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EDGE ARTICLE

A spontaneous gold(I)-azide alkyne cycloaddition reaction yields gold-peptide bioconjugates which overcome cisplatin resistance in a p53-mutant cancer cell line†

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Solid-phase peptide synthesis (SPPS) is a versatile technique for the assembly of small to medium size peptides, that can help in the delivery of bound metal complexes to certain cellular compartments, for example in cancer cells. This work shows a new route to gold-peptide bioconjugates *via* a non-catalyzed [3 + 2] cycloaddition reaction of gold azides with alkynyl peptides. Gold(I) tetrapeptide conjugates with a mitochondria-targeting sequence were synthesized and display prolonged stability in the presence of thiol-containing biological media. Their antiproliferative potency against selected cancer cells (2–50 μM) corresponds to the lipophilicity of the conjugates. The cellular uptake of Au, determined by atomic absorption spectroscopy (AAS), shows that high initial uptake equals strong cytotoxicity. Respiration and acidification rates react immediately upon treatment with the Au-peptide conjugates, and a terminal breakdown of essential cellular functions is complete within *ca.* 12 h at most, as observed by online monitoring of the cancer cell metabolism in a microfluidic biosensor device (Bionas sensorchip system). The mode of action of these Au-peptide bioconjugates was elucidated by a variety of biochemical and cell biological experiments. First, a strong selective inhibition of the enzyme thioredoxin reductase (TrxR), a regulator of cellular redox processes, was found. In this context, elevated levels of reactive oxygen species (ROS) and strong effects on the respiration of isolated mouse liver mitochondria were found. These finally lead to cell death *via* apoptotic pathways, as indicated by flow cytometry, low mitochondrial membrane potential (MMP) and DNA fragmentation. Intriguingly, cisplatin-resistance in p53-mutant MDA-MB231 breast cancer cells could be overcome by the Au-peptide conjugates presented herein.

Introduction

Cancer is a leading cause of death worldwide. According to the World Health Organization (WHO) it accounts for 7.9 million deaths (or 13% of all deaths) in 2007.

Although in the last decades a lot of progress has been made in understanding the cause of cancer on the cellular and molecular level, most of the treatments in clinical use target the whole organism and can cause severe side-effects. For example, nephrotoxicity (kidney damage) is one of the major issues in cancer treatment with cisplatin.[1] Since the discovery of antiproliferative effects of Auranofin, an established clinical drug for the treatment of rheumatoid arthritis with relatively mild side effects,[2] a huge variety of Au(I) complexes derived from Auranofin has been synthesized and tested for anticancer activity. Although many of these compounds showed promising results against a panel of cancer cell lines *in vitro*, their *in vivo* efficacy was limited.[3] This can be at least partially attributed to rapid ligand exchange reactions of the thiolate and chloride ligands *in vivo*.[3,4] As an example for improved kinetic stability, Berners-Price *et al.* recently introduced cationic gold(I) N-heterocyclic carbene complexes that accumulate in mitochondria and exhibit anti-mitochondrial activity by selectively inducing apoptosis in malignant over normal breast cancer cells. These compounds displayed high kinetic stability in the presence of thiols.[5]

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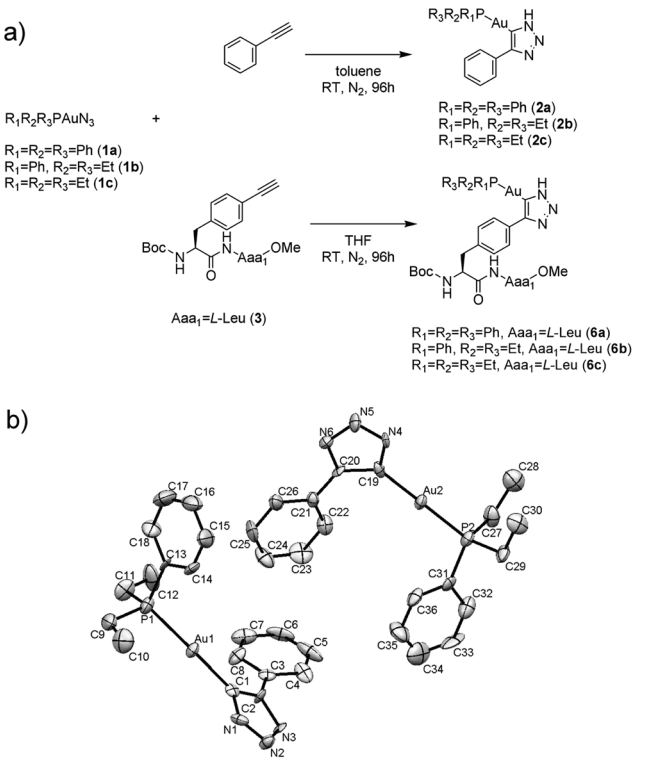
† Electronic supplementary information (ESI) available: HPLC data for **8a–c**, **9**; full experimental procedures for ^{31}P NMR stability measurements and biological *in vitro* studies. CCDC reference number 865056 (for **2b**). For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2sc01127a

Recently, cytotoxic gold(I) thiourea complexes acting as very potent TrxR inhibitors were reported.[6] However, by their molecular architecture they do not offer possibilities for rational targeting in cancer cells. Quite surprisingly, despite the vast number of gold(I) complexes examined for their activity in biological systems,[7–9] only a few examples of gold complexes bound to biomolecules such as amino acids and peptides exist.[10,11] One of the main challenges in the synthesis of metal bioconjugates is the chemoselective insertion of the metal complex into the biomolecule. This can be achieved by employing bioorthogonal reaction schemes both in solution and on the solid phase, as shown by recent examples for metal peptide conjugates.[12–15] Bioorthogonal reactions have become very popular over the last decade due to their high chemoselectivity and recent reviews highlight their importance in the modification of biomolecules such as peptides, proteins, oligonucleotides and DNA.[16,17] The power of this approach has been elegantly demonstrated by Bertozzi *et al.* through *in vivo* [3 + 2] cycloadditions and Staudinger ligations.[18] In a recent study, gold(III) complexes were also used as catalysts for the bifunctional modification of oligosaccharides.[19] Based on the considerations on activity and stability mentioned above, we synthesized new organometallic (phosphine)gold(I) peptide conjugates by a [3 + 2] cycloaddition reaction,[20,21] resulting in a kinetically more stable yet biologically active class of (phosphine)gold(I) bioconjugates. The selected peptide sequences are essentially based on the tetrapeptides used in the groups of Szeto[22] and Kelley,[23] which were shown to efficiently cross cell membranes and accumulate in the mitochondria of live Caco-2 cells as well as of live HeLa and MCF-7 cancer cells, respectively.



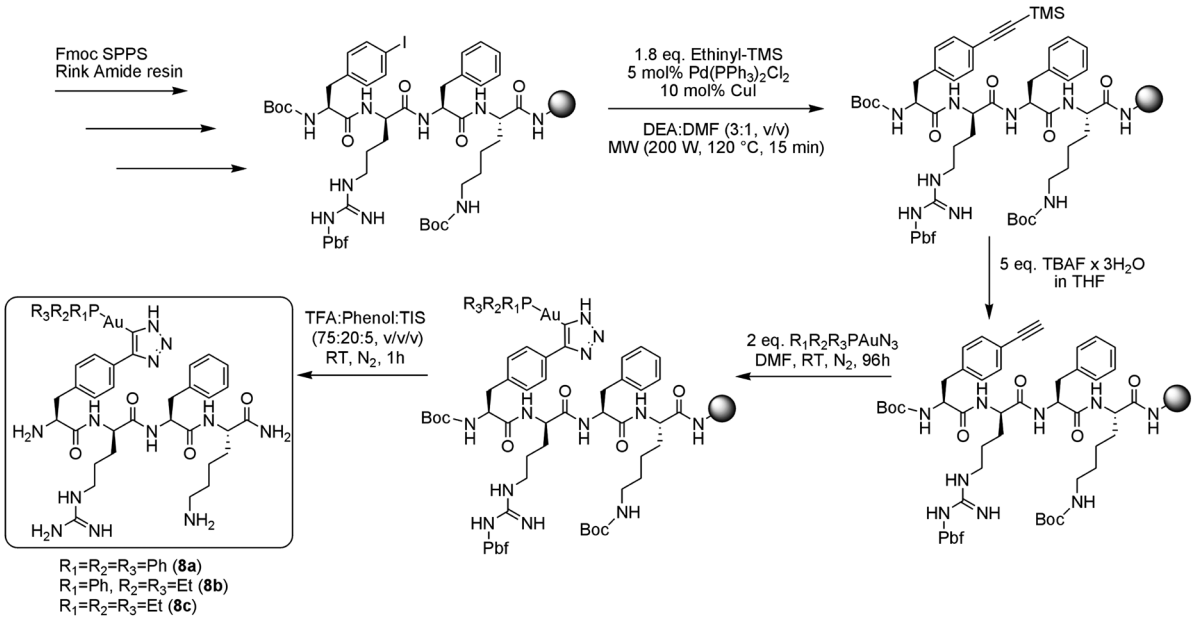
Scheme 1 (a) Synthesis of (phosphine)gold(I) phenyl triazoles **2a–c** and dipeptide triazoles **6a–c** in solution. (b) ORTEP view of **2b**. Thermal ellipsoids drawn at the 50% probability level. Hydrogen atoms are omitted for clarity. Selected bond lengths (Å) and angles (°) (two independent molecules): Au1–C1 2.016(2), Au2–C19 2.022(1), Au1–P1 2.280(4), Au2–P2 2.287(5); C1–Au1–P1 179.20(4), C19–Au2–P2 178.92(4).

