Extracellular palladium-catalysed dealkylation of 5-fluoro-1-propargyl-uracil as a bioorthogradly activated prodrug approach

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A bioorthogonal organometallic reaction is a biocompatible transformation undergone by a synthetic material exclusively through the mediation of a non-biotic metal source; a selective process used to label biomolecules and activate probes in biological environs. Here we report the in vitro bioorthogonal generation of 5-fluorouracil from a biologically inert precursor by heterogeneous Pd⁰ catalysis. Although independently harmless, combined treatment of 5-fluoro-1-propargyl-uracil and Pd⁰-functionalized resins exhibits comparable antiproliferative properties to the unmodified drug in colorectal and pancreatic cancer cells. Live-cell imaging and immunoassay studies demonstrate that the cytotoxic activity of the prodrug/Pd⁰-resin combination is due to the in situ generation of 5-fluorouracil. Pd⁰-resins can be carefully implanted in the yolk sac of zebrafish embryos and display excellent biocompatibility and local catalytic activity. The in vitro efficacy shown by this masking/activation strategy underlines its potential to develop a bioorthogradly activated prodrug approach and supports further in vivo investigations.

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Bertozzi’s landmark works in the early 2000s (refs 1,2) demonstrated that artificial chemistry and biological chemistry can take place within the same physical space, yet do not interfere with each other. Through the development of labelling strategies based on the chemoselective conjugation of two biologically-inert reactive partners, this revolutionary chemical paradigm—so-called bioorthogonal chemistry—has enabled the non-intrusive real-time study of a wide range of biomolecules in their native environs3,4. Because of the exclusive nature of the bioorthogonal concept, the range of chemical reactions that meet its requirements has been limited in practice to the Staudinger ligation1 and cycloadditions between spring-loaded reactive partners (metal-free click chemistry5–7). Nevertheless, the bioorthogonal concept may integrate additional bio-independent chemical processes that are promoted by abiotic activators such as bio-compatible electromagnetic radiations5–7 and non-biological transition metals5,9.

Although the application of transition metals in medicine have been investigated for many centuries, its use to mediate chemoselective transformations in cells—for example, bioorthogonal organometallic (BOOM) reactions8—is a recent addition to the chemical biology field8–14. In 2006, Streu and Meggers10 described the first application of a ruthenium catalyst to carry out Alloc deprotection of a caged fluorophore inside human cells. The aim to minimize the inherent toxicity of the catalyst motivated the Bradley group to investigate heterogeneous catalysis to mediate palladium chemistry inside cells. Pd0-functionalized microspheres operated as a dual catalytic/cell delivery system able to enter cells in vitro and catalyze allylcarbamate cleavage and Suzuki-Miyaura cross-coupling in the cytoplasm without cytotoxicity9,11. Notable examples of palladium-based applications in cell culture includes copper-free Sonogashira coupling of an alkyne-encoded cytoplasmic ubiquitin protein12, extracellular Suzuki reaction to label Escherichia coli cell surface components13 and the detection of intracellular CO based on the reactivity of a cyclopalladated probe towards carbonylation14.

Owing to its biological inertness, metallic Pd0 shows the safest toxicity profile among all palladium species15 and is frequently used in metal alloys for dental restoration16. Based on the unique catalytic properties and biocompatibility of this metal, we hypothesized that extracellular heterogeneous catalysis mediated by a palladium-functionalized implant could enable the chemoselective conversion of a systemically administered prodrug into its active form in a spatially controlled manner. As opposed to biolabile prodrugs, whose activation process relies on metabolic pathways17, an efficient palladium-activated prodrug therapy would be entirely dependent on the distinct catalytic properties of this transition metal and therefore the prodrug would remain intact in the absence of a palladium source. By intratumoural implantation of a metal-functionalized device, palladium-labile prodrugs would be exclusively activated within the cancerous tissue. Locoregional generation of cytotoxic agents would enable increasing dosing while reducing adverse side effects in distant tissues and organs, an optimal strategy to treat advanced tumours that cannot be resected by surgery. Notably, this spatially targeted approach would serve to expand the therapeutic window and scope of potent cytotoxic drugs such as 5-fluorouracil (5FU), which has a long history in oncology practice but a clinical activity limited by its safety profile18.

