Bioorg. Med. Chem. 44 (2021) 116298

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Attaching palladium catalysts to antibodies

Paul Cowling, Mark Bradley *, Annamaria Lilienkampf *

EaStCHEM School of Chemistry, University of Edinburgh, Joseph Black Building, David Brewster Road, Edinburgh EH9 3FJ, UK

ARTICLE INFO

Keywords:
Antibody
Bioconjugation
Catalysis
Herceptin
Palladium
Prodrug

ABSTRACT

Antibody-directed enzyme prodrug therapy (ADEPT) is a powerful concept in which antibody targeting is linked to enzymatic prodrug activation. The work herein describes the first steps in the development of a technology analogous to ADEPT but in which a palladium catalyst is attached of an antibody rather than an enzyme. Antibody–metal conjugates have been used in a variety of contexts including for radiotherapy; however, none of the metals attached to the antibodies have been used for catalytic purposes. This work represents the first example a metal being attached to an antibody for the purposes of carrying a functional catalyst.

1. Introduction

Monoclonal antibodies (mAbs) have become an integral part of modern-day research and medicine due to their high specificity and affinity for their target antigens. Over the past few decades, the use of mAbs in disease therapeutics has increased because they can specifically target biomolecules. mAbs can impart a therapeutic effect through complement-dependent cytotoxicity or antibody-dependent cell-mediated cytotoxicity,[1] with both requiring interaction with the host's immune system. They can also have therapeutic effect by inhibiting the function of the target antigens (e.g. Trastuzumab, Bevacizumab and Ceruxitmab);[1,2] however, few monoclonal antibodies have significant therapeutic activity against cancer and in clinical trials most only increase patient survival rates by a few months.[1,3] To improve therapeutic potency of mAbs and reduce the side effects of common chemotherapy drugs, the two have been combined to create antibody–drug conjugates (ADCs).[4,5,6] ADCs aim to overcome the issues of chemotherapy side effects by targeting the delivery of a drug to cells that present the target antigen, *i.e.*, the cancerous cells, with compounds attached by a variety of linking strategies [7,8].

Antibody–directed enzyme prodrug therapy (ADEPT) is a powerful concept based on a mAb linked to an enzyme to create a conjugate.[9,10,11] These conjugates bind to cell surface receptors that are overexpressed on cancerous cells but minimally expressed on healthy cells. After accumulation of the conjugate on the target cells, a prodrug is administered whereupon the prodrug is chemically activated by the enzyme. The overall effect is similar to that of ADCs, a cytotoxic drug is delivered with spatial control to cancer cells but with enhanced dosing levels. The problem for ADEPT is that, whilst some candidates made it to stage II clinical trials,[9,10] they ultimately failed due to the immunogenicity of the treatment. This is because the conjugates studied in a clinical setting used carboxypeptidase G2, an enzyme from *Pseudomonas* with no known human analogues.[12] This was an important feature as it meant that prodrug activation was selective and allowed specific prodrugs to be designed, without cross-reactivity to the host's own enzymes, thus increasing specificity. However, this non-human enzyme elicited an immunogenic response and ultimately led to the requirement of co-administration of the immunosuppressant cyclosporine.[13] Thus, changing the enzyme to a synthetic, non-immunogenic catalytic entity, such as a metal, could be the key to allowing this technology to progress, while broadening its scope. In addition, antibody–metal conjugates could potentially have other applications in biotechnology, for example new metal-linked immunosorbent assays (analogous to enzyme-linked immunosorbent assays) could be developed.

Here, were report a palladium-catalytic antibody that can be used to activate prodrugs in situ (Fig. 1). The key need was to generate Pd-catalysts designed with the express intent of having a bioconjugation handle such that it could be coupled onto a targeting molecule, here an antibody, and be fully biocompatible and chemically active. The Pd-catalyst synthesized here was based on the *N*-heterocyclic carbenes (NHC) devised by Meldal[14] and further developed by ourselves,[15,16] and was generated using solid-phase methodologies.

* Corresponding authors.
E-mail addresses: mark.bradley@ed.ac.uk (M. Bradley), annamaria.lilienkampf@ed.ac.uk (A. Lilienkampf).

https://doi.org/10.1016/j.bmc.2021.116298
Received 30 April 2021; Received in revised form 18 June 2021; Accepted 20 June 2021
Available online 28 June 2021

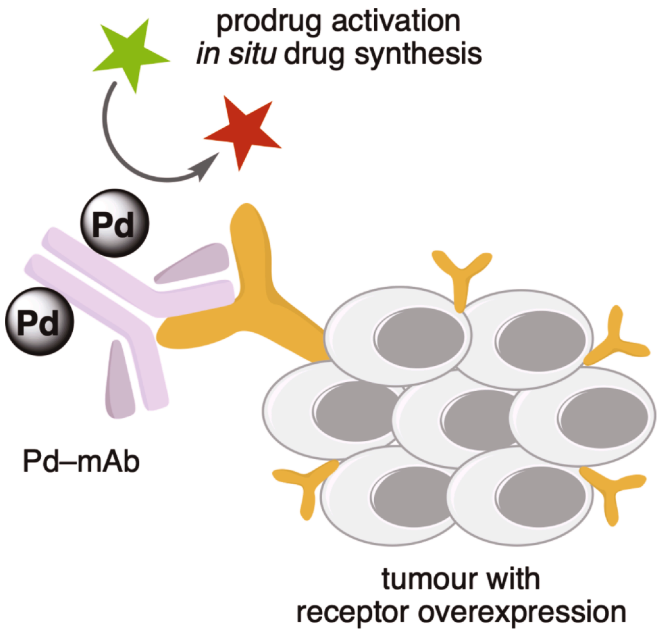


Fig. 1. The concept of this work, where the enzyme used in ADEPT is replaced by a synthetic metal-catalyst. A monoclonal antibody–Palladium (Pd-mAb) conjugate binds to an overexpressed receptor on the tumour cell surface, allowing "organic chemistry" to be "unleashed on the cancer" be it classical activation of a prodrug or in situ drug synthesis.

