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In-Cell Dual Drug Synthesis by Cancer-Targeting Palladium Catalysts

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S1. Supporting Figures

Fig. S1: Pd functionalised fluorescent microspheres. (A) Copolymerisation of aminomethyl styrene, a vinyl styrene fluorescein derivative ($\lambda_{\text{ex/em}} = 498/521 \text{ nm}$) and divinylbenzene to give amine-functionalised microspheres ($207.7 \pm 5.9 \text{ nm}$ by DLS analysis). (B) TEM image of fluorescent microspheres loaded with Pd nanoparticles ($8.3 \pm 1.7 \text{ nm}$). The white arrows show Pd nanoparticles in the fluorescent microspheres. (C) Flow cytometry histogram showing the fluorescence intensity of the microspheres (laser excitation at 488 nm and emission filter of 530/30 nm). Grey: microspheres without polymerisable fluorescein derivative; black: fluorescent microspheres loaded with Pd.

Fig. S2. Receptor-mediated cellular uptake of cRGDfE-PdNP. U87-MG cells were pre-incubated with c(RGDfE)-OH (0 μM to 10 μM) for 30 min and cRGDfE-PdNP (50 μg/mL) added with uptake analysed by flow cytometry after 30 min. (A) Percentage uptake based on intracellular mean fluorescence intensity of the cell population and normalised to cells incubated with 0 μM c(RGDfE)-OH, Cells incubated with varying concentrations of the peptide c(RGDfE)-OH and cRGDfE-PdNP, showing a decrease in uptake with increasing c(RGDfE)-OH concentration. (B) Flow cytograms showing: (i) untreated U87-MG cells (control), (ii) cells incubated with cRGDfE-PdNP, and (iii) cells incubated with 5 μM cRGDfE-OH peptide and cRGDfE-PdNP (50 μg/mL).

Fig. S3. (A) Normalised absorbance of **1** ($\lambda_{\text{abs}} = 472$ nm) and **2** ($\lambda_{\text{abs}} = 583$ nm) in H_2O . (B) Fluorescence emission spectra of **1** and **2** (10 μM) in H_2O ($\lambda_{\text{ex}} = 583$ nm).

Fig. S4. Pd catalysed decaging of **1**. A solution of **1** (20 μM) and cRGDfE-PdNP (2 μM Pd) was shaken at 37 $^{\circ}\text{C}$ for 18 h in PBS/EtOH (4:1, 0.5 mL) or 5 % FBS and the increase in fluorescence was measured (with an excitation filter 530/25 nm and emission filter 590/35 nm) and the relative fluorescence increase displayed relative to a control solution of **1** in the absence of cRGDfE-PdNP.

Fig. S5. (A) Pd catalysed decaging of **3**: (B) A solution of **3** (250 μM) and cRGDfE-PdNP (25 μM Pd) was shaken at 37 $^{\circ}\text{C}$ for 24 h in PBS (0.5 mL) and the formation of **4** monitored by HPLC (254 nm). In the HPLC chromatogram, the black trace shows the formation of **4**; the dotted trace is a control solution of **3** in the absence of cRGDfE-PdNP.

Fig. S6. (A) Pd catalysed Suzuki Miyaura cross-coupling of **5** and **6**. (B) A solution of **5** (250 μM), **6** (5 eq, 1 mM) and cRGDfE-PdNP (156 μL , 3.1 mg, 25 μM Pd) was shaken at 37 $^{\circ}\text{C}$ for 24 h in PBS (0.5 mL) and formation of **7** monitored by HPLC (254 nm), showing deboronation product of **6** (15 %) based on integration of **6**.

Fig. S7. (A) Dual Pd catalysed decaging of **3** and Suzuki Miyaura cross-coupling of **5** and **6**. A solution of **3** (250 μ M), **5** (250 μ M), **6** (5 eq, 1 mM), and cRGDfE-PdNP (25 μ M Pd) was shaken at 37 $^{\circ}$ C for 24 h in PBS (0.5 mL) and formation of **4** and **7** monitored by HPLC (254 nm).

Fig. S8. Cell viability of U87-MG cells treated with cRGDfE-PdNP (50 μ g/mL, 0.2 μ M Pd) for 1 h, washed, and treated with **3** (20 μ M), **5** (4 μ M), and **6** (10 μ M) for 3 d (MTT assay, n = 3). *** P<0.001 by one-way ANOVA with Dunnett post-test, compared to the untreated control group.

