Development and Bioorthogonal Activation of Palladium-Labile Prodrugs of Gemcitabine

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Supporting Information

ABSTRACT: Bioorthogonal chemistry has become one of the main driving forces in current chemical biology, inspiring the search for novel biocompatible chemospecific reactions for the past decade. Alongside the well-established labeling strategies that originated the bioorthogonal paradigm, we have recently proposed the use of heterogeneous palladium chemistry and bioorthogonal Pd0-labile prodrugs to develop spatially targeted therapies. Herein, we report the generation of biologically inert precursors of cytotoxic gemcitabine by introducing Pd0-cleavable groups in positions that are mechanistically relevant for gemcitabine’s pharmacological activity. Cell viability studies in pancreatic cancer cells showed that carbamate functionalization of the 4-amino group of gemcitabine significantly reduced (>23-fold) the prodrugs’ cytotoxicity. The N-propargyloxycarbonyl (N-Poc) promoiety displayed the highest sensitivity to heterogeneous palladium catalysis under biocompatible conditions, with a reaction half-life of less than 6 h. Zebrafish studies with allyl, propargyl, and benzyl carbamate-protected rhodamines implementing in vivo bioorthogonal organometallic chemistry confirmed N-Poc as the most suitable masking group for implementing in vivo bioorthogonal organometallic chemistry.

INTRODUCTION

Bioorthogonal chemistry is an emerging field of research that focuses on the development and application of selective chemical reactions susceptible to occurring in a biological environment without interfering with the normal function of its components.1,2 These reactions are used as labeling methods to study cell constituents in their native state, including proteins and biomolecules that cannot be monitored by genetically encoded reporters (e.g., glycans, lipids, DNA, etc.).3–4 Looking beyond the well-established labeling strategies that originated the bioorthogonal field, the induction of local chemotherapy by biologically inert means could allow for the reduction of adverse pharmacological effects in distant organs and tissues. This therapeutic paradigm has so far reached the clinic with the so-called photodynamic therapy, a method based on the local generation of cytotoxic reactive oxygen species by spatially controlled excitation (and subsequent energy transfer to the molecular oxygen present in the tissue) of a nontoxic systemically administered photosensitizer with a benign light source.5 Following the same principle of bioorthogonality, novel strategies are being currently investigated by several groups to enable local conversion of chemically masked prodrugs into cytotoxic small molecules using either harmless electromagnetic radiations6,7 or biocompatible heterogeneous catalysts.8

Technically speaking, for a bioorthogonal reaction to be optimal to living systems, the corresponding nonbiotic reactive partners need to display (i) high chemical stability to enzymatic processing and (ii) selective reciprocal reactivity in biocompatible conditions (water, pH 7.4, isotonicity, and 37 °C). Importantly, the reactants and the resulting product/s should be nontoxic, unless this is the intended outcome of the process. The Staudinger ligation and the copper-free azide alkyne cycloaddition, originally developed by the Bertozzi group,9–11 are representative examples of such chemistry. In addition to catalyst-free ligations, the use of transition metals to function- alize biomolecules or activate fluorogenic probes in living cells has recently emerged as a new alternative in the field.12–13 These biocompatible processes, also known as bioorthogonal organometallic (BOOM) reactions,14 are mediated by nonbiotic transition metals such as copper(I),15 ruthenium-(II),16–18 or palladium species8,14,18–23 and have expanded...
the diversity of chemical groups and transformations suitable for use bioorthogonally in cell culture.

Aiming to modulate drug activity by an internal self-renewable control element rather than an external radiation source (e.g., light in photopharmacology), we have recently proposed the development of nonbiological transition metals into biocompatible catalytic devices to allow the activation of systemically administered prodrugs at the location determined by the device. This bioorthogonally activated chemotherapeutic strategy would be optimal to implement focalized treatment of unresectable primary or metastatic tumors and could also find application in the treatment of other disorders such as local infections, Parkinson’s disease, cardiovascular disorders, etc. In the seminal investigation of such an approach, we reported the development of a selective metallo-substrate (5-fluoro-1-propargyluracil) and a solid-phase palladium catalyst (Pd0-resins) as a highly efficient bioorthogonal prodrug/activator system. On the basis of their complementary chemical roles, cytotoxic 5-fluorouracil was efficiently generated via extracellular BOOM catalysis in cell culture. Importantly, when each of these reagents was cultured separately, neither of them exhibited cytotoxicity, demonstrating the feasibility of the method.

To explore this paradigm further, herein we report a comprehensive study on the development and evaluation of novel Pd0-labile prodrugs of gemcitabine (deoxycytidine analogue clinically used to treat several types of cancers) using two masking strategies: (i) carbonate formation at the 5′-OH group of the sugar moiety and (ii) carbamate masking of the 4-NH2 group of the cytosine base (Figure 1).

