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Trastuzumab gold-conjugates: synthetic approach and *in vitro* evaluation of anticancer activities in breast cancer cell lines.

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Abstract

We describe the preparation of gold(I)-compounds that are amenable to efficient bioconjugation with monoclonal antibodies via activated ester or maleimide linkers. New Trastuzumab-gold conjugates were synthesized and fully chracterized. These bioconjugates are significantly more cytotoxic (sub-micromolar range) to HER2-positive breast cancer cells than the gold complexes and Trastuzumab.

Antibody-drug conjugates (ADCs) represent a promising therapeutic approach for cancer chemotherapy.[1–8] These conjugates combine the antigen-targeting specificity of monoclonal antibodies (mAbs) with the cytotoxic potency of chemotherapeutics. Human tumor cells have unique or overexpressed tumor-specific antigens and mAbs can specifically bind to these antigens. Monoclonal antibodies have been used as anticancer agents as they can

Conflicts of interest

"There are no conflicts to declare".

Electronic Supplementary Information (ESI) available: Experimental section (synthesis and characterization of all compounds and antibody gold conjugates), NMR and MS-ESI spectra for new compounds **1–4** and their stability studies by NMR spectroscopy; HPLC and MALDI-TOF spectra for **Tras-1** and **Tras-4** as well as ELISA assays for the binding affinity of **Tras-1**, **Tras-4** and **Trastuzumab** to HER2. It also includes details on the cell viability assays for all compounds and AGCs described. See DOI: 10.1039/x0xx00000x

induce an immunological response or inhibit cellular signalling pathways. However, therapeutic efficacy is limited by the cell death effect that the mAb may generate and addition of small molecule cytotoxic payloads have shown to increase the efficacy.[1]

Since the vast majority of cytotoxic drugs do not discriminate tumor and healthy tissues, the use of mAbs as targeting vehicles give rise to more selective chemotherapeutic treatments. Two ADCs were approved by the US Food and Drug Administration (FDA) and the European Medicine Agency (EMA), Adcetris® (brentuximab vedotin) in 2011 and Kadcyla® (Trastuzumab emtansine or T-DM1) in 2013. More recently in 2017, inotuzumab (Besponsa™) and *gemtuzumab ozogamicin* (Mylotarg™) have also been approved by the FDA. In addition, over 65 ADCs (in 2017) are in the clinical pipeline.[1–8] The current research focus is on new generations of ADCs based on site-specific conjugation and a different type of payload which would minimize their high cost-of-goods.

In terms of cytotoxic payloads, very few ADCs based on metal-compounds as cytotoxic loads have been described.[9,10] Gold(I) compounds have emerged as potential anticancer chemotherapeutics[11–14] with Auranofin([(2R,3R,4S,5R,6S)-3,4,5-tris(acetyloxy)-6-{[(triethyl-λ5-phosphanylidene)aurio]sulfanyl} oxan-2-yl]methyl acetate, a compound containing the $[AuPEt_3]^+$ fragment)[15] being evaluated in clinical trials.[16,17] AF is known for its redox enzymes inhibitory and ROS scavenging properties.[15] The first ADC based on a N-heterocyclic carbene gold fragment conjugated to an engineered Trastuzumab antibody, Thiomab, was reported recently by Bernardes *et al.*[18] With an GI_{50} in the low micromolar range, the anti-proliferative activity of this ADC in HER2 positive breast cancer cell line showed a promising moderate improvement as compared to the gold-complex drug. However, the direct conjugation of the gold atom to the free cysteine of Thiomab (Au-S-cysteine bond) may pose *in vivo* stability issues.[19] Linkers in ADC constructs do not only serve the purpose of bridging the antibody and the cytotoxic drug, but also play a relevant role in the ADC stability during preparation, storage and systemic circulation period.[19] They can also facilitate intracellular targeted release by inclusion of appropriate functionalities.[19]

We describe here the preparation of gold compounds containing the $[Au(PPh_3)]^+$ motif and a linker. This study stands as a proof-of-concept for the efficient and versatile bioconjugation of gold complexes to mAbs.