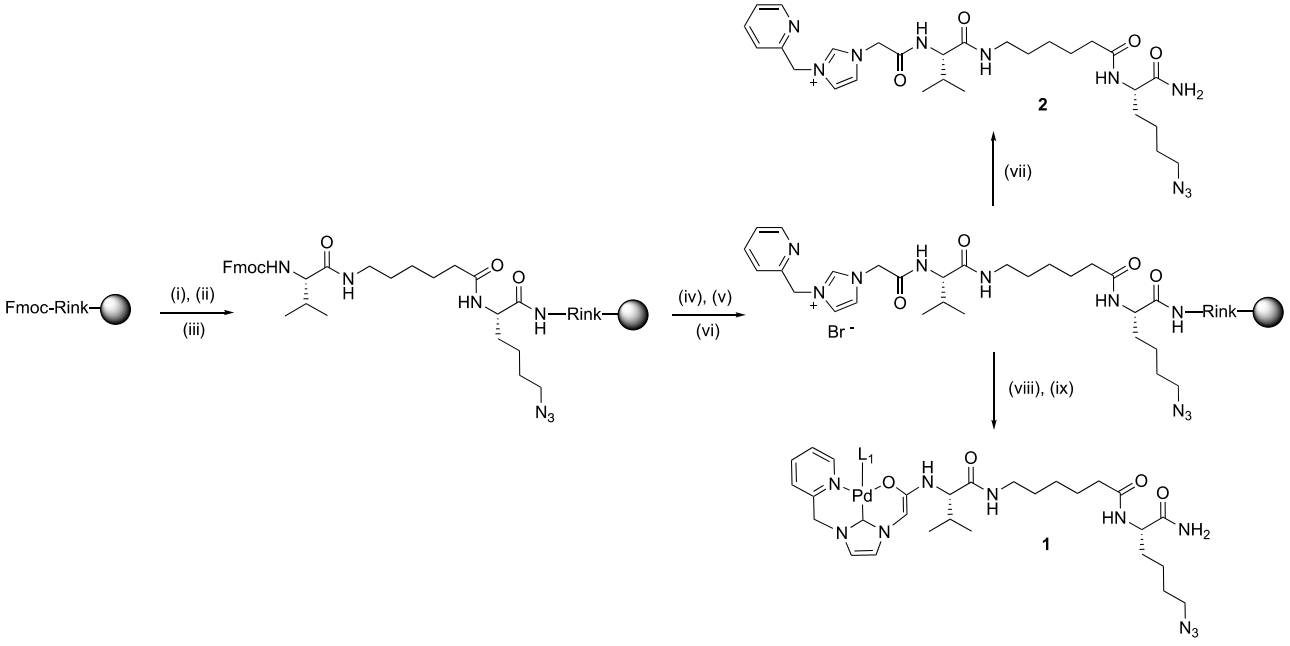
2. Results and discussion

2.1. Synthesis of a conjugatable NHC–Pd catalyst

The catalyst **1** was synthesized using a solid-phase approach on an aminomethyl polystyrene resin and a Rink-amide linker (Scheme 1). An azide-derivative of lysine (Fmoc-Lys(N_3)–OH) was firstly coupled (using DIC/Oxyma) to the linker to introduce a reactive handle for subsequent antibody attachment via a strain promoted cycloaddition reaction. Next, an aminohexanoic acid spacer was coupled, followed by a valine residue, which has been shown to promote the catalytic activity of similar ligands.[16] The key steps in the generation of the NHC ligand were acylation of the free amino group with bromoacetic acid, followed by alkylation using imidazole (promoted by the use of $AgNO_3$) and a second alkylation reaction using 2-(bromomethyl)pyridine, with all reactions microwave assisted and driven by the use of 1–2 M reagent concentrations.[16] The Pd was loaded onto the ligand using $Pd(COD)Cl_2$ and the catalyst was cleaved off the Rink linker using TFA in DCM, and purified by reverse-phase HPLC. The ligand (compound **2**) was fully characterised before loading with Pd using NMR, HPLC (ELS and 254 nm detection), and HRMS, and the final catalyst **1** characterised by HPLC and HRMS (Pd-loading broadens the resonances of the complex such that NMR analysis was not possible). Remarkably, the azide moiety tolerated the microwave-assisted synthesis, the silver chemistry, and the palladium loading conditions, and the metal centre was resistant to the acidic cleavage conditions.

2.2. Catalytic activity of the conjugatable NHC–Pd catalyst

The catalytic activity of NHC–Pd **1** was initially evaluated against two protected fluorophores, *bis*-propargylcarbamate rhodamine 110[17,18] **3** and *O*-propargylated 2-hydroxmethyl-2′,7′-dichlorofluorescein[19] **5**, with analysis of their decaging, as well the ability of **1** to mediate cross-coupling chemistry under aqueous conditions, monitored at 37 °C (Fig. 2A). Catalyst **1** was able to generate a > 10-fold increase in fluorescence upon activation of the two profluorophores **3** and **5** under biologically relevant conditions, with formation of **4** ($\lambda_{Ex/Em}$ 495/518 nm) and **6** ($\lambda_{Ex/Em}$ 500/530 nm), respectively (Fig. 2B). Catalyst **1** was also able to carry out a Suzuki-Miyaura coupling[20] in water forming



Scheme 1. The microwave assisted solid-phase synthesis of the catalyst **1** and ligand **2** on an aminomethyl polystyrene resin loaded with a Rink-amide linker. As drawn, the catalyst **1** is Pd(II) with L_1 most likely formate (from the HPLC purification buffer). All reactions were monitored by TFA cleavage from a small sample of the resin and analysis by HPLC and NMR. (i) 20% piperidine in DMF, 2 × 10 min; Fmoc-Lys(N_3)–OH, DIC, Oxyma (all at 0.33 M, 5 equiv.) in DMF (3 mL); (ii) 20% piperidine in DMF, 2 × 10 min; Fmoc-6-amino hexanoic acid, DIC, Oxyma (0.33 M, 5 equiv.) in DMF (3 mL); (iii) 20% piperidine in DMF, 2 × 10 min; Fmoc-Val-OH, DIC, Oxyma (0.33 M, 5 equiv.) in DMF (3 mL); (iv) 20% piperidine in DMF, 2 × 10 min; 2 M $BrCH_2CO_2H$, 1 M DIC, anhyd. DMF, 20 min, 60 °C, μw; (v) 2 M imidazole, 0.5 M $AgNO_3$ in anhyd. DMSO, 40 min, 60 °C, μw; (vi) 1 M 2-(bromomethyl)pyridine, 1 M Et_3N, 0.5 M $AgNO_3$, anhydrous DMF, 90 min, 60 °C, μw; (vii) TFA–H_2O (19:1), 2 h; (viii); BEMP, anhyd. DMF, N_2, 45 min, then $Pd(COD)Cl_2$ overnight; (ix) 50% TFA in DCM, 2 h.