Results and discussion

The (phosphine)gold(I) azides **1a**, **1b** and **1c** were synthesized following a published procedure.[21] The [3 + 2] cycloaddition of the three (phosphine)gold(I) azides with phenylacetylene was straightforward in all cases and yielded the expected derivatives **2a** (72%, lit. 78%[21]), **2b** (35%) and **2c** (45%) (Scheme 1a). The ^{1}H and $^{31}P\{^{1}H\}$ NMR spectra show distinct characteristics of this class of compounds. In coordinating H-bond acceptor solvents such as dimethyl sulfoxide or acetone the resonance of the triazole NH proton is clearly visible around 14 ppm.[24] The ^{31}P nucleus of **1b** resonates at 34.4 ppm in DMSO-d^6, while the signal is shifted downfield to 44.8 ppm in **2b**. X-Ray diffraction analysis of crystals obtained for the complete series of (phosphine)gold(I) phenyl triazoles corroborate the successful conversion to the desired complexes. As a representative example, the X-ray single-crystal structure of **2b** is shown, confirming the linear P–Au–C binding mode (Scheme 1b).

To investigate the scope of the reaction for bioconjugation purposes, N- and C-terminally protected dipeptides containing L-leucine and L-histidine were reacted with the (phosphine)gold(I) azides in solution. While the L-leucine derivatives **6a–c** (Scheme 1a) could be isolated in good yields (62–68%) after column chromatography on silica, reactions with the dipeptide containing L-histidine resulted in complex product mixtures. Based on these results, the synthesis of oligopeptide conjugates containing amino acids with unprotected soft donor functional groups (*i.e.* Arg, Lys, His) was considered unfavourable in solution. This challenge was met by incorporating the [3 + 2] cycloaddition into the Fmoc SPPS strategy, thus demonstrating a new and facile approach to gold(I) peptide bioconjugates assembled entirely on solid support.

The peptide sequence was assembled on a Rink Amide resin by standard solid-phase peptide synthesis. The iodophenylalanine-containing peptide was then placed in a microwave reactor and transformed into the alkyne by Pd-catalyzed Sonogashira coupling with an excess of ethinyl-TMS in a DEA–DMF mixture.[25] Following the on-resin TMS deprotection with TBAF,[26] the alkyne modified resin-bound peptide was reacted with an excess of (phosphine)gold(I) azide in DMF under an atmosphere of nitrogen for 96 h. Cleavage from the resin and isolation by precipitation yielded the fully deprotected crude peptide. After purification by reversed-phase column chromatography **8a–c** were isolated in 16–18% yield (Scheme 2). The ESI-MS spectra (positive mode) show the $[M + H]^+$, the $[M + 2H]^{2+}$ and the $[M + 3H]^{3+}$ ions as the main peaks in accordance with the three protonable amine functionalities in the peptide. HPLC and NMR analysis of the gold tetrapeptides and the gold-free reference peptide **9** show >95% purity (see Fig. S1, ESI†). ^{31}P NMR spectroscopy offers a valuable tool for the investigation of the stability of (phosphine)gold(I) complexes in solution[27] The compounds under study exhibit only one phosphorus resonance in various NMR solvents (including methylene chloride-d^2, acetone-d^6 and DMSO-d^6) and show no signs of degradation



Scheme 2 Fmoc solid-phase peptide synthesis of (phosphine)gold(I) tetrapeptide triazoles **8a–c**. Cleavage was effected with TFA–phenol–TIS (75 : 20 : 5) for 1 h in a Schlenk flask under nitrogen. Phenol was added to avoid oxidation of the gold(I) centre under the strongly acidic conditions.

for up to 72 h. Time-dependent $^{31}P\{^{1}H\}$ NMR spectroscopy of **7b** in a 1 : 1 (v/v) mixture of DMSO-d^6 and DMEM (containing 10% v/v FCS) was used to obtain information on complex stability in cell culture medium (see Fig. S2, ESI†).

Furthermore, the reactivity towards thiols was investigated by monitoring the reaction of an equimolar mixture of **6b** and cysteine under inert conditions in aqueous solution.[5] In order to obtain quantitative data of the decomposition of **6b**, the respective (phosphine)gold(I) chloride served as an internal reference, using a double-walled NMR tube (see Fig. S3, ESI†). Both experiments show that the (phosphine)gold(I) conjugate **6b** gradually decomposes in the presence of thiol-containing biomolecules. For the second experiment, the data could be fitted to an exponential decay curve ($R^2 = 0.998$). As a result, the rate law follows first-order reaction kinetics and the rate constant $k = 4.31 \times 10^{-2}\ M^{-1}\ s^{-1}$ was deduced, from which a half-life $t_{1/2} = 16.1$ h could be calculated (see Fig. S4, ESI†). Consequently, the kinetics of the decomposition should be slow enough to ensure a sufficiently high concentration of intact compound within the cell culture medium for the duration of cell uptake. In contrast to kinetically less stable chloride- or thiol-substituted auranofin analogues,[28] these new gold conjugates are thus amenable to detailed structure–activity relationship studies.

Besides the important issue of compound stability under physiological conditions, lipophilicity plays a key role in biodistribution and activity of drug candidates.[29] In this regard, cell uptake of small, often neutral, molecules by passive diffusion is of particular interest, as their membrane penetrability is facilitated by higher lipophilicity.[30] The *n*-octanol/PBS (pH 7.4) partition coefficients log $D_{7.4}$ for all (phosphine)gold(I) peptide conjugates were determined by the "shake-flask" method to assess their bioavailability (Table 1).[31] Compared to the established anticancer drug cisplatin all conjugates are considerably more lipophilic. The Au-dipeptide conjugates are most lipophilic, while the tetrapeptide conjugates' lipophilicity is more or less comparable to the gold phosphine lead compound auranofin. Effects of the (phosphine)gold(I) peptide conjugates on cell growth and viability of MCF-7 (human breast adenocarcinoma) and HT-29 (colon carcinoma) cell lines as well as of normal human skin cell fibroblasts (GM5756) were investigated using the crystal violet (CV) assay.[41] IC_{50} values between 2.2 and 49.5 μM were obtained for the malignant cells, which are comparable to the established clinical drugs cisplatin and auranofin (Table 1).

At first glance, the more hydrophilic dipeptide conjugates tend to be less cytotoxic than the more lipophilic dipeptide conjugates, while the hydrophilic tetrapeptide conjugates are least cytotoxic. Yet, there are exceptions to this trend, such as the comparatively low cytotoxicity of **6a** and **7c** in HT-29 cells. In general, MCF-7 cells are more sensitive than HT-29 cells, which is mirrored by the lower IC_{50} values found in the proliferation assays (Table 1). The gold-free reference peptide **9** is non-toxic on both cancer cell lines, which is in accordance with earlier reports on this class of peptides.[42] IC_{50} values for the human skin cell fibroblasts were determined for **6a** (5.17 ± 0.2 μM), **6b** (15.76 ± 0.09 μM) and **6c** (6.09 ± 0.25 μM). The IC_{50} value of **6b** is about 7-fold higher for the normal skin cells compared to MCF-7 cells, indicating a modest selectivity of the tested compounds for cancer cells over normal fibroblast cells. Cellular uptake into HT-29 cells was studied by electrothermal atomic absorption spectroscopy (ETAAS), which represents a highly selective and sensitive analytical method for the detection and quantification of metal compounds in biological matrices.[38,43] Drug concentrations of 20 μM were used for the uptake experiments and cells were harvested, lysed and measured after 2 h of incubation with the gold-peptide conjugates. After protein quantification using the Bradford assay,[44] the amount of gold in the cellular lysates was calculated as ng Au/mg protein (Fig. 1a).

The dipeptide **6c** displays the highest accumulation, followed by **6a** with less than half the gold content. Finally, the gold

Table 1 Lipophilicity, antiproliferative effects and enzyme inhibition. Values are reported as means (±SE, $n \geq 2$)

Compound	log $D_{7.4}$	IC_{50}/μM (HT-29)	IC_{50}/μM (MCF-7)	EC_{50}/μM (TrxR)	EC_{50}/μM (GR)
Ph_3PAuCl	n.d.	4.2 ± 0.9[a]	2.6 ± 0.1[a]	0.256 ± 0.002[f]	4.16 ± 0.7[f]
$Et_2PhPAuCl$	n.d.	7.9 ± 2.2	6.9 ± 2.0	0.57 ± 0.03	13.3 ± 3.1
Et_3PAuCl	n.d.	5.3 ± 1.9[a]	3.2 ± 1.3[a]	0.037 ± 0.005[f]	7.9 ± 0.4[f]
6a	1.53 ± 0.08	29.8 ± 3.7	2.24 ± 0.04	0.583 ± 0.103	>50
6b	1.6 ± 0.25	6.3 ± 0.08	2.2 ± 0.26	0.279 ± 0.002	>50
6c	1.2 ± 0.05	4.85 ± 1.0	2.35 ± 0.49	0.181 ± 0.056	>50
8a	−0.08 ± 0.02	23.0 ± 3.4	12.9 ± 0.4	0.44 ± 0.07	11.6 ± 2.5
8b	−0.74 ± 0.06	32.0 ± 14.0	12.4 ± 2.9	0.021 ± 0.008	0.465 ± 0.002
8c	−0.99 ± 0.1	49.5 ± 12.0	12.2 ± 6.2	4.1 ± 0.08	21.1 ± 3.3
5	1.17 ± 0.04	82.7 ± 11.6	62.9 ± 0.8	>50	>50
9	n.d.	>400	>400	n.d.	n.d.
Auranofin	−0.53[i]	2.6 ± 0.4[g]	1.1 ± 0.3[h]	0.009 ± 0.0[f]	15 ± 0.1[f]
$[Au(dppe)_2]Cl$	1.41[c]	<0.1	<0.1	—	—
Cisplatin	−2.53 ± 0.28[e]	7.0 ± 2.0[b]	2.0 ± 0.3[b]	~15[d]	—

[a] Ref. 32. [b] Ref. 33. [c] Ref. 34. [d] Ref. 35. [e] Ref. 36. [f] Ref. 37. [g] Ref. 38. [h] Ref. 39. [i] Ref. 40.