5FU is converted intracellularly into cytotoxic nucleotides metabolites via functionalization on its N1 position. Its active metabolites either inhibit directly thymidylate synthase or become incorporated into RNA and DNA molecules to disrupt normal cell functions (Fig. 1)19. Based on this mode of action, modification of the N1 position of 5FU with biochemically stable groups would hinder its intracellular activation and result in a significant loss of cytotoxic activity. Here we report the introduction of allyl, propargyl and benzyl moieties at the N1 position of 5FU as a means to minimize prodrug’s susceptibility to cleavage by hydrolytic enzymes, while, at the same time, rendering them potentially sensitive to palladium catalysis. These alkyl functional groups have been widely used in organic

Figure 1 | Bioorthogonally activated prodrug strategy and toxigenic mode of action. Chemical masking of the N1 position of 5-fluorouracil (5FU) with a biochemically inert palladium-sensitive group would impede 5FU intracellular activation and, consequently, prevent its pharmacological activity. Such a prodrug will be selectively converted into 5FU by the catalysis of Pd0, for instance, in the affected area, organ or tissue where a Pd0-functionalized device has been surgically implanted, thus inducing a local treatment of the disease and reducing side effects of drug in distant tissues and organs. dTMP, thymidine monophosphate; dUMP, deoxyuridine monophosphate.
synthesis as OH protection strategies\textsuperscript{20} and, importantly, recent studies have shown the sensitivity of allyl and propargyl phenolic ethers to oxidative cleavage by palladium species in biocompatible conditions\textsuperscript{21–23}. Although palladium-mediated N-dealkylations typically require temperatures incompatible with cell survival (>80 °C)\textsuperscript{24–26}, we hypothesized that the particular features of the 5FU heterocyclic system (relatively low pK\textsubscript{a} values because of the charge delocalization of 5FU aromatic conjugate bases\textsuperscript{27}, Supplementary Fig. 1a) could promote its properties as a leaving group relative to more basic amino groups.

To activate prodrugs in the extracellular space, we investigate the application of a robust heterogeneous catalytic system based on Pd\textsuperscript{0}-functionalized polyethylene glycol (PEG)-polystyrene resins\textsuperscript{28} (Pd\textsuperscript{0}-resins) that are larger than human cells (150 μm in diameter). As previously reported\textsuperscript{9,11}, Pd\textsuperscript{0} nanoparticles display high catalytic activity, can be readily generated and trapped in an amino-functionalized polymer matrix, and are safe for biological applications. Our results show that alkylation of the N1 position of 5FU suppresses the drug’s cytotoxic properties and exhibits full stability in cell culture. Remarkably, the propargyl derivative (5-fluoro-1-propargyl-uracil (Pro-5FU)) displays high sensitivity to Pd\textsuperscript{0}-mediated heterogeneous catalysis, thus allowing the bioorthogonal generation of 5FU under biocompatible conditions (37 °C, isotonic media and physiologic pH). Although independently harmless, combined treatment of Pro-5FU and Pd\textsuperscript{0}-resins exhibits equivalent antiproliferative properties to unmodified 5FU in colorectal and pancreatic cancer cells, underlining the in vitro efficacy of this unprecedented masking/activation strategy.

**Results**

**Preparation of Pd\textsuperscript{0}-resins and 5FU prodrugs.** Pd\textsuperscript{0}-resins (Fig. 2a,b) were prepared from NovaSyn TG amino resin HL using the procedure developed in the Bradley group\textsuperscript{29} with minor modifications. Transmission electron microscope images showed dark nanoparticles (5 nm) regularly distributed across the resins (Fig. 2b), whereas the powder X-ray diffraction spectra presented the distinctive pattern of the face-centred cubic lattice of Pd\textsuperscript{0} (Supplementary Fig. 1b). Pd\textsuperscript{0}-resins were visibly differentiated from non-functionalized resins (naked resins) because of the dark colour of the former (Supplementary Fig. 1c). Palladium content was determined to be 2.83% (w/w) by inductively coupled plasma-optical emission spectrometry.

1-Allyl-5FU (All-5FU), Pro-5FU and 1-benzyl-5FU (Bn-5FU; Fig. 2c) were synthesized in a single step by reaction of 5FU with the corresponding alkyl bromide in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene. N-propargyl-2-pyridone (1a) and N-propargyl-4-pyridone (1b) were synthesized following the same procedure (Supplementary Methods).

