06	>25	1.2 ± 0.02
Racemic mixture of **2** and **27**	17.8 ± 0.83	16.1 ± 0.47	4.6 ± 0.10	4.5 ± 0.10	8.8 ± 0.28
28	>50	>50	NT	>25	10.7 ± 0.29
29	4.1 ± 0.11	1.2 ± 0.04	0.9 ± 0.07	4.5 ± 0.14	0.8 ± 0.02

The complexes that present a dipeptide, tertiary amide or D-amino esters in their structures were less cytotoxic than their respective analogues in all the cases. Then, the increment in the proportion of amino acids in the molecule does not increase the potency of the complex, since the optimal biological activity is achieved with one single amino acid coupled. The interaction with biomolecules, such as proteins or enzymes, is a very important process that determines the transport, mode of action and activity of the compound. The ability to form hydrogen bonds or the stereospecific recognition seems to have a great influence on the final cytotoxicity of the complex, and we can see experimentally that the optimal activity is shown with molecules that can establish hydrogen bonds (like secondary amides) and natural amino acids with the L-configuration.

In view of these data, better cytotoxicity was achieved by the compounds functionalised as esters, with two gold(I) atoms per molecule and with conformationally-restricted or rigid amino acids, as in the case of proline. Following this structure–activity relationship observed, complex **29** was prepared. Complex **29** showed excellent antiproliferative activity, with IC_{50} values in the low micromolar range even in the submicromolar range as in the case of the Jurkat cell line. This compound is the most potent among all complexes prepared in these series, and it is significantly more active than the precursors **6** and **21** in all the cell lines. Furthermore, complex **29** is much more active than cisplatin in all studied cell lines. This result confirms our hypothesis and provides access to the design and preparation of more potent and selective gold(I) compounds of this type.

Determination of the mechanism and type of death

Cell and nuclear morphological changes. In order to determine the mechanism of molecular death (apoptosis or necrosis) and potential targets of our compounds, several biological studies were performed.

We carried out the analysis of the cell death and nuclear morphology in A549 and Jurkat cells in the presence of the selected compounds **6** and **21**. Nuclear staining with Hoechst 333248, a membrane-permeable dye, which binds to nucleic acids, did not show chromatin condensation and fragmentation in A549 cells, typical features of apoptosis, so in this case our complexes induced non-apoptotic cell death, while in Jurkat ones cell death proceeds, both by apoptosis and necrosis (Fig. 4).

Cell death was also evaluated by annexin V-PE (phycoerythrin-conjugated recombinant annexin V) labelling and subsequent quantification of fluorescent cells by flow cytometry. The loss of membrane asymmetry and phosphatidylserine (PS) exposition is a typical feature observed in apoptosis and annexin V is able to bind to PS exposed in the outer cell membrane. The data show that a significant percentage of cells leads to apoptosis, especially in A549 cells with complex **6** (Fig. 5). However, the staining with 7-AAD, which is not able to cross the cell membrane, revealed that a great percentage of cells lost the cell membrane integrity, suggesting that cell death proceeds, by both apoptosis and necrosis. It is known

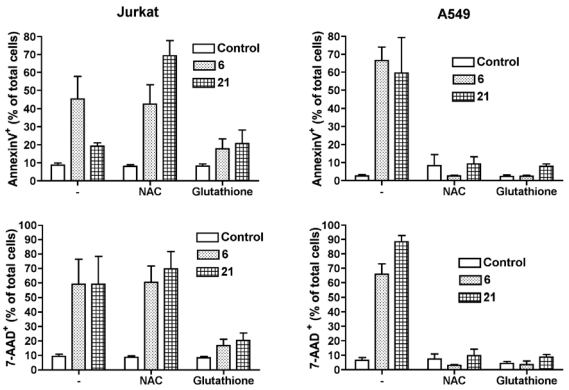


Fig. 5 Annexin V-PE labeling and 7-AAD staining assays. Data indicate that our complexes induced cell death both by apoptosis and necrosis. Antioxidants such as NAC and GSH protect the cells from cytotoxic effects of **6** and/or **21**.

that thiols like *N*-acetyl cysteine (NAC) or glutathione (GSH) are employed by the organism as antioxidants and chemoprotective agents,[18] and high levels of this type of compound have been observed in certain types of cancer cells that developed resistance to chemotherapeutic agents. The addition of NAC and GSH protected A549 from cell death induced by our compounds, but only GSH inhibited cell death in Jurkat cells. These results indicate that **6** and **21** activate different mechanisms of cell death in Jurkat and A549 cells.

Depolarization of mitochondria and ROS production. It is well-known that mitochondria plays the main role in the apoptotic process[19] because it can start or amplify the cell signals that result in apoptosis, by coordinating caspase activation through the release of apoptogenic factors such as cytochrome c. Moreover, in the last few years several studies indicate that gold compounds might exert their cytotoxic effect at the mitochondria.[20] For all these reasons, the determination of the mitochondrial membrane potential was performed. This parameter is closely connected with the correct performance of mitochondria and is very important because the existing potential gradient makes possible vital cell events like oxidative phosphorylation (ATP generation). Complexes **6** and **21** caused a strong decrease in the mitochondrial membrane potential (MMP). Although in all cases our complexes induced mitochondrial toxicity, the effect was more marked in Jurkat than in A549 cells, and complex **6** was more effective than **21**. With the addition of GSH, the potential gradient recovery was achieved, specifically in Jurkat cells, an effect that did not occur after addition of NAC (Fig. 6).

Reactive Oxygen Species (ROS) have attracted great attention because of their relationship with many diseases like cancer.[21] These radicals, whose main source is mitochondria, are able to damage proteins, enzymes or DNA in an irreversible way leading to cell death. In addition to this, some chemotherapeutic agents exert their cytotoxic effect by ROS production and the deregulation of the cellular redox state inducing oxidative stress. The generation of ROS by our compounds was analysed by flow cytometry after staining with 2-hydroxy-

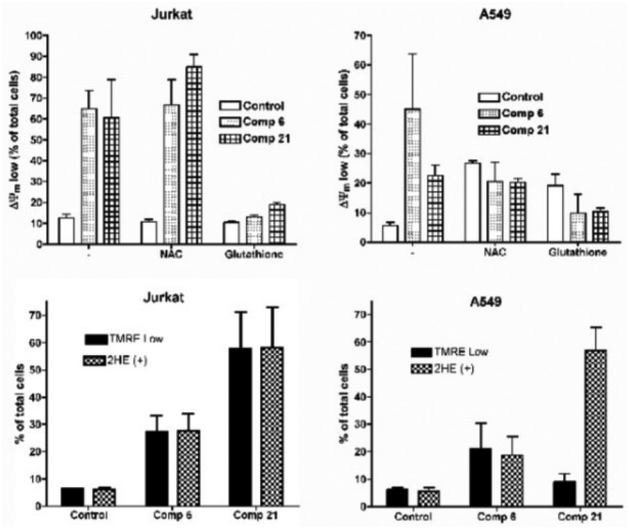


Fig. 6 Determination of the mitochondrial membrane potential and ROS formation after addition of complexes **6** and **21**.

ethidium. The assay revealed that higher levels of these radicals are generated in Jurkat cells than in A549 cells and complex **21** induced the generation of higher levels of ROS than **6**. The ROS levels produced showed good correlation with the loss of mitochondrial membrane potential. These data suggest that the damage to the mitochondria could lead to an increase in the production of ROS that in final instance triggers cell death.

Thioredoxin reductase (TrX) inhibition assay. Recently, some selenol-containing enzymes like TrX have been postulated as possible targets for gold(I) compounds,[5a,7d,15] due to the high affinity of gold for selenium. In fact, some gold(I) complexes inhibited strongly this type of enzyme even in the presence of other thiol-containing enzymes.[7e] The TrX system plays a key role in the cellular redox state (reducing oxidised proteins) and others, and inhibition of this enzyme is thought to cause apoptosis and cell death, which could explain the mechanism of action of some gold(I) complexes. The inhibition of the TrX assay revealed that complexes **6** and **21** at concentrations near IC_{50} values are potent inhibitors of this enzyme, by decreasing the enzyme activity at least in 50%. Complex **21** inhibited more efficiently the TrX than **6** (65% against 50% of total inhibition, respectively). The inhibition of TrX by our complexes would prevent the removal of ROS and may be the reason for the high levels of ROS observed in the previous assay.