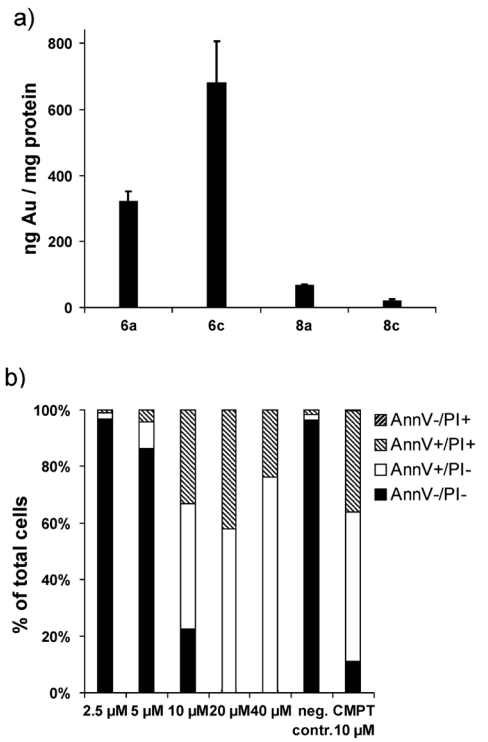


Fig. 1 (a) Cellular uptake of 20 μM (phosphine)gold(I) dipeptides **6a**, **6c** and tetrapeptides **8a**, **8c** in HT-29 cells after 2 h of incubation at 37 °C/5% CO_2. Values are means (±SE) of two independent experiments. (b) Annexin V/PI staining of Jurkat cells incubated with **8c** for 48 h. The assay discriminates between viable cells (Ann V−/PI−, "neg. control"), early apoptotic (Ann V+/PI−), late apoptotic (Ann V+/PI+) and necrotic cells (Ann V−/PI+). CMPT is included as a positive control.

tetrapeptides **8a** and to an even lesser extent **8c**, were enriched only by a fractional amount compared to the dipeptides. Taking into account a mean cellular volume, diameter and protein content of HT-29 cells, the cellular molar gold concentrations can be estimated.[38] For the dipeptides **6a** and **6c**, the ng Au/mg protein values correspond to 320 and 680 μM respectively, for the tetrapeptides **8a** and **8c** the values are considerably lower with 68 and 21 μM. In other words, the cellular gold concentrations were 16- to 34-fold higher than the concentrations used for incubation in the case of the dipeptides, while they were only marginally higher for the tetrapeptides (3.4- and 1.1-fold). Two conclusions can be drawn from these results. First, the rapid uptake of the gold dipeptides, most likely governed by a passive diffusion mechanism, seems to be greatly facilitated by their lipophilic character (*vide supra*), leading to a significantly higher accumulation than observed for the respective (phosphine) gold(I) chlorides (3- to 8-fold).[45] The uptake of the gold tetrapeptides is much lower, which can be explained by a hampered passive diffusion due to a higher hydrophilicity of the compounds, a different uptake mechanism,[46] or a combination of both. Also, it has been suggested for this class of small peptides to freely cross the cell membrane by direct potential-driven diffusion.[22,23] Second, the IC_{50} values obtained for the four gold peptide conjugates can be correlated to their cellular uptake. Most prominently, the much stronger uptake of **6c**, compared to the other peptide conjugates, is reflected by its more potent cytotoxic effects (see also Table 1).

To gain more insight into the possible mode(s) of action of the gold peptide conjugates, the Bionas sensor chip system, was employed for the continuous online measurement of essential cellular parameters, such as respiration rate, extracellular acidification rate and cell impedance.[33,37] The respiration rate is a direct measure of ATP production within functional mitochondria, while the acidification rate mainly indicates the presence of lactic acid, a product from the anaerobic oxidation of NADH to NAD^+ during glycolysis. Cell impedance renders information on changes of the cell morphology, *i.e.* membrane reorganization and cellular adhesion.

Immediate effects on respiration rate and acidification rate of MCF-7 cells become obvious after incubation with 20 μM Et_3PAuCl, **6c** and **8c**, while changes in cellular impedance set in only a few hours post administration (Fig. 2a–c). Complete breakdown of all cellular function measured by the Bionas system is complete within 5 h (Et_3PAuCl and **6c**) and 13 h (**8c**). The gold chloride Et_3PAuCl produces a rapid and complete breakdown of essential cellular functions, with no detectable recovery of the cells after removal of the compound, which points to an unspecific toxicity of Et_3PAuCl. Similar results were found in related studies on the acute cytotoxicity of Et_3PAuCl on

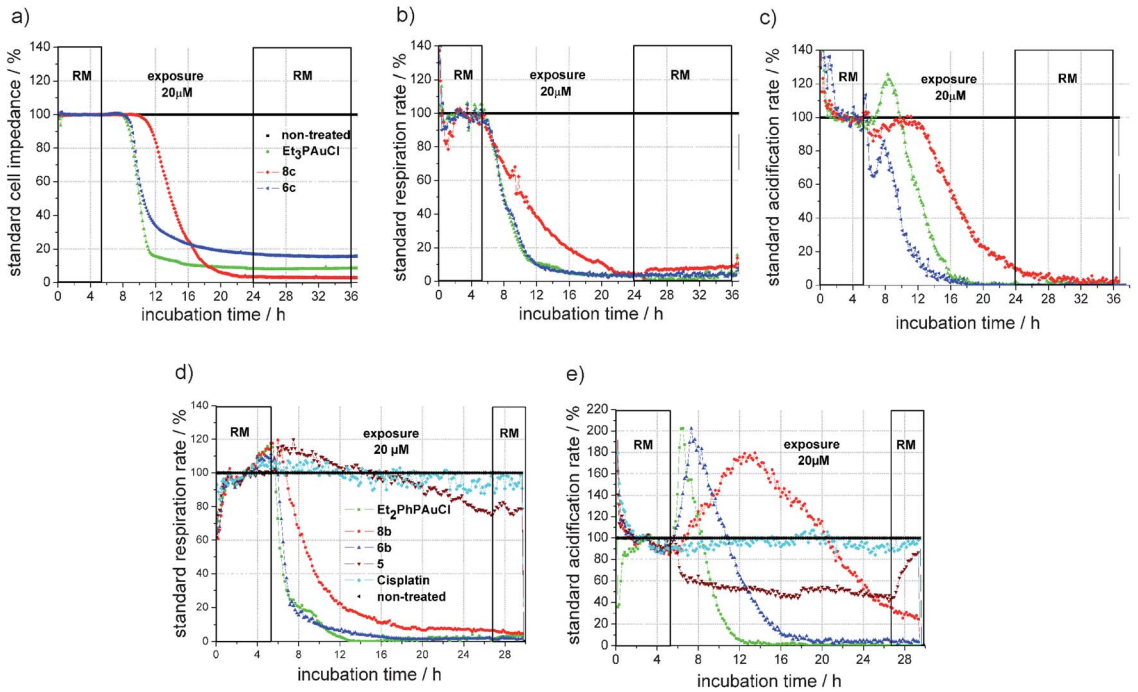


Fig. 2 (a) Standard cell impedance, (b) standard acidification rate and (c) standard respiration rate of MCF-7 cells incubated with 20 μM Et_3PAuCl (green line), **6c** (blue line) and **8c** (red line). "RM" ("running medium") denotes compound free cell culture medium. "Exposure/20 μM" indicates the window of incubation with the respective compound and concentration. (d) Standard respiration rate and (e) standard acidification rate of p53-mutant MDA-MB231 cells incubated with 20 μM $Et_2PhPAuCl$ (green line), **6b** (blue line), **8b** (red line), **5** (brown line) and cisplatin (turquoise line).

isolated rat hepatocytes, suggesting the primary involvement of mitochondria with a complex interplay of effects, including electron transport chain inhibition, mitochondrial swelling and dissipation of the mitochondrial inner membrane potential.[47] Although the cell damage evoked by **6c** and **8c** is also irreversible, the decline in cellular respiration and acidification is more gradual, especially for **8c**. In comparison, the triazole dipeptide **5** has a much less pronounced impact on the cell parameters over all, with a stabilized respiration and an almost complete recovery of the acidification rate after compound removal (see Fig. S5, ESI†). Additionally, the effect of the gold-peptide conjugates on p53-mutant MDA-MB231 breast cancer cells was evaluated. Besides high aggressiveness and poor prognosis, mutant-p53 expressing tumours also display increased resistance against various anticancer treatments.[48] Accordingly, incubation of p53-mutant MDA-MB231 cells with 20 μM cisplatin did not significantly alter the respiration and acidification rate compared to the non-treated control (Fig. 2d–e). In contrast, exposure to 20 μM **6b** and **8b** strongly affects both cellular parameters. The standard respiration rate drops to 20% within just 3–7 h after the addition of **6b**, **8b** and $Et_2PhPAuCl$. The strong decrease in cellular respiration is mirrored by an initially steep increase of the standard acidification rate for **6b**, **8b** and $Et_2PhPAuCl$, which can be explained by an enhanced compensatory glycolysis. Ultimately, as the cells perish, all parameters irreversibly drop to basal levels. Again the gold-free dipeptide **5** has almost no effect on cell respiration and reversibly decreases the acidification rate to about 50–60% only during compound administration. The results show that the (phosphine)gold(I) conjugates are able to break the resistance of the p53-mutant MDA-MB231 cells against cisplatin, which also points to a different mode of action for these gold drugs compared to the former.