Cell-free Pd\textsuperscript{0}-mediated prodrug-into-drug conversion. To recreate a biocompatible scenario, reactions were carried out at 37 °C in an isotonic solution with a physiologic pH. Prodrugs (100 μM) and Pd\textsuperscript{0}-resins (1 mg ml\textsuperscript{−1}, [Pd\textsuperscript{0}] = 266 μM) were dispersed in PBS, incubated for 6–48 h and analysed by high-performance liquid chromatography (HPLC). Figure 2d shows Pro-5FU completely disappeared from the crude mixture after 24 h, with 5FU being the major reaction product. In accordance with previous observations\textsuperscript{26}, mass spectrometry analysis of the reaction crude indicated the formation of nontoxic 1-hydroxyacetone—a natural lipid metabolite also known as acetol\textsuperscript{30}—as a byproduct from the oxidative cleavage of the propargyl group (Supplementary Fig. 2a,b). In contrast, conversion analysis of All-5FU and Bn-5FU did not show substantial levels of 5FU after 48 h (Supplementary Fig. 3a). N-Depropargylation of Pro-5FU using sub-stoichiometric amounts of Pd\textsuperscript{0} confirmed the catalytic nature of the reaction, albeit 72 h were required to achieve completion (Supplementary Fig. 3b). To determine whether Pro-5FU dealkylation is mediated by heterogeneous catalysis, Pd\textsuperscript{0}-resins were incubated in PBS at 37 °C for 24 h, micro-filtered to eliminate solid contents and Pro-5FU added to the solid-free solution for additional 24 h incubation. HPLC analysis detected unreacted Pro-5FU as major mixture component and relatively small quantities of 5FU (Supplementary Fig. 3c), indicating that Pd\textsuperscript{0} is in part released from the resins into the solution, although this amount contributes

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**Figure 2 | Pd\textsuperscript{0}-mediated prodrug-into-drug conversion assays in biocompatible environment.** (a) Scanning electron microscope image of Pd\textsuperscript{0}-resins at approximately 200 magnification. Scale bar, 50 μm. (b) Transmission electron microscope image of a Pd\textsuperscript{0}-resin cross-section at approximately 190,000 magnification. Scale bar, 50 nm. (c) Pd\textsuperscript{0}-mediated dealkylation of 5-fluorouracil (5FU) prodrugs under biocompatible conditions (37 °C, isotonic media and physiologic pH). (d) Representative HPLC traces of Pd\textsuperscript{0}-mediated conversion of Pro-5FU into 5FU. Reaction crudes were analysed using an ultraviolet detector (280 nm) at t = 0 h, t = 6 h and t = 24 h. Conversion studies were performed in triplicate. All-5FU, 1-allyl-5FU; Bn-5FU, 1-benzyl-5FU; Pro-5FU, 5-fluoro-1-propargyl-uracil.
minimally to the reaction catalysis. Treatment of two additional N-propargylated heterocycles, N-propargyl-2-pyridone (1a) and N-propargyl-4-pyridone (1b), under the same conditions afforded the dealkylated derivatives in 24 h (Supplementary Fig. 3d).

Pro-5FU bioorthogonality. Based on the primary malignancies against which 5FU is currently prescribed18,19, colorectal HCT116 and pancreatic BxPC-3 cancer cell lines were chosen as models for antiproliferative and mechanism-of-action studies. To evaluate the efficacy of the masking strategy in suppressing 5FU cytotoxic properties, dose response studies were carried out with unmodified drug and Pro-5FU (0.01 μM to 1 mM). Cell viability was determined at day 5 using PrestoBlue reagent and analysed by spectrofluorometry to calculate EC50 (5FU) values of 2 and 0.14 μM for HCT116 and BxPC-3 cells, respectively (Supplementary Fig. 4a,b). Notably, Pro-5FU displayed no cytotoxicity at any concentration used (EC50 [Pro-5FU]/EC50 [5FU] > 500), proving the effectiveness of the deactivation strategy. To test whether Pro-5FU could be biotransformed into 5FU in cells with higher metabolic capacity, cell viability assays were carried out in liver HepG2 cells30. As shown in Supplementary Fig. 4c, although 5FU was significantly cytotoxic at low micromolar concentration (EC50 = 1.4 μM), Pro-5FU did not exhibit a toxic effect (EC50 > 1 mM), illustrating the high biochemical stability of the propargyl group to enzymatic cleavage. Because of the close structural similarities between Pro-5FU and 5FU, it was considered that the prodrug could function as a competitive inhibitor of the enzymatic routes followed by 5FU to generate its cytotoxic metabolites. This potential drawback of the prodrug strategy was ruled out by incubating 5FU with increasing concentrations of Pro-5FU up to a prodrug/drug ratio of 100:1, which exhibited no antagonistic effect (Supplementary Fig. 5a,b). In addition, dose response studies of 1-hydroxyacetone (main reaction byproduct) with HCT116 and BxPC-3 cancer cell lines showed no effect in cell viability (Supplementary Fig. 5c,d).