Experimental section

Materials and methods

All manipulations were routinely carried out under Ar using common Schlenk techniques. Solvents were purified by standard procedures immediately prior to use. 2-Mercaptonicotinic acid, 2-diphenylphosphino-pyridine, L-H-Lys(Boc)-OMe·HCl, L-H-Pro-OMe·HCl, L-H-Gly-OMe·HCl, L-H-Ala-OMe·HCl, D-H-Ala-OMe·HCl, D/L-H-Ala-OMe·HCl and diethylamine were purchased from Aldrich, Fluka and Bachem and used without further purification. C, H, and N analyses were carried out with a Perkin-Elmer 2400 microanalyser. Mass spectra were recorded on a Bruker Esquire 3000 Plus, with the electrospray (ESI) technique and on a Bruker Microflex (MALDI-TOF). ^{1}H, ^{13}C{H} and ^{19}F NMR, including 2D experiments, were performed at room temperature on a Bruker Avance 400 spectrometer (^{1}H, 400 MHz, ^{13}C, 100.6 MHz) or on a Bruker Avance II 300 spectrometer (^{1}H, 300 MHz, ^{13}C, 75.5 MHz), with chemical shifts (δ, ppm) reported relative to the solvent peaks of the deuterated solvent. The RPMI 1640 (Roswell Park Memorial Institute) cell culture medium and fetal bovine serum (FBS) were purchased from LONZA Co. MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Sigma Chemical. MTT was dissolved (5 mg ml^{-1}) in phosphate buffer at pH 7.2. Fluorescence intensity measurements were carried out on a PTI QM-4/206 SE Spectrofluorometer (PTI, Birmingham, NJ) with right angle detection of fluorescence using a 1 cm path length quartz cuvette.

Starting materials

$[AuCl(PPh_3)]$,[22] $[AuCl(PPh_2Py)]$[22] and $[O(AuPPh_3)_3]BF_4$ [23] were prepared according to published procedures. All other reagents were commercially available.

Synthesis

Compounds **1–18** were synthesised as reported elsewhere.[14]

[Au(SPyCOOH)(PPh$_2$Py)] (19). To a suspension of 2-mercaptonicotinic acid (0.155 g, 1 mmol) in acetone (10 ml) was added an excess of K_2CO_3 (0.276 g, 2 mmol) and [AuCl-(PPh$_2$Py)] (0.496 g, 1 mmol). The mixture was stirred for 24 h at room temperature. The solution was concentrated to *ca.* 5 ml and addition of hexane (20 ml) afforded **19** as a white solid (0.456 g, 74.2%). ^{1}H-NMR ($CDCl_3$, 400 MHz δ (ppm), J (Hz)): 8.79 (d, 1H, J = 4.4, *H*12), 8.50 (dd, 1H, J = 8.0 and 2.0, *H*1), 8.48 (dd, 1H, J = 4.6 and 2.0, *H*3), 7.95 ("t", 1H, J = 7.6, *H*9), 7.80 (m, 1H, *H*10), 7.72 (m, 4H, *ArH*), 7.50 (m, 6H, *ArH*), 7.39 (m, 1H, *H*11), 7.11 (dd, 1H, J = 8.0 and 4.6, *H*2). ^{31}P-NMR ($CDCl_3$, 400 MHz, δ (ppm)): 35.9. ^{13}C-NMR (d_6-DMSO, 400 MHz, δ (ppm), J (Hz)): 171.1 (*C*OOH), 163.3 (C, *Py*), 154.2 (d, C, J = 78.7, *C*8), 151.3 (d, CH, J = 15.2, *C*12), 144.9 (CH, *C*1), 140.9 (C, *C*13), 137.3 (d, CH, J = 10.4, *C*10), 134.3 (d, CH, J = 14.0, *C*5), 130.9 (d, CH, J = 31.7, *C*9), 131.8 (CH, *C*7), 129.7 (C, *C*4), 129.3 (d, CH, J = 11.3, *C*6), 133.7 (CH, *C*3), 125.7 (CH, *C*11), 117.7 (CH, *C*2). MS (ESI+) m/z: $[M + H]^+$ = 615.0 (calcd), 615.0 (found). IR (cm^{-1}): 3400–3049 (br, *COOH*), 1570, 1480 and 1435 (w, *Ar*), 1099 and 1067 (s, *C–O*), 741, 710 and 690 (w, *Ar*). Anal. Calcd for $C_{23}H_{18}AuN_2O_2PS$ (614.41): C, 44.96; H, 2.95; N, 4.56; S, 5.22. Found: C, 45.07; H, 3.01; N, 4.65; S, 5.40.

General procedure for the synthesis of complexes 20, 22, 24, 26 and 27

To a suspension of [Au(SPyCOOH)(PPh$_3$)] or [Au(SPyCOOH)-(PPh$_2$Py)] (1 mmol) in acetonitrile (6 mL) was added DIPEA (2.2 mmol). The mixture was stirred for 5 min at room temperature and then PyBOP was added (1.2 mmol), and the result-

ing solution was stirred for an additional 45 min. To this solution was added dropwise at 0 °C a solution of the corresponding amino acid or dipeptide methyl ester (1.5 mmol) in acetonitrile (4 mL) and DIPEA (1.5 mmol). After the addition the mixture was stirred for 48 h at room temperature. The acetonitrile was evaporated and dichloromethane (40 mL) was added. This solution was washed with a saturated $NaHCO_3$ solution in water (3 × 15 mL) and a saturated solution of NaCl (3 × 15 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered off and evaporated to dryness. The complexes were purified by column chromatography on silica gel using as the eluent a mixture of acetone–hexane 3 : 7.

[Au(SPyCOAlaOMe)(PPh$_2$Py)] (20). Yield. 67.3%. ^{1}H-NMR ($CDCl_3$, 400 MHz δ (ppm), J(Hz)): 9.15 (d, 1H, J = 6.8, CONH_{Ala}), 8.65 (dd, 1H, J = 4.8 and 0.8, *H*12), 8.22 (d, 1H, J = 3.2, *H*1), 8.14 (dd, 1H, J = 8.0 and 2.0, *H*3), 7.90 ("t", 1H, J = 7.7, *H*9), 7.56 (m, 4H, *Ar*), 7.64 (m, 1H, *H*11), 7.36 (m, 6H, *ArH*), 7.27 (m, 1H, *H*10), 6.88 (dd, 1H, J = 8.0 and 5.0, *H*2), 4.70 ("q", 1H, J = 7.1, $C_{\alpha,Ala}H$), 3.63 (s, 3H, OCH_3), 1.45 (d, 3H, J = 7.2, $C_{\beta,Ala}H_3$). ^{31}P-NMR ($CDCl_3$, 400 MHz, δ (ppm)): 36,1. ^{13}C-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): 173.4 (*C*OOMe), 166.0 (*C*ONH_{Ala}), 165.5 (C, *Py*), 151.2 (d, CH, J = 14.7, *C*12), 148.6 (CH, *C*1), 147.8 (C, *C*8), 139.5 (CH, *C*3), 136.5 (d, CH, J = 11.3, *C*9), 134.7 (d, CH, J = 13.8, *C*5), 132.2 (CH, *C*11), 131.7 (d, CH, J = 2.4, *C*7), 130.0 (d, C, J = 86.9, *C*4), 129.0 (d, CH, J = 11.7, *C*6), 126.8 (C, *Py*), 125.2 (d, CH, J = 2.5, *C*10), 118.5 (CH, *C*2), 52.3 (O*C*H$_3$), 49.1 ($C_{\alpha,Ala}$H), 18.2 ($C_{\beta,Ala}H_3$). MS (ESI+) m/z: $[M + H]^+$ = 700.1 (calcd), 700.0 (found). IR (cm^{-1}): 3280 (br, w, *CONH*), 1736 (s, *COOMe*), 1637 (s, *CONH*), 1569, 1519, 1480 and 1434 (w, *Ar*), 1099 and 1069 (s, *C–O*), 742, 711 and 691 (w, *Ar*). Anal. Calcd for $C_{27}H_{25}AuN_3O_3PS$ (699.51): C, 46.36; H, 3.60; N, 6.01; S, 4.58. Found: C, 46.44; H, 3.68; N, 6.10; S, 4.69. TLC R_f: 0.5 (acetone–hexane 1 : 1). $[\alpha]_D$: + 35.69 (c = 1.0 g ml^{-1}, $CHCl_3$).