The preparation of the new linkers containing the $[AuPPh_3]^+$ fragment (**1**-**4**) are depicted in Scheme 1. All linkers employed are commercially available with linker **b** (PTDA) already described for the preparation of ADCs.[20,21] The first strategy (A) is based on a copper-free cycloaddition reaction between a linker terminal alkyne and gold azide complex. This reaction was first described by Gray *et al.* in 2007,[22] and employed in the preparation of peptides containing gold.[23] The second strategy (B) is based on amide bond formation between a linker with a terminal amine and a gold thiolate compound containing a free carboxylate group ($[Au(mba)(PPh_3)]$) extensively used in our research group as a component of anticancer heterometallic complexes.[24,25] The second method is simpler and more environmentally friendly than the cycloaddition reactions as it involves shorter reaction times at room temperature, and less toxic reagents (see Supporting Information, SI).

Compounds **1**-**4** were obtained in moderate yields (30–60%) and were fully characterized (see SI, Section 1). Compound **3** could only be characterized by elemental analysis and partially by NMR, as it is insoluble in most organic and inorganic solvents and only slightly soluble in DMSO. Compounds **1**-**4** stability profile in solution (either in d^6-DMSO, or mixtures d^6-DMSO:PBS-D_2O in a ratio 3:1) was studied by ^1H and $^{31}P\{^1H\}$ NMR spectroscopy. Compounds **2** and **4** remained intact in d^6-DMSO for up to 14 and 40 days, respectively. Compound **4** was equally stable in d^6-DMSO:PBS-D_2O. Compound **1** (containing hygroscopic linker **a**) displayed the lowest stability, with half-lives of 6 and 5 hours in d^6-DMSO and d^6-DMSO/PBS-D_2O, respectively. However, this stability is sufficient for subsequent bioconjugation reactions.

Two compounds (**1** and **4**) with two distinct groups amenable for bioconjugation and good solubility were selected for bioconjugation reactions to the anti-HER2 antibody, Trastuzumab. Trastuzumab (Herceptin™, Genentech) is a humanised IgG1 mAb, which has been exploited successfully as a targeting vector for both therapeutic agent and radioactive isotopes. Its main application lies in the treatment of HER2 positive cancers either alone, or in combination with chemotherapy, hormone blockers or tyrosine kinase inhibitors. Overexpression of HER2 correlates with increased tumor aggression and metastatic potential.[26]

Compound **1** activated NHS ester moiety can react with Trastuzumab available lysine residues[27] (Scheme 2) while compound **4** maleimide moiety can react with Trastuzumab cysteine residues available after reduction of Trastuzumab interchain disulfide bonds.[28] The selectivity of the conjugation method is of primary importance as non-site selective methods will result in heterogeneous ADC mixtures with a broad range of properties (e.g. binding potency, stability). Due to the high number of lysine accessible in a typical antibody (up to 20), lysine conjugation results in heterogeneous ADC population with a broad range of drug-to-antibody ratio (DAR) and a plethora of potential regioisomers. Methods for site-selective conjugation were evaluated to overcome ADCs' heterogeneity and include the reduction of antibody interchain disulfide bound to provide free cysteine.[28]