Antiproliferative effect of Pro-5FU/Pd0-resin combination. After assessing the catalyst concentration range tolerated by cells and optimal for catalysis (Supplementary Fig. 6), 1 mg ml−1 (for HCT116) and 0.66 mg ml−1 (for BxPC-3) of Pd0-resins were used for the biological assays. Preliminary cell screening of palladium-mediated generation of 5FU from the three prodrugs confirmed that Pro-5FU/Pd0-resin combination was effective in producing a strong toxicogenic effect (Supplementary Fig. 7a,b). Hence, a range of concentrations of Pro-5FU and Pd0-resins was incubated independently (negative controls) and in combination (BOOM conversion assay) to study cell proliferation in comparison to unmodified 5FU (positive control). As observed in Fig. 5a,b, the toxicogenic effect generated by the prodrug/catalyst combination was comparable to 5FU cytotoxicity, exhibiting similar EC50 values (Supplementary Fig. 7c,d). Independently incubated, neither the prodrug nor the palladium source exhibited antiproliferative activity.

Cytotoxic mode of action of Pro-5FU/Pd0-resin combination. To study the phenotypic effect of the prodrug/catalyst system compared with 5FU, cell proliferation was monitored in real-time using an IncuCyte ZOOM microscope, which allows quantification of cell confluence as a function of time by image-based analysis. As shown in Fig. 5c,d, negative controls exhibited a standard growth curve, whereas the proliferation of cells treated with 5FU rapidly decreased after a few hours. Growth curves of HCT116 cells incubated with the Pd0-resin/Pro-5FU combination showed two distinct phases: a regular increment for 24 h followed by a drastic fall to reach cytotoxic levels comparable to 5FU at day 5. In the case of BxPC-3 cells, population curves of cells incubated with either 5FU or the Pd0-resin/Pro-5FU combination showed similar bell-shaped growth curves. As observed in Fig. 5e,f and Supplementary Movies 1 and 2, treatment-induced changes in cell morphology indicate analogous antiproliferative/cell death induction between 5FU and the prodrug/catalyst system.

To study these phenotypic responses at the molecular level, total expression and post-translational modifications of cancer relevant pathways were quantified using ZepTosens Reverse Phase Protein Microarray analysis31. Supplementary Fig. 8a shows that total and phosphorylated p53 (Ser15) were significantly upregulated in HCT116 cells at 24 h following exposure to Pro-5FU/Pd0-resin combination, which correlates with the response observed under 5FU treatment and the DNA damage response mechanism expected from this drug32. In contrast, no induction was observed in cells treated with either Pro-5FU or Pd0-resin on their own. These observations were corroborated by western blotting (Supplementary Fig. 9).

Pd0-Resin biocompatibility and catalytic activity in zebrafish. The biocompatibility of the palladium-functionalized device in vivo was investigated by carefully introducing a single Pd0-resin in the yolk sac of zebrafish embryos 24 h after fertilization and monitoring their early development by phase contrast microscopy. As shown in Fig. 4a, zebrafish embryos containing a Pd0-resin (indicated with a red arrow) developed normally into the larval stage with no signs of toxicity or alteration of phenotype.