[Au$_2$(SPyCOOH)(PPh$_3$)$_2$]BF$_4$ (21). To a suspension of 2-mercaptonicotinic acid (0.016 g, 0.1 mmol) in CH_2Cl_2 (10 ml) was added $[O(AuPPh_3)_3]BF_4$ (0.098 g, 0.066 mmol). The mixture was stirred for 2 h at room temperature. Then, the solution was concentrated to *ca.* 5 ml and addition of hexane (20 ml) afforded **21** as an orange solid (0.110 g, 95.0%). ^{1}H-NMR ($CDCl_3$, 400 MHz, δ ppm), J (Hz)): 8.43 (d, 1H, J = 3.2, *H*1), 8.17 (d, 1H, J = 7.6, *H*3), 7.40 (m, 30H, *ArH*), 7.19 (dd, 1H, J = 7.6 and 4.8, *H*2). ^{31}P-NMR ($CDCl_3$, 400 MHz, δ (ppm)): 33.4. ^{13}C-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): 168.5 (*C*OOH), 157.3 (C, *Py*). 150.0 (CH, *C*1), 139.9 (CH, *C*3), 134.5 (d, CH, J = 13.4, *C*5), 132.2 (CH, *C*7), 129.4 (d, CH, J = 11.9, *C*6), 128.1 (d, C, J = 60.4, *C*4), 122.2 (CH, *C*2). ^{19}F-NMR ($CDCl_3$, 400 MHz, δ ppm)): −153.9. MS (ESI+) m/z: $[M]^+$ = 1072.1 (calcd), 1072.3 (found). IR (cm^{-1}): 3300–3053 (br, *COOH*), 1713 (s, *COOH*), 1571, 1480 and 1435 (w, *Ar*), 1098 and 1051 (s, *C–O*), 744, 726, and 710 (w, *Ar*). Anal. Calcd for $C_{42}H_{34}Au_2BNO_2P_2SF_4$ (1159.48): C, 43.51; H, 2.96; N, 1.21; S, 2.77. Found: C, 43.65; H, 2.99; N, 1.26; S, 2.80.

[Au(SPyCOLys(Boc)OMe)(PPh$_3$)] (22). Yield. 60.7%. ^{1}H-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): 9.07 (d, 1H, J = 7.6, CONH_{Lys}), 8.33 (dd, 1H, J = 4.8 and 1.8, *H*1), 8.26 (dd, 1H, J = 7.6 and 1.8, *H*3), 7.58 (m, 15H, *ArH*), 7.02 (dd, 1H, J = 7.6 and 4.8, *H*2), 4.86 ("dt", 1H, J = 7.6 and 5.3, $C_{\alpha,Lys}H$), 4.61 (m, 1H, N*H*Boc), 3.76 (s, 3H, OCH_3), 3.12 (m, 2H, $C_{\varepsilon,Lys}H_2$), 1.98 and 1.90 (m, 2H, diastereotopic protons, $C_{\beta,Lys}H_2$), 1.59 (m, 2H, $C_{\gamma,Lys}H_2$), 1.53 (m, 2H, $C_{\delta,Lys}H_2$), 1.42 (s, 9H, $C_{Boc}H_3$). ^{31}P-NMR ($CDCl_3$, 300 MHz, δ (ppm)): 37.6. ^{13}C-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): 172.7 (*C*OOMe), 166.4 (*C*ONH_{Lys}), 164.9 (C, *Py*), 155.9 (NHC_{Boc}), 149.1 (CH, *C*1), 139.2 (CH, *C*3), 134.3 (d, CH, J = 13.9, *C*5), 131.7 (d, CH, J = 2.4, *C*7), 130.0 (C, *C*4), 129.1 (CH, J = 11.5, *C*6), 118.8 (CH, *C*2), 53.0 ($C_{\alpha,Lys}$H), 52.3 (OCH_3), 40.3 ($C_{\varepsilon,Lys}H_2$), 32.0 ($C_{\beta,Lys}H_2$), 29.4 ($C_{\delta,Lys}H_2$), 28.4 ($C_{Boc}H_3$), 22.8 ($C_{\gamma,Lys}H_2$). MS (ESI+) m/z: $[M + H]^+$ = 856.2 (calcd), 856.3 (found). IR (cm^{-1}): 3266 (br, w, *CONH*), 1739 (s, *COOMe*), 1703 (s, *OCONH*), 1641 (s, *CONH*), 1571, 1520, 1480 and 1435 (w, *Ar*), 1100 and 1070 (s, *C–O*), 746, 710 and 691 (w, *Ar*). Anal. Calcd for $C_{36}H_{41}AuN_3O_5PS$ (855.73): C, 50.53; H, 4.83; N, 4.91; S, 3.75. Found: C, 50.61; H, 4.89; N, 4.99; S, 3.79. TLC R_f: 0.5 (acetone–hexane 1 : 1). $[\alpha]_D$: +21.18 (c = 1.0 g ml^{-1}, $CHCl_3$).

[Au(SPyCOLys(H)OMe)(PPh$_3$)] (23). To a 1 : 1 (v/v) CH_2Cl_2–TFA solution (12 ml) was added complex **23** (0.296 g, 0.346 mmol). The mixture was stirred at room temperature for 15 minutes until completion by thin-layer chromatography (the reaction was complete when the starting compound was strongly retained at the origin of TLC, R_f = 0, acetone–hexane 1 : 1). Then, the solvent was evaporated and the residue was dissolved in water (40 ml). The resulting clear solution was washed with CH_2Cl_2 (1 × 20 ml). K_2CO_3 was added carefully to the aqueous phase until slightly basic pH (11–12). At this point, the white suspension was extracted with CH_2Cl_2 (3 × 20 ml). The organic phase was dried over anhydrous $MgSO_4$, filtered off, and the solvent was evaporated, affording **23** as a white solid (0.089 g, 34.1%). ^{1}H-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): 9.01 (d, 1H, J = 7.2, CONH_{Lys}), 8.31 (dd, 1H, J = 4.8 and 1.8, *H*1), 8.22 (dd, 1H, J = 7.8 and 1.8, *H*3), 7.56 (m, 15H, *ArH*), 7.00 (dd, 1H, J = 7.8 and 4.8, *H*2), 4.86 ("dt", 1H, J = 7.6 and 5.4, $C_{\alpha,Lys}H$), 3.76 (s, 3H, OCH_3), 2.71 (t, 2H, J = 6.5, $C_{\varepsilon,Lys}H_2$), 1.99 (m, 2H, $C_{\beta,Lys}H_2$), 1.59 (m, 2H, $C_{\gamma,Lys}H_2$), 1.52 (m, 2H, $C_{\delta,Lys}H_2$). ^{31}P-NMR ($CDCl_3$, 300 MHz, δ (ppm)): 37.6. ^{13}C-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): 172.8 (*C*OOMe), 166.4 (*C*ONH_{Lys}), 149.1 (CH, *C*1), 139.1 (CH, *C*3), 134.3 (d, CH, J = 13.9, *C*5), 131.6 (d, CH, J = 2.1, *C*7), 130.2 (C, *C*4), 129.1 (CH, J = 11.6, *C*6), 118.8 (CH, *C*2), 53.2 ($C_{\alpha,Lys}$H), 52.2 (OCH_3), 41.7 ($C_{\varepsilon,Lys}H_2$), 32.0 ($C_{\beta,Lys}H_2$), 26.4 ($C_{\delta,Lys}H_2$), 22.8 ($C_{\gamma,Lys}H_2$). MS (ESI+) m/z: $[M + H]^+$ = 756.2 (calcd), 756.2 (found). IR (cm^{-1}): 3260 (br, w, *CONH*), 2961 and 2941 (br, w, *NH$_2$*), 1739 (s, *COOMe*), 1634 (s, *CONH*), 1571, 1525, 1480 and 1435 (w, *Ar*), 1100 and 1071 (s, *C–O*), 746, 710 and 691 (w, *Ar*). Anal. Calcd for $C_{31}H_{33}AuN_3O_3PS$ (755.62): C, 49.28; H, 4.40; N, 5.56; S, 4.24. Found: C, 49.41; H, 4.51; N, 5.61; S, 4.32. $[\alpha]_D$: +9.64 (c = 1.0 g ml^{-1}, $CHCl_3$).