The Annexin V/propidium iodide assay,[49] which can be used to discriminate between healthy, necrotic and apoptotic cells, was performed on Jurkat T leukemia cells. After an exposure time of 48 h, the impact of **8c** at concentrations smaller or equal to 2.5 μM are comparable to the untreated control (Fig. 1b). A significant increase in early apoptotic cells (white bar, AnnV+/PI−) is apparent at a sub-toxic concentration of 5 μM and at 10 μM the effect is at level with the commercial cytostatic Irinotecan (Campto®, CMPT). No healthy cells remain at concentrations >20 μM. Similar results were found for **8a** and **8b**. Additionally, a strong concentration-dependent DNA fragmentation in BJAB lymphoma cells after incubation with **6a–c** for 72 h was found, which is an indicator of the late stages of apoptosis (see Fig. S6, ESI†).[50] Consequently, it can be concluded that apoptotic cell death pathways play an important role in the mode of action of these gold peptide conjugates. A highly important regulatory cellular function such as apoptosis can be triggered by a multitude of events and connected signalling pathways.[51] Although DNA damage can be a starting point, mitochondria play a pivotal role in the regulation of apoptosis.[52] Based on the huge structural diversity of biologically active gold complexes, one single intracellular target seems to be very unlikely.[4]

Although it has been shown that auranofin and analogous compounds do not bind to DNA,[53] this "usual suspect" for metal complex binding cannot be precluded as a possible target.[54] In order to evaluate their reactivity towards dsDNA, the gold dipeptide conjugates **6a**, **6b** and **6c**, together with cisplatin as

a positive control, were incubated at 25 μM concentrations with a 100 bp DNA ladder (50 μM bp^{-1}) at 37 °C for 4 h, followed by agarose gel electrophoresis (Fig. 3b).[55] While the gel mobility of the DNA ladder is strongly influenced by the interactions with cisplatin (lane 1), no significant changes can be detected for the three (phosphine)gold(I) dipeptides **6a,b,c** (lanes 2, 3 and 4), the tetrapeptide conjugate **8b** (data not shown) and for the vehicle control (lane 5), compared to DNA alone (lane 6). From these results, DNA can be excluded as a relevant target for these gold peptide conjugates.

Recently, it has been proposed that inhibition of thioredoxin reductase (TrxR), an important enzyme within the cellular and mitochondrial redox system, is the most relevant factor for the antiproliferative effects displayed by gold compounds.[56] In this picture, impairment of mitochondrial function through TrxR inhibition would finally lead to cell death *via* apoptosis. The effect on TrxR and a second thiol-containing enzyme, namely glutathione reductase (GR), was studied on the isolated enzymes following a published procedure.[32] All compounds show strong inhibitory effects on TrxR (EC_{50}: 0.021–4.1 μM), while the values for GR (EC_{50}: 0.465–21.1 μM) are at least one order of magnitude lower, indicating a certain selectivity of enzyme inhibition (Table 1).[53] The elevated generation of reactive oxygen species (ROS), produced by cellular respiration in mitochondria, indicates an imbalance of normal cell metabolism. A dysregulation of mitochondrial function for instance by TrxR inhibition would lead to increased ROS levels, inducing oxidative stress within the cell, which ultimately leads to apoptosis.[57] Indeed, ROS levels were significantly increased in a concentration-dependent manner in Jurkat cells incubated with **8b** for 48 h at concentrations well below the IC_{50} value (Fig. 3a), correlating with TrxR inhibition and induction of apoptosis by these compounds. Thus, the (phosphine)gold(I) conjugates mediate a cellular response that is opposite to the cyto-protective, antioxidant action of a very similar tetrapeptide incorporating tyrosine or dimethyltyrosine (Dmt) within the sequence.[42] The involvement of mitochondria in the onset of apoptosis was further supported by the dose-dependent breakdown of the mitochondrial membrane potential $\Delta\Psi_m$ in BJAB lymphoma cells incubated with **6a** for 48 h (Fig. 4a). Flow cytometry revealed that up to 80% of the cells had a low membrane potential at drug concentrations as low as 2.5 μM, opening up the so-called mitochondrial permeability transition (MPT) pore. This leads to swelling of the matrix, followed by outer membrane rupture and release of cytochrome c, which in turn activates the apoptotic signalling cascade.[52] Finally, the oxygen consumption of freshly isolated mouse liver mitochondria during incubation with the gold peptide conjugates was monitored to further support the theory of mitochondrial involvement in the observed biological activities. In this assay, oxygen saturation is decreased by functional, respirating mitochondria (Fig. 4b, control).

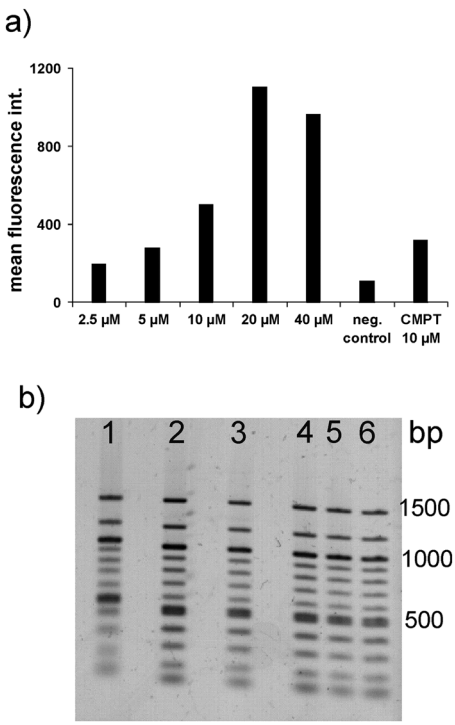


Fig. 3 (a) Concentration-dependent ROS formation in Jurkat cells incubated with **8b** for 48 h. CMPT is used as a positive control. (b) Gel electrophoresis of a 100 bp DNA ladder (50 μM bp^{-1}) on a 1% (w/v) agarose gel after 4 h of incubation with the following substances ($r = 0.5$; r = (metal complex) : (DNA base pairs)); lane 1: DNA + 25 μM cisplatin, 2: DNA + 25 μM **6c**, 3: DNA + 25 μM **6b**, 4: DNA + 25 μM **6a**, 5: DNA + 0.5% DMSO, 6: DNA only.

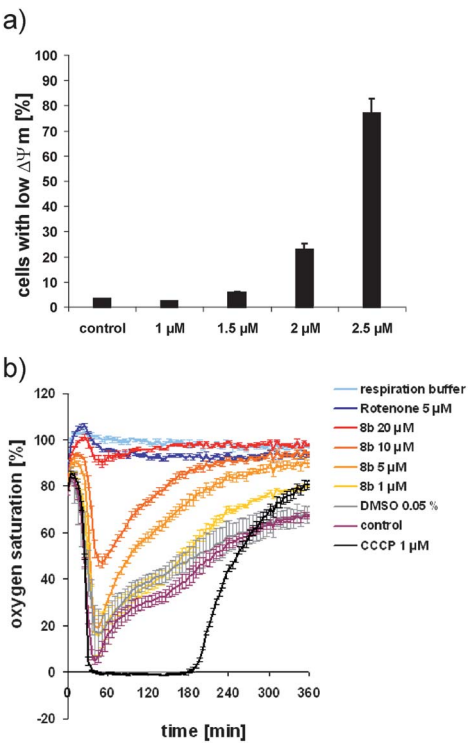


Fig. 4 (a) Mitochondrial membrane potential $\Delta\Psi_m$ of BJAB lymphoma cells measured by flow cytometry (JC-1 assay) after 48 h of incubation with **6a**. Values are means (±SE) of three independent experiments. (b) Oxygen saturation in isolated mouse liver mitochondria during the incubation with **8b**; rotenone (positive control for the inhibition of mitochondrial respiration), CCCP (positive control for enhanced mitochondrial respiration), control (untreated mitochondria), respiration buffer (no mitochondria).

Addition of rotenone, a known inhibitor of respiratory chain complex I, results in a constantly high level of oxygen saturation. Compound **8b** (Fig. 4b), as well as **8c**, **6b,c** and to a lesser extent **6a** and **8a** (data not shown), exhibit a concentration dependent reduction of mitochondrial respiration. At a concentration of 20 μM of **8b**, an almost complete shutdown of mitochondrial activity comparable to the positive control rotenone can be observed.

Conclusions

This work describes in detail the changes in cellular functions upon treatment of cancer cell lines with Au(I)-peptide bioconjugates, giving a very complete picture of the mode of action of these conjugates. The straight-forward synthesis *via* a spontaneous cycloaddition reaction opens the road to conjugates that will specifically target malignant cells, *e.g.* by overexpression of certain membrane receptors. As demonstrated for a p53-mutant cell line, resistance to other anticancer drugs may be overcome by the gold-based drug candidates presented in this work, making them very promising next-generation drug candidates.

Experimental

General remarks

Reactions were carried out under positive nitrogen pressure using standard Schlenk techniques unless stated otherwise. The (phosphine)gold(I) chlorides, **1a**, **2a**, Boc-*p*-I-phenylalanine and Boc-*p*-eth-phenylalanine were prepared following published procedures,[21,58] all other chemicals were obtained commercially and used without further purification. Enantiomerically pure amino acids and the Rink amide resin were purchased from Iris Biotech (Marktredwitz, Germany) or Novabiochem (Laufelfingen, Switzerland). Peptide-grade DMF was obtained from Roth (Karlsruhe). Elemental analyses were performed on a vario EL from Elementar Hanau in C, H, N mode. ^{1}H, ^{13}C and ^{31}P{^{1}H} NMR spectra were recorded at room temperature on a Bruker DRX 400 (400 MHz), DPX 250 (250 MHz) or DPX 200 (200 MHz) spectrometer. Chemical shifts, δ, are given in ppm relative to TMS and are referenced by using the residual undeuterated solvent signal. Coupling constants, J, are reported in Hz, multiplicities being marked as: singlet (s), doublet (d), triplet (t) or multiplet (m). IR spectra were measured as solid samples on a Bruker Tensor 27 with an ATR unit. Absorption bands are given in cm^{-1}, their intensities indicated as strong (s), medium (m) and weak (w). FAB-MS spectra were measured on a VG autospec, ESI-MS spectra on a Bruker Esquire 6000. The mass to charge relation (m/z) is given as a dimensionless number. LC-MS analyses were carried out on an Agilent 1100 instrument coupled to the Bruker Esquire 6000. An Agilent Zorbax SB-C18 column was used (2.1 × 50 mm, 1.8 μm) with Millipore^{R} water (supplemented with 0.05% TFA) and acetonitrile (Baker, HPLC grade) as eluents. Routinely, the gradient was 0–100% acetonitrile in 10 min with a flow rate of 0.3 ml min^{-1} with detection at $\lambda = 220$ or 254 nm. HPLC was performed on a Knauer smartline with a 4-wavelength detector and a Varian Dynamax Microsorb C-18 reversed-phase column (250 × 10 mm) for semi-preparative runs. Mixtures of Millipore^{R} water and acetonitrile with 0.1% TFA (v/v) were used as eluents at a flow rate of 4–5 ml min^{-1}. All chromatograms were recorded at $\lambda = 220$ and 254 nm.