To study the catalytic functionality of the Pd0-resins in the zebrafish yolk, palladium-sensitive pro-fluorophore 3 (Fig. 4b) was prepared and incubated with zebrafish embryos containing either a non-functionalized resin (naked resin) or a Pd0-resin for 24 h. N,N’-bis(propargyloxycarbonyl)-protected rhodamine 110 (3) was synthesized from rhodamine 110 chloride by reaction with propargyl chloroformate and triethylamine in dry dimethylformamide (DMF). The enhanced lipophilic properties of non-fluorescent probe 3 facilitated its diffusion across biological barriers. On the contrary, highly fluorescent rhodamine 110 generated upon propargylcarbamate cleavage was trapped in the physiological compartment where it was produced because of its high hydrophilicity, thus enabling the imaging of localized palladium-mediated catalysis8,10,11. As shown in Fig. 4c, a strong fluorescent signal was clearly observed from the area surrounding the Pd0-resin in the yolk sac of zebrafish embryos 24 h after fertilization and monitoring their early development by phase contrast microscopy. As shown in Fig. 4a, a strong fluorescent signal was also observed from the gastrointestinal system33,34 in fish incubated with pro-fluorophore 3 regardless of the presence or absence of Pd0-resin (Fig. 4c, pointed with white arrow heads, and Supplementary Fig. 10), suggesting that the N-propargyloxycarbonyl (N-Poc) group is biochemically cleaved by digestive enzymes. Although compound 3 exhibited high sensitivity to palladium catalysis, it also displayed low chemical stability in the digestive system, indicating that N-Poc masked prodrugs could not be used via oral administration in a bioorthogonal strategy.

Discussion

For more than a decade1–3, the concept of bioorthogonal chemistry (understood as performing chemical reactions by artificial biologically inert means under biocompatible conditions) has inspired the search for novel biocompatible organic reactions and found a niche application as a labelling strategy to study biomolecules in their native state1–5,35. Indeed, bioorthogonal reactions have become particularly useful to study biological small molecules and macromolecules that cannot be
monitored by genetically encoded reporters or antibodies (for example, lipids, glycans, and so on) both in cells and *in vivo*.

Although metal-free bioorthogonal reactions are considered optimal for living systems because of their biocompatibility, significant progress has been made in recent years in the development of cell-tolerated BOOM reactions, opening up new avenues for the exploitation of the bioorthogonal paradigm. However, the potential of such selective processes in medicine is yet to be determined. Within an inclusive notion of bioorthogonality, the clinical potential of bioorthogonal processes has only been explored to date with the photoinduced generation of cytotoxic reactive oxygen species through the use of a harmless visible light source and a non-toxic photosensitizer, which has become a valuable therapeutic option for local treatment of disease (so-called photodynamic therapy).

In this line of reasoning, here we report a novel palladium-mediated N-dealkylation that takes place under biocompatible conditions and its application beyond the current scope of BOOM reactions: a highly efficient masking method that achieves complete elimination of 5FU cytotoxic activity, while enabling the bioorthogonal restoration of the drug’s pharmacological properties by heterogeneous palladium chemistry in cell culture.

In accordance with 5FU mode of action (Fig. 1), modification of its N1 position with each of the three alkyl groups investigated (allyl, propargyl and benzyl; Fig. 2c) generates harmless prodrugs (Supplementary Fig. 7a,b), which demonstrates the low susceptibility of alkyl groups to cleavage by hydrolytic enzymes. However, under biocompatible conditions, only the propargyl group displays high sensitivity to Pd0-resins, thereby allowing the palladium-catalysed N-dealkylation of Pro-5FU at 37°C in PBS (isotonic, aqueous solution buffered at pH 7.4) within 24 h. Although the high stability of the benzyl moiety was anticipated because of the lack of an additional hydrogen source, the drastic difference in Pd0 sensitivity between Pro-5FU and All-5FU suggests that electronic and/or conformational aspects are critical.
for efficient metal coordination and cleavage (Supplementary Fig. 2a). To briefly examine the potential scope of the N-depropargylolation process, N-propargyl-2-pyridone (1a) and N-propargyl-4-pyridone (1b) were synthesized and treated under the same conditions as Pro-5FU (Supplementary Fig. 3d). Dealkylated products were obtained within 24 h, indicating that this environmentally friendly methodology could be translated to other heterocyclic systems with lactam/lactim tautomery. Because of its implications for the bioorthogonal use of this novel masking/activation strategy, it is important to highlight that the palladium-mediated oxidative cleavage of the N-propargyl group in aqueous media results in the formation of non-toxic 1-hydroxyacetone26 (Supplementary Fig. 2b). The generation of this natural compound29 would minimize any local off-target effects caused by side products originated from the activation process itself.