[BocGlyProOMe]. To a solution of Boc-Gly-OH (0.350 g, 2 mmol) in acetonitrile (10 ml) was added DIPEA (0.753 ml, 4.4 mmol). The mixture was stirred for 5 min at room temperature, and then PyBOP (1.25 g, 2.4 mmol) was added, and the resulting mixture was stirred for an additional 45 min. At this point, a solution of HCl·H-Pro-OMe (0.497 g, 3 mmol) and DIPEA (0.515 ml, 3 mmol) in acetonitrile was added dropwise

and at 0 °C. After the addition, the mixture was stirred for 48 h at room temperature. Acetonitrile was evaporated and CH_2Cl_2 was added (40 ml). This solution was washed with a saturated $NaHCO_3$ solution in water (3 × 15 mL) and a saturated solution of NaCl (3 × 15 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered off and evaporated to dryness. The crude of the reaction was purified by column chromatography of silica gel using as an eluent a mixture of ethyl acetate–pentane (1 : 3), affording the dipeptide as a colourless oil (0.436 g, 76.2%). ^{1}H-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): rotamer mixture (ratio: 1 : 0.2): 5.35 (m, 1H, CONH_{Gly}), 4.41 (dd, 1H, J = 8.8 and 3.6, $C_{\alpha,Pro}H$), 3.88 and 3.81 (dd, ABX system, 2H, diastereotopic protons, J = 17.2 and 4.8, J = 17.2 and 4.4, $C_{\alpha,Gly}H_2$), 3.66 and 3.62 (s, 3H, OCH_3), 3.49 and 3.38 (m, 2H, diastereotopic protons, $C_{\delta,Pro}H_2$), 2.11 and 1.89 (m, 2H, diastereotopic protons, $C_{\beta,Pro}H_2$), 1.94 (m, 2H, $C_{\gamma,Pro}H_2$), 1.33 (s, 9H, $C_{Boc}H_3$).

[HGlyProOMe]. The dipeptide BocProGlyOMe (0.436 g, 1.52 mmol) was dissolved in a solution 3 M HCl–AcOEt (6 ml). The solution was stirred at room temperature for 3 h until completion by thin-layer chromatography. The solvent was evaporated, and the residue was dissolved in water and freeze-dried, affording the desired dipeptide as a white solid (0.322 g, 95.3%). ^{1}H-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): rotamer mixture (ratio 1 : 0.2): 8.25 (m, 3H, NH_3), 4.55 (m, 1H, $C_{\alpha,Pro}H$), 4.10 (m, 2H, $C_{\alpha,Gly}H_2$), 3.77 (A) and 3.72 (B) (s, 3H, OCH_3), 3.61 (m, 2H, diastereotopic protons, $C_{\delta,Pro}H_2$), 2.25 and 1.99 (m, 2H, diastereotopic protons, $C_{\beta,Pro}H_2$), 1.99 (m, 2H, $C_{\gamma,Pro}H_2$).

[Au(SPyCOGlyProOMe)(PPh$_3$)] (24). Yield: 60.3%. ^{1}H-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): rotamer mixture ratio: 1 : 0.3; 9.06 ("t", 1H, J = 3.9, CONH_{Gly}), 8.31 (dd, 1H, J = 4.8 and 2.0, $H1$), 8.14 (dd, 1H, J = 7.6 and 2.0, $H3$), 7.54 (m, 15H, ArH), 6.99 (dd, 1H, J = 7.6 and 4.8, $H2$), 4.58 (A) and 4.51 (B) (dd, 1H, J = 8.6 and 3.4 and J = 8.0 and 2.8, $C_{\alpha,Pro}H$), 4.37 and 4.26 (dd, ABX system, 2H, diastereotopic protons, J = 17.8 and 4.6, J = 17.8 and 4.2, $C_{\alpha,Gly}H_2$), 3.71 (s, 3H, OCH_3), 3.55 (m, 2H, diastereotopic protons, $C_{\delta,Pro}H_2$), 2.19 and 2.04 (m, 2H, diastereotopic protons, $C_{\beta,Pro}H_2$), 2.08 (m, 2H, $C_{\gamma,Pro}H_2$). ^{31}P-NMR ($CDCl_3$, 400 MHz, δ (ppm)): 37.5. ^{13}C-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): 172.4 (COOMe), 167.0 (CON$_{Pro}$), 165.1 (CONH$_{Gly}$), 149.2 (CH, $C1$), 138.5 (CH, $C3$), 134.3 (d, CH, J = 13.9, $C5$), 131.7 (CH, $C7$), 130.2 (d, C, J = 74.7, $C4$), 129.2 (d, CH, J = 11.6, $C6$), 118.6 (CH, $C2$), 58.9 ($C_{\alpha,Pro}$H), 52.3 (OCH$_3$), 46.1 ($C_{\delta,Pro}$H$_2$), 43.2 ($C_{\alpha,Gly}$H$_2$), 29.1 ($C_{\beta,Pro}$H$_2$), 24.6 ($C_{\gamma,Pro}$H$_2$). MS (ESI+) m/z: $[M + H]^+$ = 782.1 (calcd), 782.1 (found). IR (cm^{-1}): 3205 (br, w, NH), 1736 (s, $COOMe$), 1628 (s, $CONH$), 1571, 1523, 1481, 1455 and 1435 (w, Ar), 1100 and 1073 (s, C–O), 749, 710 and 692 (w, Ar). Anal. Calcd for $C_{32}H_{31}AuN_3O_4PS$ (781.61): C, 49.17; H, 4.00; N, 5.38; S, 4.10. Found: C, 49.29; H, 4.07; N, 5.41; S, 4.19. TLC R_f: 0.4 (acetone–hexane 1 : 1). $[\alpha]_D$: −21.36 (c = 1.0 g ml^{-1}, $CHCl_3$).