S2. Experimental

General

Amino acids and the 2-chlorotrityl polystyrene resin were purchased from GL Biochem (Shanghai) Ltd and NovaBiochem, and all other chemicals from Sigma Aldrich and Acros. ^1H and ^{13}C spectra were recorded on a Bruker AVA-500 (at 500 and 125 MHz, respectively) at 298 K in the solvents indicated (resonances are in ppm). Low Resolution Mass Spectra (LRMS) were obtained using 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. MALDI-ToF mass spectra were obtained with a Bruker UltraflexExtreme MALDI TOF/TOF instrument and analysed with Bruker Daltonics flexAnalysis software. Analytical RP-HPLC was performed using an Agilent 1100 Chemstation equipped with a Zorbax Eclipse C18 reverse phase column (4.6 mm $\times$ 100 mm, 3.5 micron) eluting with a gradient of water/formic acid (0.1%) to MeCN/formic acid (0.1%) over 10 minutes with a flow rate of 1 mL/min, and compounds were detected by an ELS detector and multiwavelength detector. Semi-preparative RP-HPLC was performed on an Agilent 1100 system equipped with a Zorbax Eclipse XDB-C18 reverse-phase column (250 mm $\times$ 10 mm, 5 μm) with a flow rate 2 mL/min and eluting with a gradient of (a) water/formic acid (0.1%) and (b) MeCN/formic acid (0.1%) of 5 to 95% (b) over 15 min. ICP-OES were obtained on a Perkin Elmer Optima 5300 DV ICP-OES suitable for the trace analysis of metals between 0.0002–1000 ppm. UV/Vis measurements were performed on a BioTek HT Synergy multi-mode reader. Fluorescence spectra were recorded on a SPEX Fluoromax, using 1 cm path length fused silica cuvettes. Dynamic light scattering (DLS) and zeta potentials by laser Doppler velocimetry were carried out on a Malvern Zetasizer Nano-ZS. TEM analysis was carried out on a JEOL JEM-1400 Plus TEM. Representative images were collected on a GATAN OneView camera. Flow cytometry analysis was carried out on a Becton Dickinson (BD) FACSAriaTM and analysed using FlowJo.

5-fluoro-1-propargyl-uracil **3**, 1-cyclopentyl-3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine **6**, and 1-cyclopentyl-3-(1H-pyrrolo[2,3-b]pyridin-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP-121) **7** were synthesised according to previously reported procedures.^[1,2]

Synthesis of cRGDfE-Doc-OH

Scheme S1. Synthetic route for the side-chain protected cyclic(R(Pbf)GD(^tBu)fE)-Doc-OH.

The peptide was synthesised on a 2-chlorotrityl chloride polystyrene resin (loading 1 mmol/g). The spacer was coupled to the resin by adding a solution of Fmoc-Doc-OH (3 eq, 0.1 M) and DIPEA (6 eq) in anhydrous DMF to the resin (1 eq, 300 mg resin, preswollen in anhydrous DCM) and shaken for 1 h. After washing (DMF, DCM, and MeOH) and Fmoc deprotection (20% piperidine in DMF), the relevant amino acid sequence was coupled to the resin following the procedure given below. A Ninhydrin test was used to detect the presence of amines after each coupling step. Once the sequence was complete, the allyl protecting group was removed by treatment with a solution of Pd(PPh₃)₄ (0.4 eq) and PhSiH₃ (24 eq) in degassed DMF for 4 h. Fmoc deprotection of the terminal amino group was performed with 20% piperidine in DMF. Cyclisation of the peptide was performed on resin by adding a solution of HBTU (6 eq), HOBT (6 eq) and DIPEA (12 eq) in DMF and shaking for 3 h. After cleavage from the resin with HFIP/DCM (1:4), the peptide was purified by semi-preparative HPLC, and analysed by MALDI-Tof MS and HPLC. For the competition assay, the peptide c(RGDfE)-OH (without the Doc spacer) was cleaved from the resin with simultaneous side-chain protecting group removal with TFA/H₂O/TIS (95:2.5:2.5).

Coupling procedure. The appropriate Fmoc-protected amino acid (3 eq, 0.1 M) and Oxyma (3 eq) in DMF were stirred for 10 min. DIC (3 eq) was added and the mixture stirred for a further 5 min. The

coupling solution was added to the resin (1 eq), preswollen in DCM, and shaken for 45 h. After filtration, the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL), and MeOH (3 × 10 mL).