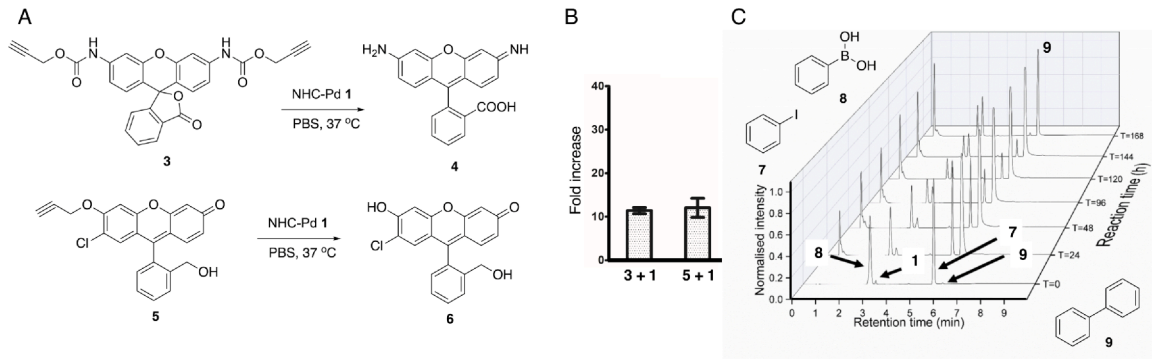


Fig. 2. Catalytic activity of the NHC-Pd catalyst **1**. A) The pro-fluorophores used in the catalyst activation process; B) The levels of activation observed with the catalyst **1** (10 μM) and 100 μM of **3** or **5** in PBS overnight; C). HPLC analysis (254 nm) over time (0 to 168 h) of the formation of biphenyl **9** from iodobenzene **7** (37.5 μM) and phenyl boronic acid **8** (75 μM) using the NHC-Pd catalyst **1** (0.1 mol %) in H_2O/ Acetonitrile at 37 °C. The retention times of catalyst **1**, **8**, **7**, and product **9** are 3.0, 3.1, 5.9 and 6.1 min, respectively.

biphenyl **9** from phenyl boronic acid **8** and iodobenzene **7** (Fig. 2C). Both, the de-propargylation reactions and Suzuki-Miyaura coupling, alongside the azide reactive handle, made **1** a feasible catalyst to carry forward for antibody bioconjugation.

2.3. Conjugation of the NHC-Pd catalyst to a monoclonal antibody

Herceptin is a clinically approved mAb used to treat breast cancer and binds the epidermal growth factor receptor (EGFR, also known as the Her2 receptor) and prevents receptor dimerisation.[21,22] This, in turn, prevents the epidermal growth factor (EGF) binding to the receptor and, as a result, there is no downstream signalling to induce cellular growth and proliferation. Here, Herceptin was reacted with the NHS active ester of the dibenzocyclooctyl stained alkyne **10** (DBCO-NHS, 5 equiv.) at 37 °C for 4 h to give **11a**. As an initial proof of concept, the modified DBCO-mAb **11a** was incubated with azide-functionalized fluorescein **12** (*N*-(3-azidopropyl)-]5-carboxamide fluorescein) at 37 °C for 4 h to allow conjugation via a stain promoted cycloaddition (Fig. 3A).[23]

Analysis of the reaction products by SDS-PAGE and MALDI-TOF MS showed clearly that Herceptin had been successfully labelled (Fig. 3B and 3C). Thus, the SDS-PAGE gel showed the fluorescently labelled product **13** generated via the addition of the stained alkyne and the subsequent azide cycloaddition chemistry. MALDI-TOF MS analysis showed an average increase in molecular mass of *ca* 1200 Da between the native Herceptin and **13** suggesting an average degree of labelling of 2.5 fluorophores per antibody.[24]

Having successfully labelled Herceptin, it was imperative to test if the antibody retained its receptor binding functionality. The fluorescently-labelled antibody **13** was incubated with two breast cancer cell lines, SK-BR-3 and MCF-7, which both express the EGFR receptor; however, the SK-BR-3 cells have a significant over-expression of the receptor compared with MCF-7 cells.[21,25] Thus, binding to MCF-7