[Au(SPyCOAlaNEt$_2$)(PPh$_3$)] (25). To a solution of complex **8** (0.433 g, 0.63 mmol) in acetonitrile (10 mL) was added DIPEA (0.237 ml, 1.39 mmol). The mixture was stirred at room temperature for 15 min and PyBOP was added (0.393 g, 0.76 mmol). After 15 min diethylamine (0.072 ml, 0.69 mmol) was added and then the mixture was stirred for 36 h. The solvent was evaporated and the crude was purified by silica gel column chromatography using as an eluent acetone–hexane 1 : 1, affording **25** as a green solid (0.106 g, 22.7%). ^{1}H-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): 9.14 (d, 1H, J = 7.2, CONH_{Ala}), 8.28 (dd, 1H, J = 4.8 and 1.8, $H1$), 8.16 (dd, 1H, J = 7.6 and 1.8, $H3$), 7.54 (m, 15H, ArH), 6.96 (dd, 1H, J = 7.6 and 4.8, $H2$), 5.12 ("q", 1H, J = 6.8, $C_{\alpha,Ala}H$), 3.54 (m, 2H, diastereotopic protons, $C_{NEt_2}H_2$), 3.29 (m, 2H, diastereotopic protons, $C_{NEt_2}H_2$), 1.49 (d, 3H, J = 6.8, $C_{\beta,Ala}H_3$), 1.26 (m, 3H, $C_{NEt_2}H_3$) and 1.13 (t, 3H, J = 7.1, $C_{NEt_2}H_3$). ^{31}P-NMR ($CDCl_3$, 300 MHz, δ (ppm)): 37.6. ^{13}C-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): 165.1 (CONEt$_2$), 164.8 (CONH$_{Ala}$), 163.4 (C, Py), 147.5 (CH, $C1$), 138.6 (CH, $C3$), 134.3 (d, CH, J = 14.0, $C5$), 131.6 (d, CH, J = 2.0, $C7$), 129.1 (d, CH, J = 11.5, $C6$), 118.3 (CH, $C2$), 46.3 ($C_{\alpha,Ala}$H), 41.7 and 40.2 (C_{NEt_2}H$_2$), 19.2 ($C_{\beta,Ala}$H$_3$), 14.6 and 12.9 (C_{NEt_2}H$_3$). MS (ESI+) m/z: $[M + H]^+$ = 740.2 (calcd), 740.2 (found). Anal. Calcd for $C_{31}H_{33}AuN_3O_2PS$ (739.62): C, 50.34; H, 4.50; N, 5.68; S, 4.34. Found: C, 50.46; H, 4.59; N, 5.70; S, 4.39. TLC R_f: 0.3 (acetone–hexane 1 : 1).

[Au(SPyCO(D)AlaOMe)(PPh$_3$)] (26). Yield: 67.7%. ^{1}H-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): 9.63 (d, 1H, J = 5.2, CONH_{Ala}), 8.38 (dd, 1H, J = 7.6 and 2.0, $H1$), 8.19 (dd, 1H, J = 5.2 and 2.0, $H3$), 7.63–7.47 (m, 15H, ArH), 6.98 (dd, 1H, J = 7.6 and 5.2, $H2$), 4.80 ("q", 1H, J = 7.2, $C_{\alpha,Ala}H$), 3.76 (s, 3H, OCH_3), 1.57 (d, 3H, J = 7.2, $C_{\beta,Ala}H_3$). ^{31}P-NMR ($CDCl_3$, 400 MHz, δ (ppm)): 37.6. ^{13}C-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): 173.3 (COOMe), 165.9 (CONH$_{Ala}$), 165.6 (C, Py), 148.4 (CH, $C1$), 139.4 (CH, $C3$), 134.2 (d, CH, J = 14.1, $C5$), 131.5 (d, CH, J = 2.3, $C7$), 130.4 (C, Py), 129.7 (d, C, J = 55.2, $C4$), 129.1 (d, CH, J = 11.5, $C6$), 118.3 (CH, $C2$), 52.4 (OCH$_3$), 49.1 ($C_{\alpha,Ala}$H), 18.1 ($C_{\beta,Ala}$H$_3$). MS (ESI+) m/z: $[M + H]^+$ = 699.1 (calcd), 699.0 (found). IR (cm^{-1}): 3273 (br, w, NH), 1739 (s, $COOMe$), 1639 (s, $CONH$), 1570, 1523, 1479 and 1434 (w, Ar), 1098 and 1069 (s, C–O), 744, 709 and 690 (w, Ar). Anal. Calcd for $C_{28}H_{26}AuN_2O_3PS$ (698.52): C, 48.14; H, 3.75; N, 4.01; S, 4.59. Found: C, 48.07; H, 3.81; N, 4.09; S, 4.32. TLC R_f: 0.5 (acetone–hexane 1 : 1). $[\alpha]_D$: −35.83 (c = 1.0 g ml^{-1}, $CHCl_3$).

[Au(SPyCO(D/L)AlaOMe)(PPh$_3$)] (racemic mixture). Yield: 68.5%. The data are the same as those for complex **27**, except for the angle of polarised light. $[\alpha]_D$: +0.04 (c = 1.0 g ml^{-1}, $CHCl_3$).

[Au(SPyCO(D)PheOMe)(PPh$_3$)] (27). Yield: 62.0%. ^{1}H-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): rotamer mixture (ratio 1 : 0.8): 9.33 (d, 1H, J = 6.8, CONH_{Phe}), 8.27–8.25 (m, 2H, $H1$ and $H3$), 7.64–7.48 (m, 15H, ArH), 7.33–7.18 (m, 5H, Ar_{Phe}H), 6.97 (m, 1H, $H2$), 5.06 (A) and 3.75 (B) ("dd", 1H, J = 13.6 and 6.8 and J = 8.0 and 5.2, $C_{\alpha,Phe}H$), 3.72 (A) and 3.69 (B) (s, 3H, OCH_3), 3.28 and 3.22 (A) and 3.10 and 2.86 (B) (dd, 2H, diastereotopic protons, J = 13.8 and 6.0 and J = 14.0 and 6.8; and J = 13.6 and 5.2 and J = 13.6 and 8.0, $C_{\beta,Phe}H_2$). ^{31}P-NMR ($CDCl_3$, 400 MHz, δ (ppm)): 37.4. ^{13}C-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): rotamer mixture: 172.0 (COOMe), 166.1 (CONH$_{Phe}$), 165.8 (C, Py), 148.4 (CH, $C1$), 139.4 (CH, $C3$), 136.4 (C, $C8$), 134. (d, CH, J = 13.9, $C5$), 131.7 (d, CH, J = 2.2, $C7$), 130.1 (d, C, J = 64.0, $C4$), 129.5 and 129.2 (CH, $C10$), 129.1 (d, CH, J = 11.5, $C6$), 128.6 and 128.4 (CH, $C9$), 126.9 and 126.8 (CH, $C11$), 118.3 (CH, $C2$), 55.8 (A) and 55.0 (B) ($C_{\alpha,Phe}$H), 52.1 (A) and

52.0 (B) (OCH_3), 41.1 (A) and 38.2 (B) ($C_{\beta,Phe}H_2$). MS (ESI+) *m*/*z*: $[M + H]^+$ = 775.1 (calcd), 775.1 (found). IR (cm^{-1}): 3281 (br, w, CO*NH*), 1736 (s, *COOMe*), 1640 (s, *CONH*), 1571, 1518, 1495, 1480, 1453 and 1435 (w, *Ar*), 1100 and 1071 (s, *C–O*), 745 and 709 (w, *Ar*). Anal. Calcd for $C_{34}H_{30}AuN_2O_3PS$ (774.62): C, 52.72; H, 3.90; N, 3.62; 4.14. Found: C, 52.81; H, 3.68; N, 3.78; S, 4.01. TLC R_f: 0.5 (acetone–hexane 1 : 1). $[\alpha]_D$: −4.65 (c = 1.0 g ml^{-1}, $CHCl_3$).