CAUTION! Be aware that heavy-metal azides are potentially explosive when exposed to heat or shock! Direct contact with acids can cause release of toxic hydrazoic acid! Even though no such incident occurred in the present study, metal azides should be handled in small amounts only and with utmost care, and all necessary safety precautions should be taken.

Syntheses

The synthesis of the (phosphine)gold(I) azides **1b** and **1c** was carried out according to the literature procedure for **1a**.[21]

(Diethylphenylphosphine)gold(I) azide (1b)

Light orange oil (780 mg, 96%). $C_{10}H_{15}AuN_3P$ (405.1 g mol^{-1}). MS (FAB^{+}): m/z 428.0 [M + Na]$^{+}$, 529.1 [$(Et_2PhP)_2Au$]$^{+}$, 363.0 [Et_2PhPAu]$^{+}$. ^{1}H NMR (CD_2Cl_2, 250 MHz): δ 7.77–7.68 (m, 2H, H_{Ar,PPh_3}), 7.58–7.48 (m, 3H, H_{Ar,PPh_3}), 2.10 (dq, 4H, $^{3}J = 7.5$ Hz, $CH_{2,Et}$), 1.12 (dt, 6H, $^{3}J = 7.5$ Hz, $CH_{3,Et}$). ^{31}P{^{1}H} NMR (DMSO-d^{6}, 101 MHz): δ 34.4. ATR-IR (solid, cm^{-1}): 3053 (w), 2966 (w), 2930 (w), 2874 (w), 2042 (s, $\nu_{as}(N_3)$), 1453 (m), 1435 (m), 1412 (m), 1379 (m), 1328 (m), 1108 (m), 1044 (m), 1015 (m), 767 (m), 751 (m), 723 (m), 692 (s).

(Triethylphosphine)gold(I) azide (1c)

Colourless solid (522 mg, 98%). $C_6H_{15}AuN_3P$ (357.1 g mol^{-1}). MS (FAB^{+}): m/z 358.1 [M + H]$^{+}$, 433.2 [$(Et_3P)_2Au$]$^{+}$, 315.1 [Et_3PAu]$^{+}$. ^{1}H NMR (CD_2Cl_2, 250 MHz): δ 1.38 (dq, 6H, $^{3}J = 7.5$ Hz, $CH_{2,Et}$), 1.18 (dt, 9H, $^{3}J = 7.5$ Hz, $CH_{3,Et}$). ^{31}P{^{1}H} NMR (DMSO-d^{6}, 101 MHz): δ 31.0. ATR-IR (solid, cm^{-1}): 2969 (w), 2937 (w), 2880 (w), 2053 (s, $\nu_{as}(N_3)$), 1447 (m), 1409 (m), 1333 (m), 1044 (m), 1012 (w), 763 (s), 737 (s), 711 (m), 642 (m).

The synthesis of the (phosphine)gold(I) phenyl triazoles **2b** and **2c** was carried out according to the procedure for **2a**.[21]

Diethylphenylphosphine(4-phenyl-1*H*,2,3-triazolyl)gold(I) (2b)

Colourless solid (11 mg, 35%). Anal. Calc. for $C_{18}H_{21}AuN_3P$ (507.1 g mol^{-1}): C, 42.61; H, 4.17; N, 8.28. Found: C, 43.08; H, 4.17; N, 8.28%. MS (FAB^{+}): m/z 507.9 [M + H]$^{+}$, 529.1 [$(Et_2PhP)_2Au$]$^{+}$, 478.8 [M − Et + H]$^{+}$, 362.8 [Et_2PhPAu]$^{+}$. ^{1}H NMR (DMSO-d^{6}, 400 MHz): δ 14.21 (s, 1H, $NH_{Triazole}$), 8.22 (pseudo-d, 2H, $H_{o,Phe}$, $^{3}J = 8.0$ Hz), 7.95–7.89 (m, 2H, H_{Ar,PPh_3}), 7.61–7.59 (m, 3H, H_{Ar,PPh_3}), 7.34 (pseudo-t, 2H, $H_{m,Phe}$, $^{3}J = 8.0$ Hz), 7.22 (pseudo-t, 1H, $H_{p,Phe}$, $^{3}J = 8.0$ Hz), 2.35–2.27 (m, 4H, $CH_{2,Et}$), 1.17–1.04 (m, 6H, $CH_{3,Et}$). ^{13}C{^{1}H} NMR (DMSO-d^{6}, 100.6 MHz): δ 162.1 ($C_{q,triazole}$, $^{2}J_{C,P} = 119.7$ Hz), 152.3 ($C_{q,triazol}$, $^{3}J_{C,P} = 7.0$ Hz), 135.1 ($C_{i,Ph}$), 133.4 ($C_{o,\ PPh_3}$, $^{2}J_{C,P} = 12.1$ Hz), 131.7 (C_{p,PPh_3}, $^{4}J_{C,P} = 1.0$ Hz), 129.9 (C_{i,PPh_3}, $^{1}J_{C,P} = 49.3$ Hz), 129.1 (C_{m,PPh_3}, $^{3}J_{C,P} = 10.1$ Hz), 128.2 ($C_{m,Ph}$), 126.2 ($C_{p,Ph}$), 125.5 ($C_{o,Ph}$), 19.4 ($C_{CH_2,Et}$, $^{1}J_{C,P} = 32.2$ Hz), 9.1 ($C_{CH_3,Et}$). ^{31}P{^{1}H} NMR (DMSO-d^{6}, 101 MHz): δ 44.8.

Triethylphosphine(4-phenyl-1*H*,2,3-triazolyl)gold(I) (2c)

Colourless solid (11.5 mg, 45%). R_f 0.21 (PE–EtOAc, 1 : 4). Anal. Calc. for $C_{14}H_{21}AuN_3P$ (459.1 g mol^{-1}): C, 36.61; H, 4.61; N, 9.15. Found: C, 36.87; H, 4.61; N, 9.05%. MS (FAB$^+$): *m*/*z* 460.1 [M + H]$^+$, 433.0 [$(Et_3P)_2Au$]$^+$, 431.1 [M − Et + H]$^+$, 315.0 [Et_3PAu]$^+$. ^{1}H NMR (DMSO-d^6, 400 MHz): δ 14.14 (s, 1H, $NH_{Triazole}$), 8.19 (pseudo-d, 2H, $H_{o,Phe}$, ^3J = 8.0 Hz), 7.33 (pseudo-t, 2H, $H_{m,Phe}$, ^3J = 8.0 Hz), 7.20 (pseudo-t, 1H, $H_{p,Phe}$, ^3J = 8.0 Hz), 1.96 (dq, 6H, ^3J = 8.0 Hz, $CH_{2,Et}$), 1.20 (dt, 9H, ^3J = 8.0 Hz, $CH_{3,Et}$). ^{13}C{^{1}H} NMR (DMSO-d^6, 100.6 MHz): δ 162.8 ($C_{q,triazole}$, $^2J_{C,P}$ = 118.7 Hz), 152.3 ($C_{q,triazole}$, $^3J_{C,P}$ = 8.0 Hz), 135.1 ($C_{i,Ph}$), 128.1 ($C_{m,Ph}$), 126.1 ($C_{p,Ph}$), 125.4 ($C_{o,Ph}$), 16.9 ($C_{CH_2,Et}$, $^1J_{C,P}$ = 32.2 Hz), 8.9 ($C_{CH_3,Et}$). ^{31}P{^{1}H} NMR (DMSO-d^6, 101 MHz): δ 41.7.

Preparation of alkynyl-modified dipeptides.[59]

To a stirred suspension of the L-amino acid methyl ester hydrochloride (1 mmol) in 20 ml THF, an equimolar amount of triethylamine was added. After 30 min., 1.1 mmol (315 mg) Boc-L-(*p*-eth)-phenylalanine,[58] 1.1 mmol (111 mg, 121 µl) *N*-methylmorpholine and 1.1 mmol (151 mg, 143 µl) isobutyl chloroformate were added and the resulting suspension was stirred at r.t. for 24 h. The colorless precipitate was removed by filtration and after evaporation of the solvent, the residual oil was redissolved in diethyl ether, successively washed with 0.1 M HCl, water and saturated $NaHCO_3$ solution and dried over Na_2SO_4. The crude products were purified by column chromatography on silica (0.062–0.2 mm), using mixtures of petroleum ether–ethyl acetate (**3**).