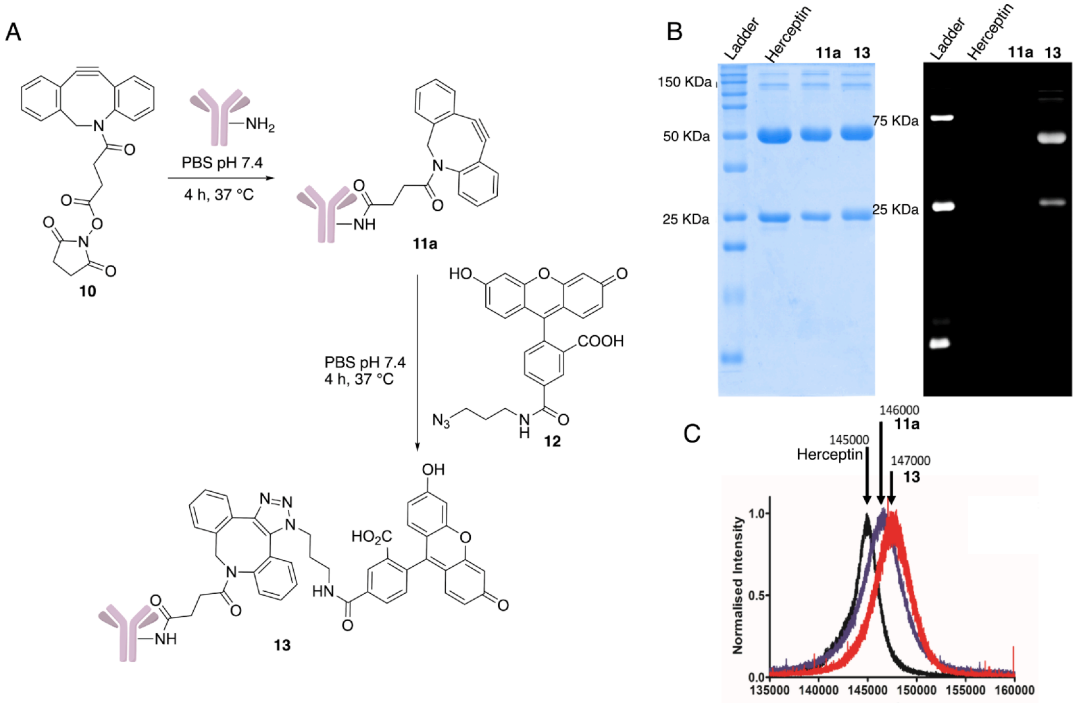


Fig. 3. A). Functionalisation of Herceptin with strained alkyne DBCO and subsequent fluorescent labelling via a strain promoted cycloaddition with FAM-azide. B). SDS-PAGE analysis of fluorescently labelled antibody **13**. Left = Coomassie staining, Right = Gel imaged under 480 nm light prior to Coomassie staining. C). MALDI-TOF MS analysis of Herceptin after each reaction step. Unmodified Herceptin is in black; **11** is in blue; **13** in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells was minimal, whereas in contrast, when **13** was incubated with the SKBR-3 cells, a clear concentration dependent shift was observed in the flow cytometry histograms, indicating successful binding of the antibody and fluorescent labelling of the cells (Fig. 4). Saturation was reached at 10 nM suggesting that the K_d of **13** is within the same order of magnitude as the native drug Herceptin.[26,27] Overall, these results shows that this method of Herceptin conjugation (via a DBCO modification and stain promoted cycloaddition) could be used for attaching an azide-bearing palladium catalyst **1**.

Following the methodology established above, Pd-catalyst **1** was attached to Herceptin. Herceptin was incubated with 5 and 25 equiv. of DBCO-NHS **10** (0.2 mM or 1 mM) at 37 °C for 4 h to generate **11a** and **11b**, respectively, with different loadings of the strained alkyne, which was followed by 5 or 25 equiv. of **1** (4 h at 37 °C). The Pd–antibody conjugates were purified using an ultra-centrifugation column (MWCO 10 kDa) to give **14a** and **14b** with the conjugation confirmed by MADLI-TOF MS, SDS-PAGE and ICP-MS analysis (Fig. 5). The SDS-PAGE showed an increase in the molecular weight of the antibody upon catalyst conjugation, with reduced mobility with bands broadened due to the heterogeneous nature of the products. MALDI-TOF MS analysis also clearly indicated conjugation of the Pd catalyst with an increase in molecular weight of 3000 and 19000 Da for **14a** and **14b**, respectively. To better quantify the Pd loading on the antibody, the conjugates were further analysed by ICP-MS, with protein concentrations determined UV–Vis analysis at 280 nm (see experimental, Table 1). ICP-MS analysis showed a loading of 2.6 and 9.0 Pd atoms per antibody for **14a** and **14b**, respectively, comparable to that calculated from MALDI-TOF MS analysis**.**

The Herceptin–Pd conjugates **14a** and **14b** were evaluated for their ability to activate the Rhodamine-based pro-fluorophore **3**, with **14a** displaying very moderate activity with an increase in fluorescence of just 1.5–2-fold (Fig. 5D). As a control, this degaging reaction was also carried out using the catalytic handle **1** in the presence of unmodified, native antibody – this, likewise, dramatically dampened down the activity of unconjugated catalyst **1**, presumably due to metal coordination to the antibody. However, when the Pd-loading on the antibody was increased by factor of 5 (**14b**), a 5-fold increase in fluorescence was observed (the molar ratios of palladium used were the same in all cases (10 μM)).

3. Conclusions

An NHC-Pd catalyst, bearing an azide bioconjugation handle, was designed and synthesised on the solid-phase. The clinically used antibody Herceptin was successfully loaded with the Pd-catalyst using the strained alkyne DBCO with differing amounts of the catalyst. The Pd–Herceptin conjugates showed catalytic activity in a depropargylation reaction by activating a pro-fluorophore. One limitation is that the reaction conversion with the Pd–Herceptin conjugates was lower than that of the "free catalyst"; however, in a clinical setting, low conversion may not matter if highly toxic prodrugs are used as in ADCs (e.g. monomethyl auristatin E) where only a few molecules per cell are needed to induce toxicity. The next steps will be to begin to test the metal-catalytic antibodies with a variety of prodrugs and evaluation in translatable models. The developed antibody conjugate could also be explored with other metals (for example Ru) or even radioactive catalytic metals for alternative therapeutic approaches. Ultimately, with the range of antibodies available bioconjugatable catalysts could become a powerful technology with potential use in a number of biotechnology-based applications.