[Au(SPyCO(D)AlaOH)(PPh_3)] (28). To a suspension of complex **2** (0.704 g, 1 mmol) in methanol (20 mL) was added LiOH (0.480 g, 20 mmol). The mixture was stirred at room temperature for a period between 24 and 48 h. The reaction is followed by thin layer chromatography and is complete when the starting compound (R_f = 0.4 (acetone–hexane 1 : 1)) is strongly retained at the origin of the thin layer chromatography (R_f = 0 (acetone–hexane 1 : 1)). At this point the methanol is evaporated and the product dissolved in water. The resulting white solution is acidified dropwise with a saturated solution of $KHSO_4$ until a slightly acidic pH (3–4). Then the solution was extracted three times with dichloromethane and the organic phase was dried over anhydrous $MgSO_4$, filtered off and evaporated to yield the desired complex **28** as a white solid (0.521 g, 76.2%). ^{1}H-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): 9.25 (d, 1H, J = 5.6, CONH_{Gly}), 8.33 (dd, 1H, J = 4.6 and 2.0, *H*1), 8.24 (dd, 1H, J = 7.6 and 2.0, *H*3), 7.59–7.42 (m, 15H, *ArH*), 7.02 (dd, 1H, J = 7.6 and 4.6, *H*2), 4.65 ("q", 1H, J = 6.8, $C_{\alpha,Ala}H$), 1.59 (d, 3H, J = 6.8, $C_{\beta,Ala}H_3$). ^{31}P-NMR ($CDCl_3$, 400 MHz, δ (ppm)): 37.2. ^{13}C-NMR ($CDCl_3$, 400 MHz, δ (ppm), J (Hz)): 173.6 (*C*OOH), 168.3 ($CONH_{Ala}$), 164.6 (C, *Py*), 149.6 (CH, *C*1), 139.1 (CH, *C*3), 134.2 (d, CH, J = 13.8, *C*5), 131.7 (d, CH, J = 1.3, *C*7), 129.6 (C, *Py*), 129.3 (d, C, J = 57.7, *C*4), 129.2 (CH, J = 11.5, *C*6), 119.1 (CH, *C*2), 50.5 ($C_{\alpha,Ala}H$), 16.9 ($C_{\beta,Ala}H_3$). MS (ESI+) *m*/*z*: $[M + H]^+$ = 685.1 (calcd), 685.0 (found). IR (cm^{-1}): 3400–2900 (br, s, *COOH*), 1731 (s, *COOH*), 1637 (s, *CONH*), 1570, 1519, 1479 and 1434 (w, *Ar*), 1098 and 1069 (s, *C–O*) 743 and 709 (w, *Ar*). Anal. Calcd for $C_{27}H_{24}AuN_2O_3PS$ (684.50): C, 47.38; H, 3.53; N, 4.09; S, 4.68. Found: C, 47.60; H, 3.90; N, 3.78; S, 4.72. $[\alpha]_D$: −5.2 (c = 1.0 g ml^{-1}, $CHCl_3$).

[Au_2(SPyCOProOMe)(PPh_3)$_2$]OTf (29). To a solution of complex **6** (0.072 g, 0.1 mmol) in CH_2Cl_2 (5 ml) was added freshly prepared [Au(OTf)(PPh_3)] (0.061 g, 0.1 mmol). The mixture was stirred for 2 h at room temperature. Then, the resulting yellow solution was filtered on Celite. The solution was concentrated to *ca.* 5 ml and addition of hexane (20 ml) afforded **29** as an orange solid (0.135 g, 92.1%). ^{1}H-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): rotamer mixture (ratio 1 : 0.8): 8.56 (m, 1H, *H*1), 7.73 (dd, 1H, J = 7.5 and 1.5, *H*3), 7.42 (m, 30H, *ArH*), 7.36 (m, 1H, *H*3), 4.57 (A) and 4.43 (B) (dd, 1H, J = 8.1 and 3.6, and J = 8.4 and 2.7, $C_{\alpha,Pro}H$), 3.67 (m, 2H, $C_{\delta,Pro}H_2$), 3.61 (A) and 3.49 (B) (s, 3H, OCH_3), 2.28 (m, 2H, $C_{\beta,Pro}H_2$), 1.92 (m, 2H, $C_{\gamma,Pro}H_2$). ^{31}P-NMR ($CDCl_3$, 300 MHz, δ (ppm)): 33.0. ^{19}F-NMR ($CDCl_3$, 300 MHz, δ (ppm)): −77.98. ^{13}C-NMR ($CDCl_3$, 300 MHz, δ (ppm), J (Hz)): rotamer mixture: 172.0 and 171.9 (*C*OOMe), 166.4 and 166.2 (CON_{Pro}), 156.0 (C, *Py*), 151.4 and 150.9 (CH, *C*1), 137.9 and 136.7 (CH, *C*3), 133.9 (d, CH, J = 13.7, *C*5), 132.3 (d, CH, J = 2.4, *C*7), 129.4 (d, CH, J = 11.9, *C*5), 127.6 (d, C, J = 61.4, *C*4), 123.1 and 122.6 (CH, *C*2), 118.8 (C, CF_3), 60.3 and 58.7 ($C_{\alpha,Pro}H$), 52.4 and 52.1 (OCH_3), 48.5 and 46.4 ($C_{\delta,Pro}H_2$), 31.1 and 29.4 ($C_{\beta,Pro}H_2$), 24.7 and 22.8 ($C_{\gamma,Pro}H_2$). HRMS (ESI+) *m*/*z*: $[M]^+$ = 1183.1795 (calcd), 1183.1859 (found). IR (cm^{-1}): 1739 (s, *COOMe*), 1631 (s, *CON*), 1570, 1545, 1479 and 1435 (w, *Ar*), 1097 and 1093 (s, *COOMe*), 746 and 709 (w, *Ar*).

Cell culture

Jurkat (leukaemia), R69 (lymphoid) and MiaPaca2 (pancreatic carcinoma) cell lines were maintained in RPMI 1640, while A549 (lung carcinoma) and 293 T cells (kidney embryonic fibroblasts) were grown in DMEM (Dulbecco's Modified Eagle's Medium). Both media were supplemented with 5% fetal bovine serum (FBS), 200 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine. The medium for A549 cells was also supplemented with 2.2 g l^{-1} Na_2CO_3, 100 μg ml^{-1} pyruvate and 5 ml non-essential amino acids (Invitrogen). Cultures were maintained under a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

Cytotoxicity assay by MTT

The MTT assay was used to determine the cell viability as an indicator for the cell sensitivity to the complexes.

Exponentially growing cells were seeded at a density of approximately 1 × 10^5 cells ml^{-1} (A549, MiaPaca2) or 3 × 10^5 cells ml^{-1} (Jurkat), in a 96-well flat-bottomed microplate and 24 h later they were incubated for 24 h with the compounds. The complexes were dissolved in DMSO (concentration 0.1 M) and tested in concentrations ranging from 0.5 to 100 μM (dilutions with 1 μl of the stock solution for 100 μM) and in quadruplicate. Cells were incubated with our compounds for 24 h at 37 °C. 10 μl of MTT (5 mg ml^{-1}) was added and plates were incubated for 1–3 h at 37 °C. Finally, 100 μl per well ^{i}PrOH (0.05 M HCl) was added. The optical density was measured at 570 nm using a 96-well multiscanner autoreader (ELISA). The IC_{50} was calculated by non-linear regression analysis using the Origin software (Origin Software, Electronic Arts, Redwood City, California, USA).

Cell death analysis

Apoptosis/necrosis hallmarks were analysed by simultaneously measuring the mitochondrial membrane potential ($\Delta\Psi_m$), exposure of phosphatidylserine (PS). Concentrations of the complexes near IC_{50} values (6 μM in A549 cells, 2 μM Jurkat) were employed. In order to evaluate the chemoprotective effects of antioxidants, *N*-acetylcysteine (10 mM) and glutathione (2 mM) were added and incubated with our compounds (10 μM A549 cells, 2 μM Jurkat cells) for 24 h. In brief, 2.5 × 10^5 cells in 200 μl were incubated in ABB (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.4), with either 5 nM $DiOC_6$ (3) or 60 nM tetramethylrhodamine (TMRE, both from Molecular Probes) at 37 °C for 10 min. Annexin V-PE or Annexin V-FITC (Invitrogen) at a concentration of 0.5 μg ml^{-1} or 7-AAD (0.5 mg μl^{-1}) was added to samples and incubated at 37 °C for an additional 15 min. In all cases, cells from each

well were diluted to 1 ml ABB to be analyzed by flow cytometry (FACScan, BD Bioscience, Spain).