Boc-L-(*p*-eth)-phenylalanine-L-leucine methyl ester (3)

Off-white solid (357 mg, 86%). R_f 0.083 (PE–EtOAc, 1 : 1). Anal. Calc. for $C_{23}H_{32}N_2O_5$: C, 66.32; H, 7.74; N, 6.73. Found: C, 66.05; H, 7.97; N, 6.51%. M_r ($C_{23}H_{32}N_2O_5$) = 416.2 g mol^{-1}. MS (FAB$^+$): *m*/*z* 439.2 [M + Na]$^+$, 417.2 [M + H]$^+$, 317.1 [M − Boc + H]$^+$. ^{1}H NMR (CD_2Cl_2, 400 MHz): δ 7.42 (d, 2H, $H_{\varepsilon,Phe}$, ^3J = 8.0 Hz), 7.18 (d, 2H, $H_{\delta,Phe}$, ^3J = 8.0 Hz), 6.30 (d, 1H, NH_{Leu}, ^3J = 8.0 Hz), 4.99 (m, 1H, NH_{Phe}), 4.55–4.49 (m, 1H, $H_{\alpha,Leu}$), 4.32–4.30 (m, 1H, $H_{\alpha,Phe}$), 3.70 (s, 3H, H_{OMe}), 3.13–2.79 (m, 2H, $H_{\beta,Phe}$), 3.11 (s, 1H, H_{Eth}), 1.61–1.45 (m, 3H, $H_{\beta,Leu}$, $H_{\gamma,Leu}$), 1.40 (s, 9H, H_{Boc}), 0.91 (m, 6H, $H_{\delta,Leu}$). ^{13}C{^{1}H} NMR (CD_2Cl_2, 100.6 MHz): δ 173.3 (CO_{Ester}), 171.2 (CO_{Amide}), 155.8 (CO_{Boc}), 138.6 ($C_{\gamma,Phe}$), 132.8 ($C_{\varepsilon,Phe}$), 130.1 ($C_{\delta,Phe}$), 121.2 ($C_{\zeta,Phe}$), 83.9 ($C_{q,Eth}$), 77.6 ($C_{q,Boc}$, C_{Eth}), 54.5 ($C_{\alpha,Phe}$), 52.7 (C_{OMe}), 51.4 ($C_{\alpha,Leu}$), 42.1 ($C_{\beta,Leu}$), 38.4 ($C_{\beta,Phe}$), 28.6 ($C_{CH_3,Boc}$), 25.3 ($C_{\gamma,Leu}$), 22.2 ($C_{\delta,Leu}$), 19.3 ($C_{\delta,Leu}$). ATR-IR (solid, cm^{-1}): 3292 (m), 2958 (m), 2872 (w), 2108 (w, ν(C≡C)), 1744 (m), 1655 (s), 1526 (m), 1470 (m), 1439 (m), 1390 (m), 1367 (m), 1249 (m), 1203 (m), 1166 (s), 1101 (s), 1050 (m), 1021 (m), 894 (w), 852 (m), 826 (m), 780 (m), 757 (m), 642 (m).

Boc-L-(p-eth)-phenylalanine-L-histidine methyl ester

Off-white solid (12 mg, 43%). R_f 0.48 (MeOH–EtOAc, 1 : 5). M_r ($C_{23}H_{28}N_4O_5$) = 440.2 g mol^{-1}. MS (FAB$^+$): *m*/*z* 463.2 [M + Na]$^+$, 441.2 [M + H]$^+$, 385.1 [M − ^{t}Bu + H]$^+$, 411.1 [M − Boc + H]$^+$. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.51 (s, 1H, $H2_{His}$), 7.39 (d, 2H, $H_{\varepsilon,Phe}$, ^3J = 7.5 Hz), 7.17 (d, 1H, NH_{His}, ^3J = 7.5 Hz), 7.16 (d, 2H, $H_{\delta,Phe}$, ^3J = 7.5 Hz), 6.13 (s, 1H, $H5_{His}$), 5.27 (d, 1H, NH_{Phe}, ^3J = 7.5 Hz), 4.76–4.69 (m, 1H, $H_{\alpha,His}$), 4.34–4.26 (m, 1H, $H_{\alpha,Phe}$), 3.69 (s, 3H, H_{OMe}), 3.13–2.92 (m, 2H, $H_{\beta,His}$), 3.13–2.92 (m, 2H, $H_{\beta,Phe}$), 3.06 (s, 1H, H_{Eth}), 1.39 (s, 9H, H_{Boc}).

Synthesis of the (1*H*,2,3-triazolyl) dipeptide (4)

To a flame-dried Schlenk flask, 0.192 mmol (80 mg) of **3**, dissolved in 2 ml of DMF–MeOH (9 : 1), were added under a flow of nitrogen. Successively, 0.01 mmol (1.8 mg, 5 mol%) copper iodide and 0.288 mmol (38 µl, 1.5 eq) trimethylsilyl azide were added and the solution was stirred at r.t. for 48 h under the exclusion of light. The solution was evaporated to dryness, the residue redissolved in DCM, filtered through Celite (coarse 545), again evaporated to dryness and finally purified by column chromatography on silica (0.062–0.2 mm), using mixtures of petroleum ether–ethyl acetate.

4-(Boc-L-phenylalanine-L-leucine methyl ester)-1*H*,2,3-triazolyl (5)

Light brown solid (46 mg, 52%). R_f 0.52 (PE–EtOAc, 1 : 2). Anal. Calc. for $C_{23}H_{33}N_5O_5$: C, 60.11; H, 7.24; N, 15.24. Found: C, 59.65; H, 7.09; N, 14.96. M_r ($C_{23}H_{33}N_5O_5$) = 459.2. MS (FAB$^+$): *m*/*z* 482.2 [M + Na]$^+$, 460.2 [M + H]$^+$, 404.1 [M − ^{t}Bu + H]$^+$, 360.1 [M − Boc + H]$^+$. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.92 (s, 1H, $H_{Triazole}$), 7.66 (pseudo-d, 2H, $H_{\varepsilon,Phe}$, ^3J = 8.0 Hz), 7.24 (pseudo-d, 2H, $H_{\delta,Phe}$, ^3J = 8.0 Hz), 6.97 (m, 1H, NH_{Leu}), 5.43 (m, 1H, NH_{Phe}), 4.60 (m, 1H, $H_{\alpha,Leu}$), 4.50 (m, 1H, $H_{\alpha,Phe}$), 3.62 (s, 3H, H_{OMe}), 3.13–3.01 (m, 2H, $H_{\beta,Phe}$), 1.57–1.52 (m, 1H, $H_{\gamma,Leu}$), 1.45–1.40 (m, 2H, $H_{\beta,Leu}$), 1.42 (s, 9H, H_{Boc}), 0.88 (d, 3H, $H_{\delta,Leu}$, ^3J = 8.0 Hz), 0.84 (d, 3H, $H_{\delta,Leu}$, ^3J = 8.0 Hz). ^{13}C{^{1}H} NMR ($CDCl_3$, 100.6 MHz): δ 173.0 (CO_{Ester}), 171.5 (CO_{Amide}), 156.0 (CO_{Boc}), 146.4 ($C_{q,triazole}$), 137.2 ($C_{\gamma,Phe}$), 130.1 ($C_{\delta,Phe}$), 128.6 ($C_{\zeta,Phe}$), 126.6 ($C_{CH,triazole}$), 126.4 ($C_{\varepsilon,Phe}$), 80.8 ($C_{q,Boc}$), 56.0 ($C_{\alpha,Phe}$), 52.4 (C_{OMe}), 51.0 ($C_{\alpha,Leu}$), 41.4 ($C_{\beta,Leu}$), 38.2 ($C_{\beta,Phe}$), 28.4 ($C_{CH_3,Boc}$), 24.8 ($C_{\gamma,Leu}$), 22.9 ($C_{\delta,Leu}$), 21.9 ($C_{\delta,Leu}$).

Synthesis of (phosphine)gold(I) dipeptides 6a–c

To a flame-dried Schlenk flask **1a** (51 mg, 0.1 mmol), alternatively **1b** (45 mg, 0.11 mmol) or **1c** (43 mg, 0.12 mmol) were added under nitrogen flow together with 1.4 molar equivalents of **3**. The mixture was suspended in 10 ml absolute THF and stirred for 4 d under a nitrogen atmosphere protected from light. After completion of the reaction, as indicated by TLC, the solution was evaporated to dryness. The crude product was purified by column chromatography on silica (0.062–0.2 mm) using petroleum ether–ethyl acetate mixtures.

Triphenylphosphine(4-(4-Boc-L-phenylalanine-L-leucine methyl ester)-1*H*,2,3-triazolyl)gold(I) (6a)

Colourless solid (62 mg, 68%). R_f 0.14 (PE–EtOAc, 1 : 2). Anal. Calc. for $C_{41}H_{47}AuN_5O_5P \cdot 1/4CH_2Cl_2$ (938.3 g mol^{-1}): C, 52.76; H, 5.10; N, 7.46. Found: C, 52.38; H, 5.22; N, 7.78%. M_r ($C_{41}H_{47}AuN_5O_5P$) = 917.8. MS (FAB$^+$): *m*/*z* 940.4 [M + Na]$^+$, 918.4 [M + H]$^+$, 721.1 [$(Ph_3P)_2Au$]$^+$, 459.0 [Ph_3PAu]$^+$. ^{1}H NMR