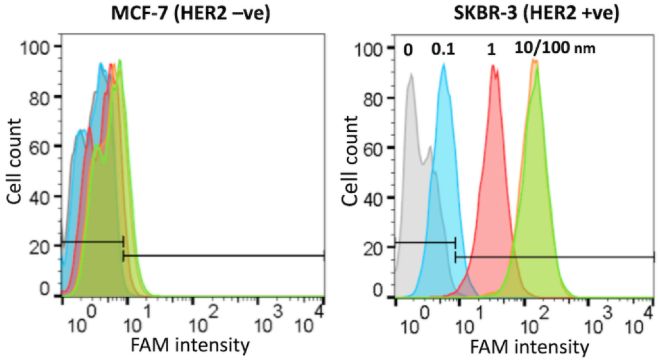


Fig. 4. Flow cytometry analysis of MCF-7 (left, very low HER2 expression) and SKBR-3 (right, high HER2 expression) cells incubated 4 h with 0.1–100 nm of **13**. Cell population colours are as follows; grey = control cells with no antibody; Blue = 0.1 nM; Red = 1 nM; Yellow = 10 nM; Green = 100 nM of **13.** (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Experimental

4.1. General information

Amino acids and aminomethylpolystyrene resin were purchased from GL Biochem (Shanghai) Ltd and NovaBiochem. 5-Carboxyfluorescein azide**12** and DBCO-NHS **10** was purchased from Jena Bioscience, and Herceptin from Carbosynth Ltd. Other chemicals and reagents were from Sigma-Aldrich or Acros.

^{1}H and ^{13}C NMR spectra were recorded on an automated Bruker AVA 500 (500 and 126 MHz, respectively) in the indicated solvents at 298 K. Chemical shifts (δ) are quoted in parts per million (ppm) using the deuterated solvent as an internal standard and all coupling constants (J) were measured in Hertz (Hz). Resonances are specified as singlets (s), doublets (d), triplets (t), multiplets (m), broad singlets (br s) or aromatics (Ar). Low Resolution Mass Spectra (LRMS) were obtained using an Agilent LCMS 1100 ChemStation with a G1946B quadrupole mass detector. High Resolution Mass Spectra (HRMS) were performed on a Bruker 3.0 T Apex II spectrometer. All HRMS data is quoted in positive mode unless specified. MALDI-TOF MS (positive mode, 2,5-Dihydroxybenzoic matrix) were obtained with a Bruker UltraflexExtreme MALDI TOF/TOF instrument (calibrated to native Herceptin) and analysed using the Bruker Daltonics flexAnalysis software. The difference in mass between the highest intensity peaks was determined and then divided by the molecular weight of the palladium ligand conjugated to give the degree of loading by MALDI-TOF MS analysis.

Analytical RP-HPLC was performed on an Agilent 1100 ChemStation equipped with a Kinetex 5 μm XB-C18 100A (50 mm × 4.6 mm) column eluting with a gradient of water and MeCN (5 to 95%) with 0.1% formic acid over 10 min with a flow rate of 1 mL/min, with compounds detected by an ELS detector and a multi-wavelength detector. Preparative RP-HPLC purifications were performed on an Agilent Technologies HP1100 Chemstation eluting with water and MeOH (5% MeOH to 95% over 25 min) with 0.1% formic acid on a Kinetex 5 μm XB-C18 100A (150 mm × 21.2 mm) column with a flow rate of 10 mL/min, or on an Eclipse XDB-C18 5 μm (9.4 mm × 250 mm) column with a flow rate of 1 mL/min.

4.2. Solid-phase synthesis of catalyst 1

Catalyst **1** was synthesised on an aminomethyl polystyrene resin (0.675 mmol/g, 100–200 mesh) functionalised with a Fmoc-Rink-amide linker. The yields were calculated based on resin loading and material recovered after HPLC purification (without exchangeable ligand L1).

Fmoc-Ahx-OH or Fmoc-Val-OH couplings. The Fmoc-protected amino acids (5 eq., 1 mmol) were dissolved in DMF (3 mL) and mixed with Oxyma (5 eq., 1 mmol) for 5 min. To this, DIC (5 eq., 1 mmol, 157 μL) was added and mixed for 5 min further. The solution was added to the resin and stirred for 45 min. The resin was then washed with DMF (3 × 5 mL), DCM (3 × 5 mL) and MeOH (3 × 5 mL).