Fmoc removal procedure. The resin was preswollen in DCM, and treated with 20 % piperidine in DMF (v/v) (3 mL, 2 × 5 min). The resin was isolated by filtration, and washed with DMF (3 × 10 mL), DCM (3 × 10 mL), and MeOH (3 × 10 mL).

Cleavage from the resin of the protected cyclic peptide c(R(Pbf)GD(^tBu)fE)-OH. (a) The resin (100–125 mg), preswollen in DCM, was treated with 2–3 mL of HFIP/DCM (1:4) for 3 h. The cleavage solution was isolated from the resin by filtration, and the compound precipitated by the addition of cold Et₂O and the product isolated by centrifugation. (b) The resin (100–125 mg), preswollen in DCM, was treated with 2–3 mL of TFA/H₂O/TIS (95:2.5:2.5) for 15 min. The cleavage solution was isolated from the resin by filtration, and the target compound precipitated by the addition of cold Et₂O and the product isolated by centrifugation.

Table S1: Characterisation of cRGDfE peptides.

	MALDI-ToF MS		HPLC	
	Calc.	Found	t _r (min), purity	
Protected c(R(Pbf)GD(^tBu)fE)-Doc-OH	C ₄₉ H ₇₂ N ₉ O ₁₅ S ⁺	1058.637	1058.486	6.84 >99%
Deprotected c(RGDfE)-OH	C ₂₆ H ₃₅ N ₈ O ₉ ⁺	604.274	604.260	4.68 >99%

Synthesis of cRGDfE-PdNP

Fluorescent microspheres were synthesised according to a previously reported procedure by Bradley^[3] and gave microspheres (207.7 ± 5.9 nm, DLS) with a solid content of 20 mg/mL and an amine loading of 0.04 μmol/mg.

Pd functionalisation was carried out according to a previously reported procedure.^[4] The Pd content was analysed by ICP-OES and gave a Pd loading of 0.004 μmol Pd / mg, with Pd nanoparticles of 8.3 ± 1.7 nm.

Coupling of c(RGDfE)-Doc-OH to Pd loaded FLMS

Scheme S2. Synthetic route to cRGDfE-Doc-OH-functionalised fluorescent Pd microspheres.

Fmoc-deprotection was carried out with 20 % piperidine/DMF (2 × 30 min) and the resulting amine-functionalised microspheres washed with DMF (3 × 1 mL). The protected peptide c(R(Pbf)GD(^tBu)fE)-Doc-OH (6 eq) was activated with dipyrrolidino(*N*-succinimidyl)oxy)carbenium hexafluorophosphate (HSPyU) (6 eq) in DMF at 50 °C for 1 h, after which the solution was added to the microspheres and shaken at 50 °C for 3 h. The coupling procedure was repeated 3 times until completion (0.02 μmol cRGDfE-Doc-OH ligand / mg microspheres) and monitored by the ninhydrin test and zeta potential measurements (shown in Table S2). The microspheres were washed with DMF (3 × 1 mL) and the side-chain protecting groups removed with HCl/HFIP (0.1 M, 3 × 1 h, monitored by the ninhydrin test). The resulting cRGDfE-PdNP were washed with DMF (3 × 1 mL), MeOH (3 × 1 mL) and H₂O (3 × 1 mL) and re-dispersed in H₂O.

Table S2: Zeta potential measurements of Pd-functionalised microspheres.

	Zeta Potential (mV)
NH ₂ -Pd microspheres	-5.2 ± 0.9
Protected cR(Pbf)GD(^t Bu)fE-PdNP	-22.8 ± 1.8
Deprotected cRGDfE-PdNP	-13.6 ± 1.5

Bis-propargylcarbamate protected Cresyl Violet (1)