Our results show that, at the concentrations tested, neither the prodrug nor the palladium source exhibits antiproliferative activity in any of the cancer cell lines (Fig. 3), thus validating the in vitro bioorthogonality of the materials. In contrast, the combined use of Pro-5FU and Pd⁰-resins led to a toxigenic effect comparable to 5FU antiproliferative activity, which demonstrates the in situ generation of 5FU. A variance in cytotoxicity was observed at several doses (Fig. 3a,b), which may be due to the time lag required for the BOOM reaction to take place and produce cytotoxic levels of drug. This difference was not significant in BxPC-3 cells at Pro-5FU doses higher than 1 μM (Fig. 3b), indicating that cytotoxic levels for this sensitive cell line are rapidly generated.

Monitoring of HCT116 cell proliferation by time-lapse imaging allowed visualizing the slower antiproliferative effect led by the Pro-5FU/Pd⁰-resins combination (BOOM conversion assay) in comparison to unmodified 5FU (Fig. 3c). This result is in accordance with the time delay required to generate cytotoxic levels of drug for this cell line. Owing to the higher sensitivity of BxPC-3 cells to 5FU, both drug and the prodrug/catalyst combination experiments showed comparable bell-shaped population curves (Fig. 3d). In agreement with these observations, our immunoassay studies show an analogous cell death induction mechanism between 5FU and the prodrug/catalyst system, corroborating that the cytotoxic activity generated by the prodrug/Pd⁰-resin combination is due to the BOOM synthesis of 5FU in cell culture.

In conclusion, the development of a biochemically resistant palladium-labile 5FU precursor and an extracellular Pd⁰-functionalized device enabled the in vitro bioorthogonal transformation of a biologically inert substance into a cytotoxic agent. Although this novel prodrug strategy is yet to be tested in disease models in vivo, the robustness of the masking strategy, the biocompatibility of the dealkylation conditions and the innocuous reaction byproduct (natural product 1-hydroxyacetone26,29) underline the therapeutic potential of this novel masking/activation approach. Finally, the biocompatibility and local catalytic activity exhibited by the Pd⁰-resin implanted within the zebrafish yolk supports the development of more comprehensive in vivo pharmacodynamics, pharmacokinetics and toxicity investigations in order to study the applicability and potential benefits of implementing a bioorthogonally activated prodrug approach.

**Methods**

**General methods.** Chemicals and solvents were purchased from Fisher Scientific, Sigma-Aldrich or VWR International Ltd. Nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature on a 500-MHz Bruker Avance III spectrometer (Bruker). Chemical shifts are reported in parts per million (p.p.m.) relative to the solvent peak. Rf values were determined on Merck TLC Silica gel 60 F254 plates (Merck) under a 254-nm ultraviolet source. Purification of compounds was carried out via manual flash column chromatography using commercially available silica gel (230–440 mesh, Sigma-Aldrich) under a 254-nm ultraviolet source. Purification of compounds was carried out via manual flash column chromatography using commercially available silica gel (230–440 mesh, Sigma-Aldrich). Cell lines were grown in culture media supplemented with serum (10% fetal bovine serum) and 1-glutamine (2 mM) and incubated in a Heracell 240i tissue culture incubator at 37°C and 5% CO₂. Human colorectal carcinoma HCT116 cells (a kind gift from Dr Van Schaeybroeck) were cultured in Dulbecco’s Modified Eagle Media (DMEM). Human pancreas adenocarcinoma BxPC-3 cells (a kind gift from Dr Mark DuSsbury) were cultured in Roswell Park Memorial Institute media. Human hepatocyte carcinoma HepG2 cells were obtained from Sigma-Aldrich and cultured in DMEM.
Synthesis and characterization of \( \text{Pd}^2 \)-resins. NovoSyn TG amino resin HL (1,000 mg, 0.39 mmol N\textsubscript{N} per g) and palladium acetate (263 mg, 1.17 mmol) were added into a 25 mL Biotage microwave vial (Biotage) with toluene (10 mL) and heated to 80°C under stirring for 10 min. The mixture was then stirred at room temperature for 2 h and the resins subsequently filtered and washed with dichloromethane (5 × 20 ml) and methanol (5 × 20 ml). Resins were dispersed in 10% hydrazine monohydrate in methanol (10 ml) and stirred at room temperature for 1 h. The resins were then filtered and washed with dichloromethane (5 × 20 ml) and dichromethane (5 × 20 ml). Resins were dispersed to a solution of Fmoc-Glu(OH)-OH (216 mg, 0.59 mmol), Oxyma (166 mg, 1.17 mmol), \( \text{N}_{\text{N}} \text{-disopropyl} \)-pyrrolidinomethide (DIPC) (148 mg, 1.17 mmol) and DCM/DMF (2:1, 5 ml) and stirred for 4 h at 40°C. The resins were filtered and washed with dichloromethane (5 × 20 ml), methanol (5 × 20 ml) and H\textsubscript{2}O (5 × 20 ml) and dried in an oven at 40°C for 3 days. Complete coupling was verified by the ninhydrin test after the last methanol wash. Scanning electron microscope/transmission electron microscope images and \( \text{Pd}^2 \) quantification (by inductively coupled plasma–optical emission spectrometry) were carried out as previously described\(^{31}\).