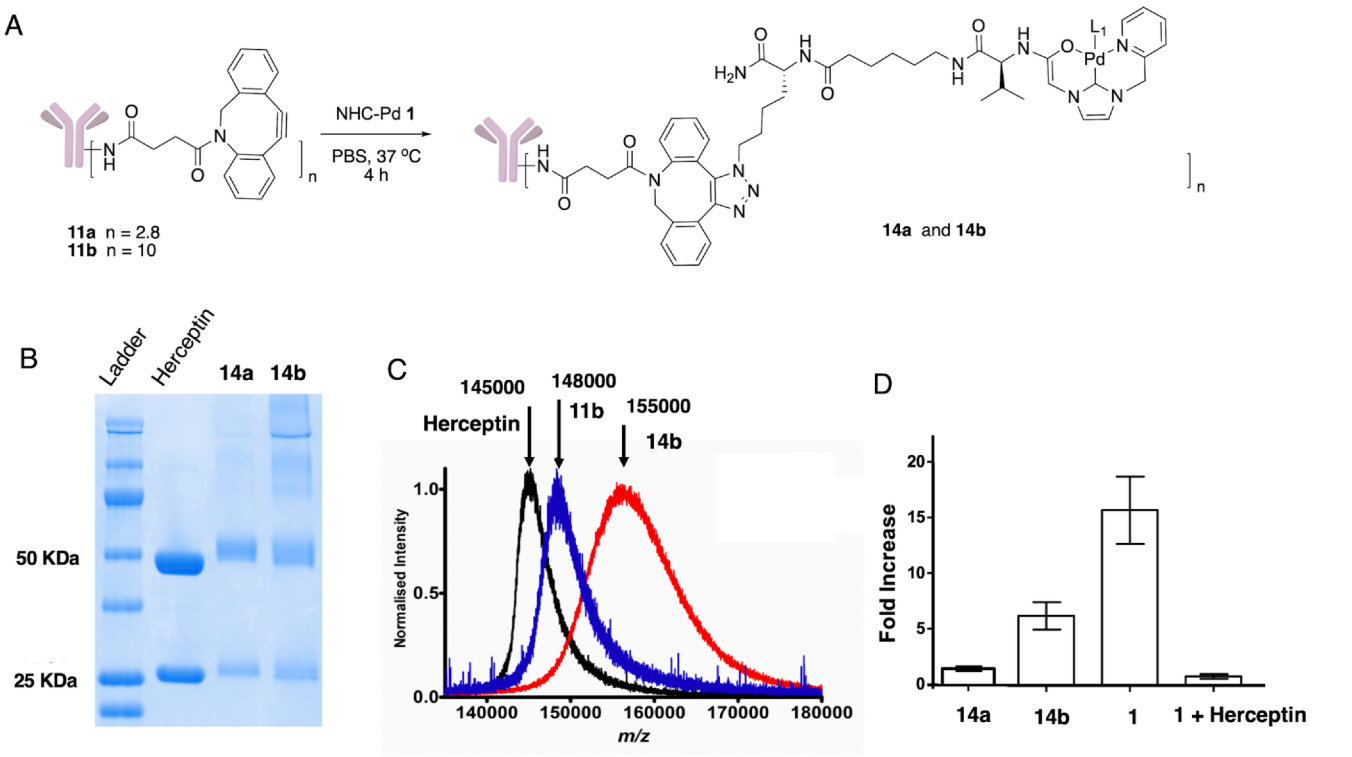


Fig. 5. A) Coupling of the NHC–Pd catalyst **1** onto Herceptin to give the Pd–Herceptin conjugates **14a** and **14b** with two different Pd loadings. B) SDS-PAGE gel of the loading of Hercepin with the catalyst **1**. C) MALDI-TOF MS analysis of the conjugation chemistry: Herceptin in black, **11b** in blue and **14b** in red (145 kDa, 148 kDa, 155 kDa respectively). D) Degaging of *bis-N,N'*-propargyloxycarbonyl rhodamine **3** (100 μM) by Pd–Herceptin catalysts **14a** and **14b**. NHC–Pd **1** and "free" **1** in solution with Herceptin were used as a control. Reactions were carried out at 37 °C, for 18 h in PBS, with 10 μM Pd concentrations in all cases (antibody in the control was 10 μM). n = 3, error bars show standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Protein concentration (based on absorbance at 280 nm) and Pd content (based on ICP-MS) of the Pd–antibody (mAb) conjugates.

	Pd (ppm)	Pd (nmol/mg mAb)	mAb (mg/mL)	Pd (μM)	mAb (μM)	Pd/mAb ratio
14a	4.22	18.6	2.21	39.8	15.2	2.6
14b	5.91	75.7	0.96	55.7	6.2	9.0

Fmoc deprotections. The pre-swollen resin (300 mg, 1 mmol/g) was treated with piperidine in DMF 20% (*v/v*) (2 × 10 min) and washed with DMF (3 × 5 mL) and then DCM (3 × 5 mL).

Bromoacetic acid coupling. The resin (300 mg, 1 mmol/g) was washed with DMF (2 × 5 mL) and a solution of bromoacetic acid (830 mg, 6 mmol) and DIC (460 μL, 3 mmol) in DMF (3 mL), pre-mixed for 20 s, was added. The mixture was heated under microwave irradiation in an SP Wave (Biotage) for 20 min at 60 °C. The resin was subsequently washed with DMF (3 × 5 mL), and DCM (3 × 5 mL).

Imidazole coupling. To the resin (300 mg, 1 mmol/g) washed with DMF (2 × 5 mL), a solution of imidazole (413 mg, 6 mmol) in DMSO (2 mL) was added and shaken for 5 min. A solution of for 40 min at 60 °C. The resin was subsequently washed with DMF (3 × 5 mL), and DCM (3 × 5 mL).

Imidazole *N*-alkylation. To the resin (300 mg, 1 mmol/g) washed with DMF (2 × 5 mL) trimethylamine (139 μL, 1 mmol) was added followed by a solution of 2-bromomethyl pyridine (759 mg, 3 mmol) in DMF (2 mL) and shaken for 5 min. A solution of $AgNO_3$ (254 mg, 1.5 mmol) in DMF (1 mL) was added and shaken for 2 min. The mixture was heated under microwave irradiation for 90 min at 60 °C. The resin bearing the ligand **2** was subsequently washed with DMF (3 × 5 mL), then piperidine in DMF 20% (*v/v*, 3 × 5 mL) and DCM (3 × 5 mL).

4.2.1. N-[2-[1-(2-Pyridinylmethyl)-1H-imidazolium-3-yl]acetyl]-L-valine-(5-carboxypentyl)-L-lysine(azide) amide 2

The compound was cleaved from the Rink-amide linker using a mixture of TFA and H_2O (19:1, 3 mL) for 2 h. The solution was drained from the resin and the solvent was removed under reduced pressure. The residue was re-dissolved in H_2O/ACN (95:5) and purified by preparative RP-HPLC to give ligand **2** for analysis. Yield: 60%; HPLC (254 nm),; t_R 2.6 min, Purity 95%; ^{1}H NMR (500 MHz, Methanol-d_4): δ 8.57 (1H, dt, J = 4.7, 1.6 Hz), 7.89 (1H, td, J = 7.7, 1.7 Hz), 7.69 (1H, d, J = 2.0 Hz), 7.62 (1H, d, J = 2.1 Hz), 7.51 (1H, dt, J = 7.8, 1.2 Hz), 7.42 (1H, ddd, J = 7.7, 4.9, 1.1 Hz), 5.57 (2H, s), 5.11 (2H, s), 4.32 (1H, dd, J = 9.0, 5.2 Hz), 4.13 (1H, d, J = 7.1 Hz), 3.30 – 3.27 (2H, m), 3.19 (2H, td, J = 6.9, 3.0 Hz), 2.25 (2H, t, J = 7.4 Hz), 2.07 (1H, h, J = 6.5 Hz), 1.85 – 1.77 (1H, m), 1.71 – 1.57 (5H, m), 1.55 – 1.40 (4H, m), 1.39 – 1.31 (2H, m), 0.98 (6H, t, J = 6.4 Hz); ^{13}C NMR (126 MHz, MeOD): δ 177.1 (C), 176.1 (C), 173.1 (C), 166.8 (C), 154.2 (C), 151.1 (CH), 139.2 (CH), 125.3 (CH), 125.1 (CH), 124.2 (CH), 123.9 (CH), 60.9 (CH), 54.9 (CH_2), 54.2 (CH), 52.3 (CH_2), 51.9 (CH_2), 40.2 (CH_2), 36.6 (CH_2), 32.8 (CH_2), 32.0 (CH), 30.0 (CH_2), 29.5 (CH_2), 27.5 (CH_2), 26.5 (CH_2), 24.2 (CH_2), 19.7 (CH_3), 18.7 (CH_3); HRMS ESI-MS calculated 583.3463 for $C_{28}H_{43}O_4N_{10}$; found 583.3483 $[M+H]^+$.