(CD_2Cl_2, 400 MHz): δ 11.86 (s, 1H, $NH_{Triazole}$), 8.27 (pseudo-d, 2H, $H_{\varepsilon,Phe}$, ^3J = 8.0 Hz), 7.63–7.53 (m, 15H, H_{Ph,PPh_3}), 7.19 (pseudo-d, 2H, $H_{\delta,Phe}$, ^3J = 8.0 Hz), 6.40 (d, 1H, NH_{Leu}, ^3J = 8.0 Hz), 5.05 (d, 1H, NH_{Phe}, ^3J = 8.0 Hz), 4.55-4-50 (m, 1H, $H_{\alpha,Leu}$), 4.35-4-33 (m, 1H, $H_{\alpha,Phe}$), 3.62 (s, 3H, H_{OCH_3}), 3.12–3.02 (m, 2H, $H_{\beta,Phe}$), 1.58–1.45 (m, 3H, $H_{\beta,Leu}$, $H_{\gamma,Leu}$), 1.38 (s, 9H, H_{Boc}), 0.86 (dd, 6H, $H_{\delta,Leu}$, ^3J = 8.0 Hz). $^{13}C\{^1H\}$ NMR (CD_2Cl_2, 100.6 MHz): δ 173.3 (CO_{Ester}), 171.6 (CO_{Amide}), 153.5 ($C_{q,triazole}$, $^3J_{C,P}$ not obs.), 135.7 ($C_{\zeta,Phe}$), 134.9 (C_{o,PPh_3}, $^2J_{C,P}$ = 14.2 Hz), 134.1 ($C_{\gamma,Phe}$), 132.4 (C_{p,PPh_3}, $^4J_{C,P}$ = 2.0 Hz), 130.4 (C_{i,PPh_3}, $^1J_{C,P}$ = 54.9 Hz), 129.9 (C_{m,PPh_3}, $^3J_{C,P}$ = 11.2 Hz), 129.8 ($C_{\delta,Phe}$), 127.0 ($C_{\varepsilon,Phe}$), 80.6 ($C_{q,Boc}$), 56.3 ($C_{\alpha,Phe}$), 52.7 (C_{OCH_3}), 51.4 ($C_{\alpha,Leu}$), 42.1 ($C_{\beta,Leu}$), 38.0 ($C_{\beta,Phe}$), 28.6 ($C_{CH_3,Boc}$), 25.2 ($C_{\gamma,Leu}$), 23.1 ($C_{\delta,Leu}$), 22.2 ($C_{\delta,Leu}$). $^{31}P\{^1H\}$ NMR (CD_2Cl_2, 101 MHz): δ 43.4.

Diethylphenylphosphine(4-(4-Boc-L-phenylalanine-L-leucine methyl ester)-1*H*,2,3-triazolyl)gold(I) (6b)

Colourless solid (73 mg, 65%). R_f 0.13 (PE–EtOAc, 1 : 2). Anal. Calc. for $C_{33}H_{47}AuN_5O_5P$ (821.3 g mol^{-1}): C, 48.24; H, 5.77; N, 8.52. Found: C, 48.63; H, 5.90; N, 8.21%. MS (FAB$^+$): *m*/*z* 822.4 [M + H]$^+$, 766.3 [M + H − ^{t}Bu]$^+$, 722.4 [M + H − Boc]$^+$, 363.1 [Et_2PhPAu]$^+$. ^{1}H NMR (CD_2Cl_2, 400 MHz): δ 12.12 (s, 1H, $NH_{Triazole}$), 8.27 (pseudo-d, 2H, $H_{\varepsilon,Phe}$, ^3J = 8.0 Hz), 7.82–7.77 (m, 2H, H_{Ph,Et_2PhP}), 7.55–7.54 (m, 3H, H_{Ph,Et_2PhP}), 7.20 (pseudo-d, 2H, $H_{\delta,Phe}$, ^3J = 8.0 Hz), 6.53 (d, 1H, NH_{Leu}, ^3J = 8.0 Hz), 5.14 (d, 1H, NH_{Phe}, ^3J = 8.0 Hz), 4.57–4.51 (m, 1H, $H_{\alpha,Leu}$), 4.35 (m, 1H, $H_{\alpha,Phe}$), 3.65 (s, 3H, H_{OCH_3}), 3.13–3.01 (m, 2H, $H_{\beta,Phe}$), 2.22–2.13 (m, 4H, CH_{2,Et_2PhP}), 1.60–1.48 (m, 3H, $H_{\beta,Leu}$, $H_{\gamma,Leu}$), 1.39 (s, 9H, H_{Boc}), 1.25–1.16 (m, 6H, CH_{3,Et_2PhP}), 0.88 (dd, 6H, $H_{\delta,Leu}$, ^3J = 8.0 Hz). $^{13}C\{^1H\}$ NMR (CD_2Cl_2, 100.6 MHz): δ 173.4 (CO_{Ester}), 171.7 (CO_{Amide}), 163.2 ($C_{q,triazole}$, $^2J_{C,P}$ = 121.7 Hz), 156.0 (CO_{Boc}), 153.6 ($C_{q,triazole}$, $^3J_{C,P}$ = 7.0 Hz), 135.6 ($C_{\zeta,Phe}$), 134.3 ($C_{\gamma,Phe}$), 134.0 ($C_{o,PPh}$, $^2J_{C,P}$ = 13.2 Hz), 132.4 ($C_{p,PPh}$, $^4J_{C,P}$ = 2.0 Hz), 130.2 ($C_{i,PPh}$, $^1J_{C,P}$ = 50.8 Hz), 129.9 ($C_{\delta,Phe}$), 129.8 ($C_{m,PPh}$, $^3J_{C,P}$ = 12.2 Hz), 126.9 ($C_{\varepsilon,Phe}$), 80.6 ($C_{q,Boc}$), 56.4 ($C_{\alpha,Phe}$), 52.7 (C_{OCH_3}), 51.4 ($C_{\alpha,Leu}$), 42.0 ($C_{\beta,Leu}$), 38.2 ($C_{\beta,Phe}$), 28.6 ($C_{CH_3,Boc}$), 25.2 ($C_{\gamma,Leu}$), 23.1 ($C_{\delta,Leu}$), 22.2 ($C_{\delta,Leu}$), 21.3 ($C_{CH_2,Et}$, $^1J_{C,P}$ = 32.5 Hz), 9.8 ($C_{CH_3,Et}$). $^{31}P\{^1H\}$ NMR (CD_2Cl_2, 101 MHz): δ 43.3.

Triethylphosphine(4-(4-Boc-L-phenylalanine-L-leucine methyl ester)-1*H*,2,3-triazolyl)gold(I) (6c)

Colourless solid (59 mg, 63%). R_f 0.12 (PE–EtOAc, 1 : 2). Anal. Calc. for $C_{29}H_{47}AuN_5O_5P\cdot 1/3C_6H_{14}$ (802.0 g mol^{-1}): C, 46.40; H, 6.49; N, 8.73. Found: C, 46.01; H, 6.21; N, 8.65%. M_r ($C_{29}H_{47}AuN_5O_5P$) = 773.3. MS (FAB$^+$): *m*/*z* 774.2 [M + H]$^+$, 718.2 [M + H − ^{t}Bu]$^+$, 433.1 [$(Et_3P)_2Au$]$^+$, 315.0 [Et_3PAu]$^+$. ^{1}H NMR (CD_2Cl_2, 400 MHz): δ 11.74 (s, 1H, $NH_{Triazole}$), 8.22 (pseudo-d, 2H, $H_{\varepsilon,Phe}$, ^3J = 8.0 Hz), 7.21 (pseudo-d, 2H, $H_{\delta,Phe}$, ^3J = 8.0 Hz), 6.38 (d, 1H, NH_{Leu}, ^3J = 8.0 Hz), 5.03 (d, 1H, NH_{Phe}, ^3J = 8.0 Hz), 4.53 (m, 1H, $H_{\alpha,Leu}$), 4.32 (m, 1H, $H_{\alpha,Phe}$), 3.66 (s, 3H, H_{OCH_3}), 3.06 (m, 2H, $H_{\beta,Phe}$), 1.91 (m, 6H, CH_{2,Et_3P}), 1.57 (m, 3H, $H_{\beta,Leu}$, $H_{\gamma,Leu}$), 1.41 (s, 9H, H_{Boc}), 1.27 (m, 9H, CH_{3,Et_3P}), 0.90 (dd, 6H, $H_{\delta,Leu}$, ^3J = 8.0 Hz). $^{13}C\{^1H\}$ NMR (CD_2Cl_2, 100.6 MHz): δ 173.3 (CO_{Ester}), 171.6 (CO_{Amide}), 164.3 ($C_{q,triazole}$), 155.9 (CO_{Boc}), 153.5 ($C_{q,triazole}$, $^3J_{C,P}$ = 7.1 Hz), 135.5 ($C_{\zeta,Phe}$), 134.3 ($C_{\gamma,Phe}$), 129.8 ($C_{\delta,Phe}$), 126.8 ($C_{\varepsilon,Phe}$), 80.6 ($C_{q,Boc}$), 56.4 ($C_{\alpha,Phe}$), 52.7 (C_{OCH_3}), 51.4 ($C_{\alpha,Leu}$), 42.1 ($C_{\beta,Leu}$), 38.1 ($C_{\beta,Phe}$), 28.6 ($C_{CH_3,Boc}$), 25.3 ($C_{\gamma,Leu}$), 23.1 ($C_{\delta,Leu}$), 22.2 ($C_{\delta,Leu}$), 18.5 ($C_{CH_2,Et}$, $^1J_{C,P}$ = 32.5 Hz), 9.4 ($C_{CH_3,Et}$). $^{31}P\{^1H\}$ NMR (CD_2Cl_2, 101 MHz): δ 40.5.