To additionally assess cell viability after treatment with the compounds, 2.5×10^5 cells were harvested and incubated in 200 μl of PBS containing 50 ng μl^{-1} of 7-amino-actinomycin D (7-AAD, Inmunostep). When analyzed simultaneously for either PS exposure or $\Delta\Psi_m$, 7-AAD was added to the samples in ABB.

Nuclear morphology

The morphology of nuclei after the treatment with the different compounds was analysed by staining cells with Hoechst 33342 (Molecular Probes) at 25 mg ml^{-1}. Cells were visualized in a fluorescence microscope (Nikon Eclipse 50i) and the ACT software was used for the acquisition of the images.

Intracellular ROS quantification

Oxidative stress was analysed by intracellular staining with the fluorescent probe 2-hydroxyethidium (2-HE, Molecular Probes). After 16 h of culture in the presence of compounds **6** and **21**, cells were incubated with 2 μM 2-HE at 37 °C for 15 min. The red fluorescence produced by reduction of 2-HE to ethidium was quantified in a flow cytometer.

Thioredoxin reductase inhibition assay

For determination of the thioredoxin reductase activity, A549 cells were incubated for 9 h with our compounds at different concentrations near IC_{50} values. Cells were collected and washed with PBS and 150 μl buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris/HCl pH 7.6, 10% v/v glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 μg ml^{-1} leupeptin, 10 mM NaF, 1 mM sodium methylsulphonium) for 30 min at 0 °C, and centrifuged at 200*g* for 10 min at 4 °C. The protein was quantified using the BCA-protein assay (Thermo Scientific) and 80 μg was added in each assay. Kinetic studies were performed in a buffer containing 0.2 M NaCl, H-phosphate pH 7.4, 2 mM EDTA, 0.25 mM NADPH and 3 mM DTNB. The increase in the absorbance was measured at 412 nm for 5 min at 25 °C.

Conclusions

The cytotoxic activity of three families of thioamino acid derivatives as esters, acids or amides was tested in various cell lines. The conclusion is that the most active compounds correspond to the ester family. Consequently, several structural modifications have been performed over the parent structure [Au{SPyCONHCH(R)COOMe}(PPh_3)], which have led to the synthesis of new thioamino acid ester species. These structural modifications include changes in the phosphine type, in the charge and number of gold(I) atoms coordinated to the sulfur center, or in the type, number, stereochemistry and functionalisation of the amino acid. All of them have been introduced in order to evaluate their effect on the cytotoxic activity and determine structure–activity relationships (SAR). All the compounds were tested against diverse tumor cell lines, showing good cytotoxicity, with IC_{50} values in the low micromolar range. The SAR established for this new bioconjugated family of compounds allowed us to prepare the complex [Au_2(SPyCO-ProOMe)(PPh_3)$_2$]OTf (**29**), the most potent of the series, confirming our hypothesis. Moreover, some important cellular events like changes in cell and nucleus morphology, loss of the mitochondrial membrane potential, production of ROS and inhibition of TrX were observed after addition of the complexes **6** and **21**. Further biological studies and refinement of our rational design might allow us shortly to prepare more cytotoxic and selective complexes.

Acknowledgements

The authors thank the Ministerio de Economía y Competitividad (MEC/FEDER CTQ2013-48635-C2-1-P, CTQ2010-17436 and SAF2010-14920) and DGA-FSE (E77, E40 and B16) for financial support. A.G. thanks the CSIC for a JAE-predoc fellowship. LGF was the recipient of an AECC fellowship.

Notes and references

1 (*a*) S. Y. Ho and E. R. T. Tiekink, in *Metallotherapeutic Drugs & Metal-based Diagnostic Agents*, ed. M. Gielen and E. R. T. Tiekink, Wiley, 2005, p. 507; (*b*) *Metals in Medicine*, ed. J. C. Dabrowiak, Wiley, 2009; (*c*) P. C. A. Bruijnincx and P. J. Sadler, *Curr. Opin. Chem. Biol.*, 2008, **12**, 197; (*d*) G. Gasser and N. Metzler-Nolte, *Curr. Opin. Chem. Biol.*, 2012, **16**, 84.

2 *Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug*, ed. B. Lippert, Wiley-VCH, 1999.

3 (*a*) E. R. T. Tiekink, *Bioinorg. Chem. Appl.*, 2003, **1**, 53; (*b*) E. R. T. Tiekink, *Inflammopharmacology*, 2008, **16**, 138; (*c*) I. Ott, *Coord. Chem. Rev.*, 2009, **253**, 1670; (*d*) A. Casini and L. Messori, *Curr. Top. Med. Chem.*, 2011, **11**, 2647; (*e*) B. Bertrand and A. Casini, *Dalton Trans.*, 2014, **43**, 4209; (*f*) M. Navarro, *Coord. Chem. Rev.*, 2009, **253**, 1619; (*g*) C. M. Che and R. W. Y. Sun, *Chem. Commun.*, 2011, **47**, 9554.

4 (*a*) T. M. Simon, D. H. Kunishima, G. J. Vibert and A. Lorber, *Cancer Res.*, 1981, **41**, 94; (*b*) C. K. Mirabelli, R. K. Johnson, D. T. Hill, L. F. Faucette, G. R. Girard, G. Y. Kuo, C. M. Sung and S. T. Crooke, *J. Med. Chem.*, 1986, **29**, 218; (*c*) E. Barreiro, J. S. Casas, M. D. Couce, A. Sánchez, A. Sánchez-González, J. Sordo, J. M. Varela and E. M. Vázquez-López, *J. Inorg. Biochem.*, 2009, **103**, 1023; (*d*) I. Ott, X. Quian, Y. Xu, D. H. V. Vlecker, I. J. Marques, D. Kubutat, J. Will, W. S. Sheldrick, P. Jesse, A. Prokop and C. P. Bagowski, *J. Med. Chem.*, 2009, **52**, 763; (*e*) E. Vergara, A. Casini, F. Sorrentino, O. Zava, E. Cerrada, M. P. Rigobello, A. Bindolli, M. Laguna and P. J. Dyson, *ChemMedChem*, 2010, **5**, 96; (*f*) D. T. Hill, A. A. Isab,

D. E. Griswold, M. J. DiMartino, E. D. Matz, A. L. Figueroa, J. E. Wawro, C. DeBrosse, W. M. Reiff, R. C. Elder, B. Jones, J. W. Webb and C. F. Shaw III, *Inorg. Chem.*, 2010, **49**, 7663.

5 (*a*) P. T. Barnard and S. J. Berners-Price, *Coord. Chem. Rev.*, 2007, **251**, 1889; (*b*) S. J. Berners-Price, G. R. Girard, D. T. Hill, B. M. Sutton, P. S. Jarrett, L. F. Faucette, R. K. Johnson, C. K. Mirabelli and P. J. Sadler, *J. Med. Chem.*, 1990, **33**, 1386; (*c*) S. Urig, K. Fritz-Wolf, R. Réau, C. Herold-Mende, K. Tóth, E. Davioud-Charvet and K. Becker, *Angew. Chem., Int. Ed.*, 2006, **45**, 1881; (*d*) C. Santini, M. Pellei, G. Papini, B. Morresi, R. Galassi, S. Ricci, F. Tisato, M. Porchia, M. P. Rigobello, V. Gandin and C. Marzano, *J. Inorg. Biochem.*, 2011, **105**, 232; (*e*) N. Pillarsetty, K. K. Katti, T. J. Hoffman, W. A. Volkert, K. V. Katti, H. Kamei and T. Koide, *J. Med. Chem.*, 2003, **46**, 1130.