Two new antibody gold-based conjugates (AGCs), **Tras-1** and **Tras-4,** were successfully obtained using the lysine and cysteine conjugation strategies with yields of 55% and 70% respectively (see Supporting Information). To our knowledge, they are the first AGCs that conjugate gold complexes to an antibody via a linker. **Tras-1** and **Tras-4** were characterized by MALDI-TOF and size exclusion HPLC (see Supporting Information). Lysine conjugation resulted in **Tras-1** with a DAR of 2.7–3.2 while conjugation through cysteine resulted in **Tras-4** with a DAR of 2.7 (see SI). DARs strongly influence the efficacy of ADCs. Low DARs can result in reduced potency of the ADC while high DARs can result in increased pharmacokinetic of the ADC.[29] To mitigate such effects, we aimed for DAR comprised between 2 and 4 for our AGCs. Furthermore, **Tras-1** and **Tras-4** were obtained with purity > 95%. Binding affinity of Tras-1 and Tras-4 for HER2 was evaluated in an ELISA assay (Figure 1). Compared to unmodified Trastuzumab (EC_{50} = 0.22 ± 0.03 nM), both **Tras-1** and **Tras-4** showed a slight decrease in binding affinity for HER2 with EC_{50} of1.13 ± 0.14 and 0.36 ± 0.01 nM, respectively. As expected, **Tras-1** showed the lowest affinity for HER2. This slight decrease in affinity is hypothesized to result from the non-site selective

modification of the Trastuzumab antibody through lysine conjugation. Modification through cysteine conjugation (**Tras-4**) resulted indeed in a much more moderate decrease in affinity. Even though, the AGCs demonstrated a decrease affinity for HER2, their binding affinity is still in the same range as unmodified Trastuzumab. The stability of the AGCs was studied in human serum over a 7-day period (see SI). The presence of unconjugated gold was evaluated by ICP-OES. No release of gold was observed up to 7 days, confirming the stability our AGCs.

Cell viability assays were carried out to investigate the antiproliferative effect of our AGCs. We evaluated the cytotoxicity of soluble gold compounds **1**, **2** and **4**, gold starting materials [AuCl(PPh_3)] and [Au(mba)(PPh_3)], unmodified Trastuzumab and new AGCs **Tras-1** and **Tras-4** on HER2-positive breast cancer cells (MCF-7 and BT-474) and on a non-cancerous human breast cell line (MCF-10A). Auranofin was used for comparative purposes. The main results are collected in Table 1 and the results for compound **2** can be found in the SI (Table S2). Cells were incubated with the compounds for 72 hours and viability was assessed with Presto Blue (see Supporting Information including statistical analysis). Starting materials [AuCl(PPh_3)] and [Au(mba)(PPh_3)], were cytotoxic to the breast cancer cell line MCF-7 in the low micromolar range (EC_{50} = 3.6 and 2.4 μM, respectively) when compared to the values for the healthy cell line MCF-10A. Compound **4** showed a comparable cytotoxicity value (EC_{50} = 2.7 μM) while compound **1** was ten-times less toxic (EC_{50} = 38.8 μM). In general, the modification of the gold starting materials by incorporation of linkers amenable to bioconjugation did not disrupt considerably the cytotoxic potential of our gold-complexes. As mentioned before, linkers bring considerable advantages in terms of ADC stability.[19]

The AGCs, **Tras-1** and **Tras-4**, displayed enhanced cytotoxicity (very low micromolar and sub-micromolar range) compared to the gold linkers (**1** and **4**) or Trastzumab in the HER2-positive MCF-7 and BT-474 cell lines. Tras-4 was a significantly more cytotoxic than starting material [Au(mba)PPh_3] in the BT-474 cell line ($P<0.01$, unpaired t-test). Conversely, all the compounds and AGCs demonstrated reduced cytotoxicity to the non-cancerous human breast epithelial cell line MCF-10A. This observation is particularly important in the case of our AGCs since it indicates a HER2-mediated toxicity. The selectivity of **Tras-4** is higher compared to **Tras-1**, all gold starting materials and Auranofin.