Cresyl violet perchlorate (100 mg, 0.276 mmol) was dissolved in anhydrous DMF (1 mL) and cooled to 0 °C. Separately, pyridine (10 eq, 2.8 mmol, 140 µL) and propargyl chloroformate (5 eq, 1.4 mmol, 116 µL) were each dissolved in anhydrous DMF (0.5 mL) and the solutions added dropwise to the cresyl violet. The reaction mixture was stirred at room temperature for 18 h, after which it was diluted with ⁱPrOH/CHCl₃ (25:75, 10 mL), washed with 5 % HCl (2 × 10 mL) and NaHCO₃ (2 × 5 mL) and dried over MgSO₄. The solvent was removed *in vacuo* and the resulting crude purified by silica gel column chromatography (EtOAc/Hexane 1:2) to give **1** as an orange solid in 16 % yield (18.8 mg, 0.044 mmol). R_f (EtOAc/Hexane 1:2) = 0.42. ¹H-NMR (500 MHz, DMSO-*d*⁶) δ (ppm) 10.50 (s, 1H), 8.61 (d, *J* = 8.0 Hz, 1H), 8.31 (d, *J* = 7.8 Hz, 1H), 7.86 (t, *J* = 7.6 Hz, 1H), 7.82 – 7.75 (m, 2H), 7.62 (d, *J* = 2.1 Hz, 1H), 7.43 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.54 (d, *J* = 1.4 Hz, 1H), 4.91 (d, *J* = 2.2 Hz, 2H), 4.83 (d, *J* = 2.3 Hz, 2H), 3.67 (t, *J* = 2.0 Hz, 1H), 3.61 (t, *J* = 2.0 Hz, 1H). ¹³C-NMR (125 MHz, DMSO-*d*⁶) δ (ppm) 162.2, 159.3, 152.4, 148.8, 144.5, 142.2, 139.0, 131.8, 131.5, 130.9, 130.0, 128.5, 124.9, 124.2, 124.1, 115.4, 103.9, 100.2, 78.6, 78.5, 78.0, 78.0, 53.6, 52.5. IR (thin film) ν (cm⁻¹) = 3310, 3283, 2852, 1737, 1692, 1591, 1543, 1428, 1222, 1058, 874, 688. HRMS calcd for C₂₄H₁₆N₃O₅ [M+H]⁺ 426.1084, found 426.1077.

***In vitro* evaluation of proc-cresyl violet decaging**

To 500 µL of PBS/EtOH 4:1 (pH 7.3, 10 mM) or 5 % FBS, **1** (10 mM in DMSO) was added to give a final concentration of 20 µM. cRGDfE-PdNP (0.25 mg, 0.001 µmol Pd, 2 µM Pd) were added and the mixture shaken at 1400 rpm for 18 h at 37 °C. Fluorescence was recorded at λ_{ex/em} 530/633 nm and displayed relative to a control solution of **1** (20 µM).

***In vitro* evaluation of Pro-5FU decaging**

To 500 µL of PBS, **3** (10 mM in DMSO) was added to give a final concentration of 250 µM. cRGDfE-PdNP (3.1 mg, 0.0125 µmol Pd, 25 µM Pd) were added and the mixture shaken at 1400 rpm for 24 h at 37 °C, with analysis by HPLC with absorbance at 254 nm.

***In vitro* evaluation of Suzuki-Miyaura cross-coupling**

To 500 µL of PBS, **5** and **6** (10 mM in DMSO) were added to give a final concentration of 250 µM and 1 mM, respectively. cRGDfE-PdNP (3.1 mg, 0.0125 µmol Pd, 25 µM Pd) were added and the mixture shaken at 1400 rpm for 24 h at 37 °C, with analysis by HPLC with absorbance at 254 nm.

***In vitro* evaluation of simultaneous Pro-5FU decaging and Suzuki-Miyaura cross-coupling**

To 500 µL of PBS, **3**, **5** and **6** (10 mM in DMSO) were added to give a final concentration of 250 µM, 250 µM and 1 mM, respectively. cRGDfE-PdNP (3.1 mg, 0.0125 µmol Pd, 25 µM Pd) were added and the mixture shaken at 1400 rpm for 24 h at 37 °C, with analysis by HPLC with absorbance at 254 nm.

Cell culture

U87-MG and MCF-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), *L*-glutamine (4 mM) and antibiotics (penicillin and streptomycin, 100 units/mL). Cell culture was performed in a 5 % CO₂ atmosphere at 37 °C in a SteriCult 200 (Huco-Erloss) incubator. 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.

Cellular uptake studies: Flow cytometry

U87-MG and MCF-7 grown in a 24-wellplate (2×10^4 cells/well) were incubated with a solution of cRGDfE-PdNP in DMEM (50 $\mu\text{g/mL}$) and incubated. After incubation, cells were washed twice with PBS, harvested with Trypsin/EDTA and analysed by flow cytometry (Becton Dickinson (BD) FACS AriaTM). For the competition assay, U87-MG cells were first incubated with a solution of cRGDfE-OH peptide (0 μM to 10 μM) for 30 min, then cRGDfE-PdNP were added (50 $\mu\text{g/mL}$, 2.5 $\mu\text{L/mL}$) and incubated for 30 min at 37 °C. After incubation, the cells were analysed as described above.