Cell viability studies of 5FU versus Pro-5FU. Cells were seeded in a 96-well plate format at the appropriate cell concentration (3,000 cells per well for HCT116 and BxPC-3 cells, and 4,000 cells per well for HepG2 cells) and incubated for 48 h before treatment. Each well was then replaced with fresh media containing: 5FU (0.01 μM to 1 mM) with DMSO (0.1% v/v), or Pro-5FU (0.01–100 μM) with DMSO (0.1% v/v) or combination of \( \text{Pd}^2 \)-resin + Pro-5FU (0.01–100 μM) with DMSO (0.1% v/v). Untreated cells were incubated with DMSO (0.1% v/v). For the cell viability assay, cells were incubated with secondary Alexa Fluor-conjugated antibody detection reagent (10% v/v) was added to each well and the plate incubated for 45 min. Fluorescence emission was detected using a PerkinElmer EnVision 2101 multilabel reader (Perkin Elmer; excitation filter at 540 nm and emissions filter at 590 nm). All conditions were normalized to the untreated cells (100%) and curves fitted using GraphPad Prism using a sigmoidal variable slope curve.

Reverse phase protein array analysis. Cells were plated in a 6-well plate at a density of 480,000 cells per well and incubated for 48 h. Before adding the produg to the HCT116 cells, Pro-5FU (100 μM) and \( \text{Pd}^2 \)-resins (1 mg ml\(^{-1} \)) were incubated in media at 37°C for 24 h to overcome the time delay required to convert Pro-5FU into 5FU, which was seen to have a relevant effect in this cell type. This pre-incubation procedure was not carried out for the study with BxPC-3 cells (30 μM). \( \text{Pd}^2 \)-resin/Pro-5FU combination and the controls (as before, untreated cells; \( \text{Pd}^2 \)-resins; Pro-5FU and 5FU) were incubated with each cell line for 6 and 24 h, respectively, with the cells washed with PBS (2 × 3 ml) with DMSO (0.1% v/v) before fixation with 10% neutral buffered formalin for 15 min. Arrays were subsequently washed in double-distilled \( \text{H}_2\text{O} \) and dried before being incubated in media at 37°C for 24 h to overcome the time delay required to convert Pro-5FU into 5FU. In this study, the dilution was pre-fractionalized by loading 10 μl of each sample at a final concentration of 0.2, 0.15, 0.1 and 0.05 mg ml\(^{-1} \). The diluted concentration series of each sample was printed onto Zeptosens protein microarray chips (ZeptoChip, Zeptosens-Bayer) under environmentally controlled conditions (constant 50% humidity and 14°C temperature) using a non-contact printer (Nanoplotter 2.1e, GeMS). A single 400 Pico litre droplet of each lysate/standard was deposited on each chip; four spots were printed at a reference grid of Alexa Fluor-647 conjugate BSA consisting of 4 columns × 22 rows was spotted onto each sub-array. Each sample concentration series were spotted in between reference columns. After array printing, the arrays were blocked with an aerosol of BSA solution using a custom designed nebulizer device (ZeptoFOG, Zeptosens-Bayer) for 1 h. The array chips were subsequently washed in double-distilled \( \text{H}_2\text{O} \) and dried before preparing a dual antibody immunosassay comprising of a 24-h incubation of primary antibody (the library of primary antibodies used is in Supplementary Table 1 and were purchased from Cell Signaling Technologies) followed by 2 h incubation with secondary Alexa Fluor-conjugated antibody detection reagent (10% v/v; anti-rabbit A647 Fab; Invitrogen). Following secondary antibody incubation and a final wash step in BSA solution, the immunostained arrays were imaged using the ZeptoReader instrument (Zeptosens-Bayer). For each sub-array, five separate images were acquired using different exposure times ranging from 0.5 to 10 s. Manual intensity images representing the longest exposure for each sub-array were saved. Automatic fluorescent signal detection were automatically selected for analysis using the ZeptoView 3.1 software (Zeptosens-Bayer). A weighted linear fit through the
fourfold concentration series was used to calculate relative fluorescence intensity (RFI) value for each sample replicate. Local normalization of sample signal to the reference ‘blank’ grid was used to compensate for intra- or inter-plate variation. Local normalized RFI values were used for all subsequent analysis. Abundance levels of total p53 protein, phosphorylated p53 (Serine 15) and phosphorylated Akt (Ser 473) were plotted as RFI data calculated by the ZeptoView 3.1 software.