Preparation of (phosphine)gold(I) tetrapeptides 8a–8c by Fmoc SPPS

Solid-phase synthesis was performed manually in 5 ml plastic syringes with a porous disc as filter. The peptide sequence (I-L-Phe-D-Arg-L-Phe-L-Lys) was assembled on Fmoc-protected Rink amide resin (Novabiochem, loading 0.62 mmol g^{-1}).[14,60,61] 464 mg (0.288 mmol) of the peptide-loaded resin were transferred to a microwave glass tube and placed in a nitrogen-filled Schlenk tube. The resin was swollen in 1 ml DMF (peptide grade, Roth) for 15 min, then 3 ml DEA (p.a.) were added. The mixture was flushed with nitrogen for 15 min. 5.5 mg (0.0288 mmol) CuI, 72 µl (0.518 mmol) TMS-acetylene and 12 mg (0.017 mmol) $(Ph_3P)_2PdCl_2$ were added.[25] The suspension was reacted in a microwave (200 W, 120 °C, 30 min), transferred into a fritted 5 ml plastic syringe, followed by washing with DMF and DCM. TMS-deprotection was accomplished by aspiring 376 mg (1.44 mmol, 5 eq) of tetrabutylammonium fluoride (TBAF·$3H_2O$) and 59 µl (mmol, 4.5 eq) of acetic acid, dissolved in 1.5 ml THF, into the syringe.[26] After 2.5 h on a laboratory shaker, the resin was washed (5 × THF, 5 × H_2O, 5 × DMF, 5 × DCM) and dried *in vacuo*. The resin (synthesis of: **8a** (130 mg, 0.08 mmol), **8b** (170 mg, 0.1 mmol) and **8c** (150 mg, 0.09 mmol)) was weighed into a fritted 5 ml plastic syringe and swollen in DMF for 20 min. DMF was discarded, the respective (phosphine)gold(I) azide (**1a** (71 mg, 0.14 mmol, 1.8 eq), **1b** (85 mg, 0.21 mmol, 2 eq) and **1c** (66 mg, 0.19 mmol, 2 eq)) transferred into the syringe. 4 ml of degassed DMF were aspired into the syringe which was placed into a nitrogen-flushed Schlenk flask and mounted on a laboratory shaker protected from light. After 4 d, the resin was washed (5 × DMF, 5 × DCM, MeOH) and dried *in vacuo*. Cleavage, then precipitation in ice-cold diethyl ether yielded the crude product, which was purified by semi-preparative RP-HPLC (see Fig. S1, ESI†). Fractions containing the pure product were pooled, frozen in liquid nitrogen and lyophilized.

Triphenylphosphine(4-(4-L-phenylalanine-D-arginine-L-phenylalanine-L-lysine-amide)-1*H*,2,3-triazolyl)gold(I) (8a)

Colourless solid (14 mg, 16%). $C_{50}H_{60}AuN_{12}O_4P$ (1120.4 g mol^{-1}). LC-MS (ESI$^+$): t_R = 7.9 min (λ = 220 nm); *m*/*z* 1121.1 [M + H]$^+$, 721.0 [$(Ph_3P)_2Au$]$^+$, 561.2 [M + 2H]$^{2+}$, 374.5 [M + 3H]$^{3+}$. ^{1}H NMR (DMSO-d^6, 400 MHz): δ 14.38 (s, 1H, $NH_{triazole}$), 8.62 (d, 1H, ^3J = 8.0 Hz, NH_{Lys}), 8.41 (d, 1H, ^3J = 8.0 Hz, $NH_{Phe,B}$), 8.18 (d, 2H, $H_{\varepsilon,Phe,A}$, ^3J = 8.0 Hz), 8.11–8.04 (m, 4H, $NH_{Phe,A}$, NH_{Arg}), 7.65–7.62 (m, 15H, H_{Ar,PPh_3}), 7.35–7.17 (m, 9H, $H_{\delta,Phe,A}$, $H_{Phe,B}$, $NH_{2,Lys}$), 7.07 (br, 1H, $NH_{2,Arg}$), 4.63–4.58 (m, 1H, $H_{\alpha,Phe,B}$), 4.42–4.37 (m, 1H, $H_{\alpha,Lys}$), 4.19–4.14 (m, 1H, $H_{\alpha,Arg}$), 4.12–4.06 (m, 1H, $H_{\alpha,Phe,A}$), 3.13–3.04 (m, 2H, $H_{\beta,Phe,A}$), 2.95–2.86 (m, 2H, $H_{\gamma,Arg}$), 2.79–2.70 (m, 4H, $H_{\beta,Phe,B}$, $H_{\varepsilon,Lys}$), 1.70–1.50 (m, 4H, $H_{\beta,Arg}$, $H_{\beta,Lys}$), 1.33–1.16 (m, 4H, $H_{\gamma,Arg}$, $H_{\delta,Lys}$), 1.02–0.96 (m, 2H, $H_{\gamma,Lys}$). $^{31}P\{^1H\}$ NMR (DMSO-d^6, 101 MHz): δ 43.2.

Diethylphenylphosphine(4-(4-L-phenylalanine-D-arginine-L-phenylalanine-L-lysine-amide)-1*H*,2,3-triazolyl)gold(I) (8b)

Colourless solid (19 mg, 17%). $C_{42}H_{60}AuN_{12}O_4P$ (1024.4 g mol^{-1}). LC-MS (ESI$^+$): t_R = 7.0 min (λ = 220 nm); *m/z* 1025.2 [M + H]$^+$, 513.2 [M + 2H]$^{2+}$, 342.4 [M + 3H]$^{3+}$. ^{1}H NMR (DMSO-d^6, 400 MHz): δ 14.29 (s, 1H, $NH_{Triazol}$), 8.60 (d, 1H, ^3J = 8.0 Hz, NH_{Lys}), 8.40 (d, 1H, ^3J = 8.0 Hz, $NH_{Phe,B}$), 8.18 (d, 2H, $H_{\varepsilon,Phe,A}$, ^3J = 8.0 Hz), 8.11–8.09 (br, 1H, NH_{Arg}), 7.95–7.90 (m, 2H, H_{Ph,PEt_2Ph}), 7.81 (d, 1H, ^3J = 8.0 Hz, $NH_{Phe,A}$), 7.66–7.62 (m, 5H, $NH_{2,Lys}$,$H_{Ph,\ PEt_2Ph}$), 7.36–7.17 (m, 9H, $H_{\delta,Phe,A}$, $H_{Phe,B}$, NH_2), 7.07 (br, 2H, $NH_{2,Arg}$), 4.62–4.58 (m, 1H, $H_{\alpha,Phe,B}$), 4.40–4.37 (m, 1H, $H_{\alpha,Lys}$), 4.19–4.13 (m, 1H, $H_{\alpha,Arg}$), 4.12–4.06 (m, 1H, $H_{\alpha,Phe,A}$), 3.13–3.02 (m, 2H, $H_{\beta,Phe,A}$), 2.96–2.86 (m, 2H, $H_{\gamma,Arg}$), 2.79–2.70 (m, 4H, $H_{\beta,Phe,B}$, $H_{\varepsilon,Lys}$), 2.35–2.24 (m, 4H, CH_{2,PEt_2Ph}) 1.70–1.49 (m, 4H, $H_{\beta,Arg}$, $H_{\beta,Lys}$), 1.33–1.24 (m, 4H, $H_{\gamma,Arg}$, $H_{\delta,Lys}$), 1.17–1.08 (m, 6H, CH_{3,PEt_2Ph}), 1.01–0.92 (m, 2H, $H_{\gamma,Lys}$). ^{31}P{^{1}H} NMR (DMSO-d^6, 101 MHz): δ 44.8.

Triethylphosphine(4-(4-L-phenylalanine-D-arginine-L-phenylalanine-L-lysine-amide)-1*H*,2,3-triazolyl)gold(I) (8c)

Colourless solid (16 mg, 18%). $C_{38}H_{60}AuN_{12}O_4P$ (976.4 g mol^{-1}). LC-MS (ESI$^+$): t_R = 6.6 min (λ = 220 nm), *m/z* 977.2 [M + H]$^+$, 489.1 [M + 2H]$^{2+}$, 433.1 [$(Et_3P)_2Au$]$^+$, 326.5 [M + 3H]$^{3+}$. ^{1}H NMR (DMSO-d^6, 400 MHz): δ 14.22 (s, 1H, $NH_{triazole}$), 8.60 (d, 1H, ^3J = 8.0 Hz, NH_{Lys}), 8.40 (d, 1H, ^3J = 8.0 Hz, $NH_{Phe,B}$), 8.15 (d, 2H, $H_{\varepsilon,Phe,A}$, ^3J = 8.0 Hz), 8.11–8.09 (br, 1H, NH_{Arg}), 7.81 (d, 1H, ^3J = 8.0 Hz, $NH_{Phe,A}$), 7.67 (br, 2H, $NH_{2,Lys}$), 7.38–7.15 (m, 9H, $H_{\delta,Phe,A}$, $H_{Phe,B}$, NH_2), 7.07 (br, 1H, $NH_{2,Arg}$), 4.63–4.57 (m, 1H, $H_{\alpha,Phe,B}$), 4.40–4.36 (m, 1H, $H_{\alpha,Lys}$), 4.18–4.14 (m, 1H, $H_{\alpha,Arg}$), 4.12–4.06 (m, 1H, $H_{\alpha,Phe,A}$), 3.13–3.02 (m, 2H, $H_{\beta,Phe,A}$), 2.95–2.84 (m, 2H, $H_{\gamma,Arg}$), 2.79–2.70 (m, 4H, $H_{\beta,Phe,B}$, $H_{\varepsilon,Lys}$), 2.00–1.92 (m, 6H, CH_{2,Et_3P}), 1.70–1.49 (m, 4H, $H_{\beta,Arg}$, $H_{\beta,Lys}$), 1.33–1.16 (m, 13H, $H_{\gamma,Arg}$, $H_{\delta,Lys}$, CH_{3,Et_3P})), 1.01–0.90 (m, 2H, $H_{\gamma,Lys}$). ^{31}P{^{1}H} NMR (DMSO-d^6, 101 MHz): δ 41.7.

Abbreviations

Boc	*tert*-Butyloxycarbonyl
Fmoc	9-Fluorenylmethyloxycarbonyl
SPPS	Solid phase peptide synthesis
ET-AAS	Electrothermal atomic absorption spectroscopy
TrxR	Thioredoxin reductase
GR	Glutathione reductase
ROS	reactive oxygen species
PI	propidium iodide
IC_{50}	half maximal inhibitory concentration
EC_{50}	half maximal effective concentration
TBAF	tetrabutylammonium fluoride
TFA	trifluoroacetic acid
TIS	triisopropylsilane
PBS	phosphate-buffered saline
ATP	adenosine triphosphate
NADH	reduced nicotinamide adenine dinucleotide
NAD+	oxidized nicotinamide adenine dinucleotide

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