Cellular uptake studies: Live cell confocal microscopy

U87-MG cells were plated on an 8-well μ -Slide (with ibiTreat, ibidi GmbH, Germany) with 1×10^4 cells/well and grown for 24 h at 37 °C. The medium was removed and a solution of cRGDfE-PdNP in DMEM (50 $\mu\text{g/mL}$) and CellLight[®] early Endosomes-RFP, BacMam 2.0 (ThermoFisher) solution, according to the manufacturer's instructions, was added and incubated for 18 h at 37 °C. After incubation, cells were washed twice with PBS, stained with Hoechst 33342 for 10 min and imaged with a Leica SP5 confocal microscope. Microscope lasers settings were: excitation laser lines at 405 nm, 488 nm, and 543 nm with emission filters of 385–470 nm for Hoechst 33342 (nuclei stain), 505–530 nm for cRGDfE-PdNP, and 595–615nm for RFP (CellLight[®] early Endosomes-RFP).

Intracellular proc-cresyl violet decaging

U87-MG cells grown in a 24-wellplate (2×10^4 cells/well) were incubated with a solution of cRGDfE-PdNP (50 $\mu\text{g/mL}$, 0.2 μM Pd) in DMEM and incubated for 1 h at 37 °C. The cells were washed twice with PBS and a solution of **1** (20 μM) was added and incubated for 1–18 h. After incubation, cells were washed twice with PBS, harvested with Trypsin/EDTA, and analysed by flow cytometry (Becton Dickinson (BD) FACS AriaTM).

Intracellular proc-cresyl violet decaging: Live cell confocal microscopy

U87-MG cells were plated on an 8-well μ -Slide (with ibiTreat, ibidi GmbH, Germany) with 1×10^4 cells/well and grown for 24 h at 37 °C. The medium was removed and a solution of cRGDfE-PdNP in DMEM (50 $\mu\text{g/mL}$, 0.2 μM Pd) was added and the cells incubated for 1 h at 37 °C. The cells were washed twice with PBS and a solution of **1** (20 μM) was added and incubated for 6 h. After incubation, cells were washed twice with PBS, stained with Hoechst 33342 for 10 min and imaged with a Leica SP5 confocal microscope. Microscope lasers settings were: excitation laser lines at 405 nm, 488 nm, and 595 nm with emission filters of 385–470 nm for Hoechst 33342 (nuclei stain), 505–530 nm for cRGDfE-PdNP, and 590–650 nm for synthesised compound **2**.

Cell viability studies

U87-MG cells grown in a 96-well plate (5,000 cells/well) were incubated with a solution of **3**, **4**, **5**, **6**, or **7** at desired concentrations and the cells incubated for 5 d. The media was then replaced with 100 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL) in PBS and the cells incubated for 3 h at 37 °C. After incubation, the resulting formazan crystals were dissolved by adding 100 μ L of MTT solubilisation solution (10 % Triton-X 100 in 0.1 N HCl in isopropanol). The absorbance was measured at a wavelength of 570 nm (BioTek HT Synergy multi-mode reader using the Gen5 microplate and imaging software 2.0.) and the results compared to untreated cells.

Intracellular Pro-5FU decaging and Suzuki-Miyaura cross-coupling

U87-MG cells grown in a 96-well plate (5,000 cells/well) were incubated with a solution of cRGDfE-PdNP (50 μ g/mL, 0.2 μ M Pd) in DMEM and the cells incubated for 1 h at 37 °C. The cells were then washed twice with PBS and a solution of either **3** (20 μ M), **5** and **6** (4 μ M and 10 μ M, respectively), or **3**, **5**, and **6** (20 μ M, 4 μ M, and 10 μ M, respectively) was added to the wells and incubated for 5 d. The media was then replaced with 100 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL) in PBS and the cells incubated for 3 h at 37 °C. After incubation, the resulting formazan crystals were dissolved by adding 100 μ L of MTT solubilisation solution (10 % Triton-X 100 in 0.1 N HCl in isopropanol). The absorbance was measured at a wavelength of 570 nm and results compared to untreated cells.

S3. References

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