In conclusion, we have prepared antibody-gold based conjugates that incorporate linkers. Two bioconjugation strategies were investigated to offer site-specific or random functionalization of the antibody. Importantly, the conjugation of gold compounds to Trastuzumab maintains their affinity toward HER2. The two generated AGCs are significantly more cytotoxic to HER2-positive breast cancer cell lines than the gold-containing linkers and Trastuzumab. For ADCs to be efficacious, their cytotoxicity in cells has to be in the low or sub-nanomolar range.[1] The very promising EC_{50} values (low micromolar and sub-micromolar range) obtained for the new AGCs with a non-optimized payload, the fragment $[Au(PPh_3)]^+$, warrant the study of new antibody drug conjugates based on gold-cytotoxic payloads displaying low nanomolar EC_{50} values (such as specific gold-N-heterocyclic carbenes).[30] The synthesis described here are much less complex than those described for the incorporation of conventional payloads. Gold payloads may therefore become competitive in terms of the high cost-of-goods of ADCs. The versatility of the

strategies developed will allow the synthesis of a library of gold-AGCs with a range of linkers with variable pharmacological profiles or intracellular release functionalities, enabling the preparation of gold-based biological imaging probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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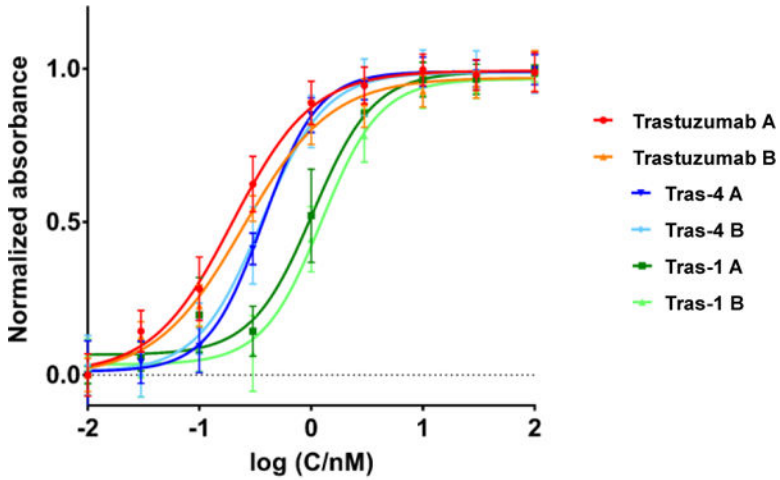
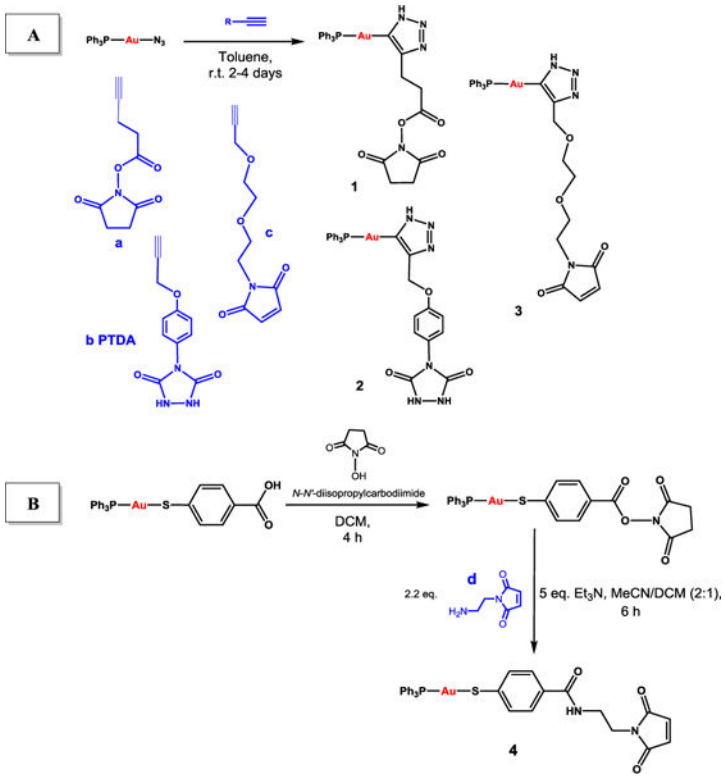