**Western blot analysis.** Cell lysates for HCT116 and BxPC-3 cells were prepared as described above for Zetosens analysis. Protein samples (35 μg) and SeelBlue Plus2 Pre-Stained Standard (7.5 μg) were separated by SDS-PAGE (Bio-Rad, 4–15%) and transferred to PVDF membranes (GE). Western blotting was performed using rabbit monoclonal antibodies against human p53 and Phospho-p53 (Ser 15) ((1:1,000 for each one, Cell Signaling Technologies, cat. no. 9282 and 9284, respectively) at 4 °C overnight. This was followed by 1 h incubation at room temperature with secondary horseradish peroxidase (HRP)-linked antibody (1:10,000, anti-rabbit IgG, Sigma). The loading control, actin, was detected using a mouse monoclonal antibody (1:40,000, Calbiochem, cat. no. CP01) followed by a secondary HRP-linked antibody treatment (1:40,000, anti-mouse IgM, Calbiochem). HRP was detected by addition of POD ECL (Roche) and bands visualized using a ChemiDoc MP Imager (Bio-Rad).

**Zebrafish studies.** For the biocompatibility assay, wild-type zebrafish embryos were collected from AB-TPL breeding pairs and reared at 28 °C in E3 embryo medium. Twenty-four hours post fertilization, embryos were treated with tricaine (anesthetic) and pierced in the yolk with a fine needle. Either a naked resin or a Pd–resin was then rapidly inserted into the yolk opening to minimize loss of yolk. Embryos that lost significant yolk in the procedure were removed from the experiment. Embryos were then gently transferred to fresh E3 medium (n = 5), returned to 28 °C and monitored over 3 days by microscopy. For the catalytic activity assay, either a naked resin or a Pd–resin was implanted in 24 h.p.f. zebrafish embryos (n = 5) following the above procedure and gently transferred to fresh E3 medium. Embryos were incubated for 24 h at 28 °C to ensure the yolk wound was closed. Embryos that lost significant yolk in the procedure were removed from the experiment. Compound 3 was added to the embryo medium (final concentration 5 μM) and fish incubated for additional 24 h at 31 °C to increment the probe activation rate. Fish were imaged using fluorescent microscopy (Olympus Scan-R, Olympus). It is important to note that the zebrafish embryos looked older than developmental 3 d.p.f., probably due to the increased incubation temperature (31 °C). Experiments were repeated three times. Zebrafish husbandry and experiments were performed under Home Office License in compliance with the Animals (Scientific Procedures) Act 1986, and approved by the University of Edinburgh Ethics Committee.

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performed and analysed the zebrafish assays; K.G.M. performed the Zeptosens analysis; W.R. performed the surgical insertion of resins in zebrafish embryos; C.F. prepared materials and contributed to the zebrafish studies; C.T.-S. characterized the Pd\(^{0}\)-resins by powder XRD; M.B. conceived the original concept of using palladium to synthesize probes and drugs in living systems; N.O.C. supervised the biological assays, designed and analysed the Zeptosens, Incucyte and western blot assays, and contributed to the paper preparation; A.U.-B. designed the prodrug approach and the materials, planned and supervised the research, analysed the data and wrote the paper.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare that a patent (application number GB1311107.5) is pending on Pro-5FU and its method of activation for medical use.

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