6 (*a*) M. M. Jellicoe, S. J. Nichols, B. A. Callus, M. V. Baker, P. J. Barnard, S. J. Berners-Price, J. Whelan, G. C. Yeoh and A. Filipovska, *Carcinogenesis*, 2008, **29**, 1124; (*b*) E. Schuh, C. Pflüger, A. Citta, A. Folda, M. P. Rigobello, A. Bindoli, A. Casini and F. Mohr, *J. Med. Chem.*, 2012, **55**, 5518; (*c*) L. Oehninger, R. Rubbiani and I. Ott, *Dalton Trans.*, 2013, **42**, 3269; (*d*) W. Liu and R. Gust, *Chem. Soc. Rev.*, 2013, **42**, 755; (*e*) R. Rubbiani, I. Kitanovic, H. Alborzinia, S. Can, A. Kitanovic, L. A. Onambele, M. Stefanopoulou, Y. Geldmacher, W. S. Sheldrick, G. Wolber, A. Prokop, S. Wölfl and I. Ott, *J. Med. Chem.*, 2010, **53**, 8608; (*f*) M. V. Baker, P. J. Barnard, S. J. Berners-Price, S. K. Brayshaw, J. L. Hickey, B. W. Skelton and A. H. White, *Dalton Trans.*, 2006, 3708; (*g*) A. Citta, E. Schuh, F. Mohr, A. Folda, M. L. Massimino, A. Bindoli, A. Casini and M. P. Rigobello, *Metallomics*, 2013, **5**, 1006; (*h*) F. Hackenberg, H. Muller-Bunz, R. Smith, W. Streciwilk, X. Zhu and M. Tacke, *Organometallics*, 2013, **32**, 5551; (*i*) L. Kaps, B. Biersack, H. Muller-Bunz, K. Mahal, J. Munzner, M. Tacke, T. Mueller and R. Schobert, *J. Inorg. Biochem.*, 2012, **106**, 52.

7 (*a*) G. Marcon, S. Carotti, M. Coronnello, L. Messori, E. Mini, P. Orioli, T. Mazzei, M. A. Cinellu and G. Minguetti, *J. Med. Chem.*, 2002, **45**, 1672; (*b*) C. M. Che, R. W. Y. Sun, C. B. Ko, N. Zhu and H. Sun, *Chem. Commun.*, 2003, 1718; (*c*) L. Ronconi, C. Marzano, P. Zanello, M. Corsini, C. Macca, A. Trevisan and D. Fregona, *J. Med. Chem.*, 2006, **49**, 1648; (*d*) A. Casini, C. Hartinger, C. Gabbiani, E. Mini, P. J. Dyson, B. K. Keppler and L. Messori, *J. Inorg. Biochem.*, 2008, **102**, 564; (*e*) M. Coronnello, E. Mini, B. Caciagli, M. A. Cinellu, A. Bindoli, C. Gabbiani and L. Messori, *J. Med. Chem.*, 2005, **48**, 6761; (*f*) J.-J. Zhang, W. Lu, R. W.-Y. Sun and C.-M. Che, *Angew. Chem., Int. Ed.*, 2012, **51**, 4882.

8 (*a*) A. A. Isab and P. J. Sadler, *J. Chem. Soc., Dalton Trans.*, 1982, 135; (*b*) C. F. Shaw III, M. T. Coffer, J. Klingbeil and C. K. Mirabelli, *J. Am. Chem. Soc.*, 1988, **110**, 729; (*c*) G. Lewis and C. F. Shaw III, *Inorg. Chem.*, 1986, **25**, 58; (*d*) R. C. Elder, W. B. Jones, R. Floyd, Z. Zhao, J. G. Dorsey and K. Tepperman, *Met. Based Drugs*, 1994, **1**, 363; (*e*) J. Lenke, A. Pinto, P. Nichoff, V. Vasylyeva and N. Metzler-Nolte, *Dalton Trans.*, 2009, 7063; (*f*) T. Zhou, C. T. Lum, S. S.-Y. Chui and C.-M. Che, *Angew. Chem., Int. Ed.*, 2013, **52**, 2930.

9 (*a*) J. Becher, T. Johansen and M. A. Michael, *J. Heterocycl. Chem.*, 1984, **21**, 41; (*b*) B. Blank, N. W. DiTullio, C. K. Miao, F. F. Owings, J. G. Gleason, S. T. Ross, C. E. Berkoff, H. L. Saunders, J. Delarge and C. L. Lapiere, *J. Med. Chem.*, 1974, **17**, 1065; (*c*) M. W. Whitehouse, P. D. Cookson, G. Siasios and E. R. T. Tiekink, *Met. Based Drugs*, 1998, **5**, 245.

10 *Peptides: Chemistry and Biology*, ed. N. Sewald and H. Jakubke, Wiley-VCH, Weinheim, Germany, 2002.

11 A. F. A. Peacock, G. A. Bullen, L. A. Gethings, J. P. Williams, F. H. Kriel and J. Coates, *J. Inorg. Biochem.*, 2012, **117**, 298.

12 M. N. Kouodom, L. Ronconi, M. Celegato, C. Nardon, L. Marchiò, Q. P. Dou, D. Aldinucci, F. Formaggio and D. Fregona, *J. Med. Chem.*, 2012, **55**, 2212.

13 (*a*) D. J. Ecker, J. C. Hempel, B. M. Sutton, R. Kirsch and S. T. Crooke, *Inorg. Chem.*, 1986, **25**, 3139; (*b*) S. Ahmad and A. A. Isab, *J. Inorg. Biochem.*, 2002, **88**, 44; (*c*) R. C. Elder and M. K. Eidsness, *Chem. Rev.*, 1987, **87**, 1027; (*d*) S. J. Berners-Price and P. J. Sadler, *Coord. Chem. Rev.*, 1996, **151**, 1.

14 A. Gutiérrez, J. Bernal, M. D. Villacampa, C. Cativiela, A. Laguna and M. C. Gimeno, *Inorg. Chem.*, 2013, **52**, 6473.

15 (*a*) S. Nobili, E. Mine, I. Landini, C. Gabbiani, A. Casini and L. Messori, *Med. Res. Rev.*, 2010, **30**, 550; (*b*) A. Bindoli, M. P. Rigobello, G. Scutari, C. Gabbiani, A. Casini and L. Messori, *Coord. Chem. Rev.*, 2009, **253**, 1692.

16 K. Nomiya, S. Yamamoto, R. Noguchi, H. Yokoyama, N. C. Kasuga, K. Ohyama and C. Kato, *J. Inorg. Biochem.*, 2003, **95**, 208.

17 M. C. Alley, D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czarwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Shoemaker and M. R. Boyd, *Cancer Res.*, 1988, **48**, 589.

18 (*a*) C. Carrasco-Pozo, M. E. Aliaga, C. Olea-Azar and H. Speisky, *Bioorg. Med. Chem.*, 2008, **16**, 9795; (*b*) J. C. Tsai, M. Jain, C. M. Hsieh, W. S. Lee, M. Yoshizumi, C. Patterson, M. A. Perrella, C. Cooke, H. Wang, E. Haber, R. Schlegel and M. E. Lee, *J. Biol. Chem.*, 1996, **271**, 3667; (*c*) R. Wang, Y. J. Yin, M. Li, H. M. Zhang, J. P. Zhang, S. J. Liu and W. R. Chang, *J. Biol. Chem.*, 2007, **282**, 16288.

19 O. Rackam, S. J. Nichols, P. J. Leedman, S. J. Berners-Price and A. Filipovska, *Biochem. Pharmacol.*, 2007, **74**, 992.

20 (*a*) M. J. McKeage, L. Maharaj and S. J. Berners-Price, *Coord. Chem. Rev.*, 2002, **232**, 127; (*b*) K. P. Bhabak, B. J. Bhuyan and G. Mugesh, *Dalton Trans.*, 2011, **40**, 2099.

21 A. A. Alfadda and R. M. Sallam, *J. Biomed. Biotechnol.*, 2012, **2012**, 936486.

22 R. Usón and A. Laguna, *Inorg. Synth.*, 1982, **21**, 71.

23 A. N. Nesmeyanov, E. G. Perevalova, Y. T. Struchkov, M. Y. Antipin, K. I. Grandberg and V. P. Dyadchenko, *J. Organomet. Chem.*, 1980, **201**, 343.