	Trastuzumab	Tras-1	Tras-4
EC_{50} (nM)	0.22 ± 0.03	1.13 ± 0.14	0.36 ± 0.01

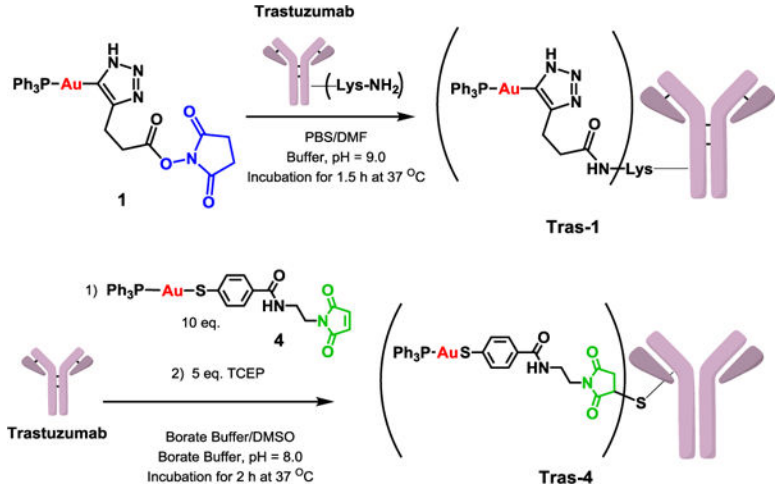
Fig. 1.

Binding affinity of AGCs **Tras-1** and **Tras-4** to HER2 as compared to unmodified Trastuzumab.



Scheme 1.

Synthetic strategies (A and B) to generate linkers containing gold(I) compounds (**1**-**4**) amenable to bioconjugation to monoclonal antibodies.



Scheme 2.

Preparation of novel Trastuzumab-gold conjugates **Tras-1** and **Tras-4.**

Table 1

Cell viability EC_{50} values (μM) in breast cancer HER2-positive MCF-7 and BT-474 cells, and non-cancerous breast MCF-10A cells for: a) gold starting materials **[AuCl(PPh$_3$)]** and **[Au(mba)(PPh$_3$)]**, b) new gold-containing linkers **1**, and **4**, and c) novel antibody gold-based conjugates **Tras-1** and **Tras-4**. Auranofin (**AF**) was used as control. [a]

	[AuCl(PPh$_3$)]	[Au(mba)(PPh$_3$)]	1	4	Trastuzumab	Tras-1	Tras-4	AF
MCF-7 (**A**)	3.57 ± 0.33	2.36 ± 0.32	38.76 ± 4.85	2.73 ± 0.86	> 60 μM	2.67 ± 0.70	0.63 ± 0.05	4.09 ± 0.07
BT-474 (**B**)	3.32 ± 0.10	3.51 ± 0.16	23.28 ± 0.31	0.81 ± 0.01	> 60 μM	1.73 ± 0.17	0.32 ± 0.01	3.94 ± 0.33
MCF-10A (**C**)	18.94 ± 1.30	5.52 ±0.36	44.57 ± 5.15	6.77 ± 0.67	> 50 μM	5.69 ± 0.45	4.04 ± 0.20	3.19 ± 0.45
EC_{50}(**C**)/EC_{50}(**A**)	5.30 ± 0.61	2.34 ± 0.35	1.15 ± 0.20	2.48 ± 0.82	0.83 ± 0.13	2.13 ± 0.58	6.41 ± 0.60	0.78 ± 0.11
EC_{50}(**C**)/EC_{50} (**B**)	5.70 ± 0.43	1.57 ± 0.13	1.91 ± 0.22	8.35 ± 0.83	0.83 ± 0.13	3.29 ± 0.41	12.63 ± 0.74	0.81 ± 0.13

[a]Compounds were dissolved in 1% of DMSO, DMF or DMSO/triethylene glycol (as described in the Supporting Information) and diluted with media before addition to cell culture medium for a 72 hour incubation period. Trastuzumab, **Tras-1** and **Tras-4** were directly diluted in media. Compounds performed in duplicate and STDEV is derived from these two separate experiments.