4.2.2. N-[2-[1-(2-Pyridinylmethyl)-1H-imidazol-2-ydiene-3-yl]acetyl]-L-valine-(5-carboxypentyl)-L-lysine (azide) amide palladium(II) 1

The resin (300 mg, 1 mmol/g) bearing ligand **2** was swollen for 30 min under Ar in anhyd. DCM. The resin was then washed with anhydrous DMF (2 × 5 mL) under Ar. The resin was swollen with a minimal amount of anhydrous DMF after which 2-*tert*-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine (BEMP) (173 μL, 0.6 mmol) was added and stirred for 30 min under Ar. The solution was then removed and dichloro(1,5-cyclooctodiene)palladium (127 mg,

0.45 mmol) in anhyd. DMF (1 mL) was added to the resin and stirred overnight under Ar at room temperature. The resin was drained, washed with DMF (2 × 5 mL) and DCM (2 × 5 mL), and 95% TFA/DCM was added for 2 h. The solution was drained from the resin and the solvent was removed under reduced pressure. The crude mixture was then re-dissolved in H_2O/ACN (95:5) and purified by preparative RP-HPLC to give catalyst **1** as a white solid in 18% yield (25 mg, 36 μmol). HPLC (254 nm),; t_R 3.0 min, purity 95%; HRMS ESI-MS calculated 687.2342 for $C_{28}H_{41}O_4N_{10}^{106}Pd$; found 687.2384 $[M+H]^+$).

4.3. Antibody conjugations

4.3.1. Herceptin-DBCO 11a and 11b

To Herceptin (50 μL, 20 mg/mL), PBS (150 μL) was added. DBCO-NHS in DMSO (5 mg/mL, 5 or 25 eq.) was added to the antibody and the solution was shaken at 37 °C for 4 h in an Eppendorf ThermoMixer®. The solution was diluted in PBS (200 μL) and purified using Amicon centrifugal spin columns (0.5 mL, MWCO 10 kDa) using a Thermo Scientific Heraeus Pico 17 benchtop centrifuge (13200 rpm, 10 min), and washed with PBS (10 × 0.5 mL) to elute unreacted DBCO-NHS. The concentrated protein solution (50 μL) was collected in an Eppendorf tube by centrifugation (1 min, 13200 rpm).

11a. MALDI-TOF MS: Intensity max at 146,000 Da; Degree of labelling: 2.8 mol of DBCO per mole of Herceptin.

11b. MALDI-TOF MS: Intensity max at 148,000 Da; Degree of labelling: 10 mol of DBCO per mole of Herceptin.

4.3.2. Herceptin-FAM 13

To **11a** (45 μL, 20 mg/mL in PBS) 5-carboxyfluorescein azide **12** (15.8 μL, 1 mg/mL in PBS, 5 eq.) was added. The solution was shaken at 37 °C for 4 h in an Eppendorf ThermoMixer® C. The solution was diluted in PBS (200 μL) and purified using Amicon centrifugal spin columns (0.5 mL, MWCO 10 kDa) using a Thermo Scientific Heraeus Pico 17 benchtop centrifuge (13200 rpm, 10 min) and washed with PBS (10 × 0.5 mL) to remove any unreacted DBCO-NHS. The concentrated protein solution (50 μL) of **13** was collected in an Eppendorf tube by centrifugation (1 min, 13200 rpm).

SDS-PAGE: Bands at 25,000 and 50,000 Da; MALDI-TOF MS: Intensity max at 147,000 Da; Absorbance: 1.41 at 280 nm, 0.97 at 492 nm; Degree of labelling: 2.8 mol of fluorophore per mole of Herceptin.

4.3.3. Herceptin–Pd catalyst 14a and 14b

To **11a** and **11b** (45 μL, 20 mg/mL in PBS), a solution of **1** (3.45 μL or 17.3 μL, 10 mM DMSO) was added. The solution was shaken at 37 °C for 4 h at 1000 rpm in an Eppendorf ThermoMixer®. The solution was diluted in PBS (200 μL) and purified using Amicon centrifugal spin columns (0.5 mL, MWCO 10 kDa) using a Thermo Scientific Heraeus Pico 17 benchtop centrifuge (13200 rpm, 10 min) and washed with PBS (10 × 0.5 mL) to elute unreacted **1**. The concentrated Herceptin–Pd solution (50 μL) was collected in an Eppendorf tube by centrifugation (1 min, 13200 rpm).

14a. SDS-PAGE: Bands at 25,000 and 50,000 Da; MALDI-TOF MS: Intensity max at 148,000 Da; ICP-MS: 4.22 ppm (15.2 μM); Degree of labelling: 2.7 mol of Palladium per mole of Herceptin.

14b. SDS-PAGE: Bands at 25,000 and 50,000 Da; MALDI-TOF MS: Intensity max at 155,000 Da; ICP-MS: 5.91 ppm (55.7 μM); Degree of labelling: 10 mol of Palladium per mole of Herceptin.

4.4. Protein analysis

SDS-PAGE. Antibody samples (1 mg/mL, 10 μL) were diluted with 2 × sample buffer (10 μL) and heated to 95 °C for 5 min. The samples were then loaded into a precast 10% polyacrylamide gel (Bio-Rad) and run at 80 V (30 min) followed by 160 V (90 min). The gel was submerged in Coomassie staining solution (~100 mL) for 4 h and then de-stained for 24 h (de-staining solution (100 mL) replaced every 6–12 h). The gel was then viewed under white light in a Bio-Rad Gel Doc XR + system and analysed on Image Lab software.

Determination of protein concentrations. Absorbance values for the antibody conjugates were measured on a Thermo Scientific NanoDrop 2000c. To determine the degree of labelling, the ε values for the fluorophore (68,000 cm^{-1} M^{-1}) and Herceptin (210000 cm^{-1} M^{-1}) at 280 nm were used.

ICP-MS analysis. ICP-MS analysis was done on an Agilent 7500ce ICP-MS suitable for the trace analysis of metals between 0.001 and 10.0 ppb. The Pd–antibody conjugates (1 μL, protein concentration measured by absorbance) was added to conc. HNO_3 (75 μL), and the solution was diluted to 5% HNO_3 with water (1.5 mL), and Pd analysis was carried out with calibration against Pd standard solutions (n = 3).

4.5. Fluorophore activation

The catalyst **14a** and **14b** solutions were freshly prepared in PBS, and **1** in PBS with 5% of acetonitrile. Assays were carried out in black 96-well plates. 50 μL of the pro-fluorophores **3**[17,18] or **5**[19] in PBS were added per well to give a final test concentration of 10 μM, followed by 50 μL of the catalyst solution (0.8 mol% Pd). The increase in fluorescence ($\lambda_{Ex/Em}$ = 485/520 nm) at 37 °C was recorded over time (4 h or 12 h) (Synergy Biotek plate reader; Gen5 software) (n = 3). The relative increase in fluorescence was compared to the PBS control.

4.6. Cell-based assays

SKBR-3 and MCF-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, L-glutamine (4 mM) and penicillin and streptomycin (100 units/mL) in a 5% CO_2 atmosphere at 37 °C. The day before the assays, the cells were washed with PBS, detached with trypsin/ EDTA (0.25% trypsin, 1 mM EDTA), counted, and diluted with DMEM to the appropriate concentration.

For flow cytometry analysis, SKBR-3 and MCF-7 cells were plated in a 24-wellplate (20000 cells/well) and grown for 24 h at 37 °C. The medium was removed and **13** in DMEM (at 0.1, 1, 10 and 100 nm) was added and incubated for 18 h at 37 °C. After incubation, cells were washed twice with PBS, harvested with trypsin/EDTA (0.25% trypsin, 1 mM EDTA) and resuspended in DMEM. Intracellular fluorescence was analysed by flow cytometry (Becton Dickinson (BD) FACSAriaTM). A total of 10,000 events were analysed per sample.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors thank the EPSRC/MRC CDT OPTIMA (EP/L016559/1) for funding.[28]

References

1 Scott AM, Wolchok JD, Old LJ. *Nat. Rev. Cancer*. 2012;12:278–287.
2 Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC. *Nat. Biotechnol.* 2005;23:1073–1078.
3 Kubota T, Niwa R, Satoh M, Akinaga S, Shitara K, Hanai N. *Cancer Sci.* 2009;100:1566–1572.
4 Polakis P. *Pharmacol Rev*. 2016;68:3–19.
5 Drago JZ, Modi S, Chandarlapaty S. *Nat. Rev. Clin. Oncol.*. 2021 https://doi.org/10.1038/s41571-021-00470-8.
6 Beck A, Goetsch L, Dumontet C, Corvaïa N. *Nat. Rev. Drug Discov.* 2017;16:315–333.
7 Bargh JD, Isidro-Llobet A, Parker JS, Spring DR. *Chem. Soc. Rev.* 2019;48:4361–4374.
8 Nicolaou KC, Rigol S. *Angew. Chemie*. 2019;58:11206–11241.
9 Sharma SK, Bagshawe KD. *Adv. Drug Deliv. Rev.* 2017;118:2–7.
10 Bagshawe KD, Sharma SK, Begent RHJ. *Expert Opin. Biol. Ther.* 2004;4:1777–1789.
11 Niculescu-Duvaz I, Springer CJ. *Adv. Drug Deliv. Rev.* 1997;26:151–172.

12 Sherwood RF, Melton RG, Alwan SM, Hughes P. *Eur. J. Biochem.* 1985;148:447–453.
13 Bagshawe KD, Sharma SK. *Transplant. Proc.* 1996;28:3156–3158.
14 Worm-Leonhard K, Meldal M. European. *J. Org. Chem.*. 2008:5244–5253.
15 Indrigo E, Clavadetscher J, Chankeshwara SV, Megia-Fernandez A, Lilienkampf A, Bradley M. *Chem. Commun.* 2017;53:6712–6715.
16 Cherukaraveedu D, Cowling PT, Birch GP, Bradley M, Lilienkampf A. *Org. Biomol. Chem.* 2019;17:5533–5537.
17 Yusop RM, Unciti-Broceta A, Johansson EMV, Sánchez-Martín RM, Bradley M. *Nat. Chem.* 2011;3:239–243.
18 Clavadetscher J, Hoffmann S, Lilienkampf A, et al. *Angew. Chemie Int. Ed.* 2016;55:15662–15666.
19 Santra M, Ko SK, Shin I, Ahn KH. *Chem. Commun.* 2010;46:3964–3966.
20 Cho K, Najman R, Dean TW, Ichihara O, Muller C, Bradley M. *J. Am. Chem. Soc.* 2006;128(19):6276–6277.
21 Hudis CA, Engl N. *J. Med.* 2007;357:39–51.
22 Vu T, Claret FX. *Front. Oncol.* 2012;2:62. https://doi.org/10.3389/fonc.2012.00062.
23 Jewett JC, Sletten EM, Bertozzi CR. *J. Am. Chem. Soc.* 2010;132(11):3688–3690.
24 Cho HS, Mason K, Ramyar KX, et al. *Nature*. 2003;421:756–760.
25 Clarke R, Brünner N, Katzenellenbogen BS, et al. *Proc. Natl. Acad. Sci. USA*. 1989;86:3649–3653.
26 Slamon DJ, Godolphin W, Jones LA, et al. *Science*. 1989;244:707–712.
27 Coussens L, Yang-Feng TL, Liao YC, et al. *Science*. 1985;230:1132–1139.
28 Cowling P. *PhD Thesis, Palladium Catalysts for Cancer Imaging*. University of Edinburgh; 2020.