![Figure 1. Gemcitabine and its cytotoxic mode of action. Following cell entry, gemcitabine is successively phosphorylated by deoxycytidine kinase at its 5′-OH position to generate cytotoxic metabolites.](image_url)

**Scheme 1. Synthesis of Gemcitabine Prodrugs**

- **Reagents and conditions:** (i) DBU (2.5 equiv), DMF, addition of alkylchloroformate (1.5 equiv) at 4 °C, then r.t. overnight (47–53%); (ii) TBS-Cl (1.1 equiv), imidazole (3.5 equiv), DMF, r.t., 2.5 h (88%); (iii) pyridine (3.2 equiv), DMF or THF, addition of alkylchloroformate (1.5 equiv) at 4 °C, then r.t. overnight 12–48 h (42–69%); (iv) TBAF (2.5 equiv), THF, r.t., overnight (62–92%).
formed the corresponding carbonate prodrugs \(2a,p,b\). TBAF-mediated deprotection yielded Cl and imidazole, followed by treatment with the corresponding primary alcohol in position 5 in moderate yields. To synthesize the 4-carbamate derivatives, it was necessary to mask the 4-NH2 group to enzymatic metabolism. To determine the stability of bioorthogonally activated prodrug approach is the robustness of each deactivation strategy in cell culture, for the generation of essential hydrogen bonding interactions with the amino acid Asp133 located within the activation site of the enzyme, thus reducing the efficacy of the phosphorylation process. Furthermore, the main detoxification route of gemcitabine is via hydrolytic deamination of the 4-NH2 group. This is mediated in the liver by cytidine deaminase, which irreversibly converts gemcitabine into its inactive metabolite difluoro-deoxyuridine. Consequently, masking the 4-NH2 group would result in a doubly beneficial effect to the bioorthogonal strategy: it would reduce the cytotoxic properties of the resulting prodrug and protect the circulating drug precursor from premature liver deactivation before reaching the target tissue.

Allyl and propargyloxycarbonyl groups (Alloc and Poc, respectively) have been widely employed as OH and NH2 protection strategies and have shown sensitivity to palladium catalysis in biocompatible conditions. Therefore, both groups were chosen to derivatize gemcitabine to palladium-labile prodrugs. Because of the requirement of an additional hydrogen source to undergo palladium-mediated cleavage, the carboxybenzyl group (Cbz) was used to generate control prodrugs resistant to oxidative catalysis. Prodrugs were synthesized using a semisynthetic strategy by functionalization of gemcitabine, Scheme 1. Treatment of 1 with allyl, propargyl, or benzyl chloroformate in the presence of DBU formed the corresponding carbonate prodrugs \(2a,p,b\) in moderate yields. To synthesize the 4-carbamate derivatives, the primary alcohol in position S’ was first silylated using TBS-Cl and imidazole, followed by treatment with the corresponding alkyl chloroformates. TBDA-mediated deprotection yielded the final carbamate prodrugs \(5a,p,b\) in moderate to good yields.

Bioorthogonality Study: Carbonate vs Carbamate Gemcitabine Prodrugs. Although, in principle, the masking of either the S’-OH or the 4-NH2 group of gemcitabine could both have a significant impact on the drug’s antiproliferative properties, the limiting factor in the development of any bioorthogonally activated prodrug approach is the robustness of the protecting group to enzymatic metabolism. To determine the robustness of each deactivation strategy in cell culture, dose–response studies with gemcitabine (1) and prodrugs \(2a,p,b\) and \(5a,p,b\) were carried out in two human cancer cell lines. Pancreas adenocarcinoma BxPC-3 and Mia PaCa-2 cells were chosen as cell models due to the clinical relevance of gemcitabine as a first-line drug in pancreatic cancer therapy. As shown in Figure 2, carbamate-protected prodrugs \(5a,p,b\) showed significant reduction of cytotoxicity relative to that of gemcitabine \(\text{EC}_{50} \left(5a,p,b\right)/\text{EC}_{50} \left(1\right) \geq 23\) in both cell lines, verifying the efficacy of the deactivation strategy. On the contrary, prodrugs \(2a,p,b\) displayed cytotoxicity similar to that of gemcitabine (1), suggesting that these prodrugs are rapidly bioactivated inside cells. These results are in accordance with the weaker nature of the carbonate bond and strongly indicate that carbonate-based masking strategies are not suitable for bioorthogonal applications. Consequently, only carbamate prodrugs \(5a,p,b\) were moved forward in this study.

PD\(^0\)-Mediated Conversion of Prodrugs \(5a,p,b\) into Gemcitabine. Polystyrene-supported reagents have been employed for a wide range of cell-based applications and have shown excellent biocompatibility both in vitro and in vivo. As catalytically active PD\(^0\) nanoparticles can be readily generated and trapped in an amino-functionalized polystyrene matrix, PD\(^0\)-functionalized resins (150 \(\mu\)m in average diameter, Figure 3a) were prepared from NovaSyn TG amino resin HL following the procedure previously reported and used as the heterogeneous palladium source (4.4% w/w in Pd). To test the susceptibility of the different carbamates to PD\(^0\)-mediated catalysis in a biocompatible environment, compounds \(5a,p,b\) (100 \(\mu\)M) and PD\(^0\)-resins (1 mg/mL, [PD\(^0\)] = 400 \(\mu\)M) were dispersed in PBS (300 mOsm/kg, pH 7.4) and incubated at 37 °C for 24 h (Figure 3b). Reactions were analyzed by HPLC at different time points (0, 6, and 24 h), with prodrugs \(5a,p,b\) being retained for 2.56, 2.45, and 2.96 min, respectively, and the gemcitabine peak appearing at 0.93 min (Figure 3c–f). Treatment of Cbz-protected compound \(5b\) with the palladium source produced negligible levels of gemcitabine (1) after 24 h (Figure 3f), which corresponds for the need of an additional hydrogen source to achieve reductive cleavage of Cbz groups. On the contrary, incubation of compounds \(5a\) and \(5p\) with PD\(^0\)-resins led to the generation of significant levels of gemcitabine (1) in less than 24 h (Figure 3d,e). Interestingly, deprotection of \(5p\) proceeded in a faster and cleaner manner, with a reaction half...

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Study of the prodrugs’ bioorthogonality. Semilog dose–response curves and calculated EC\(_{50}\) values of prodrugs \(2a,p,b\) (in gray) and \(5a,p,b\) (in blue) in comparison to those of unmodified gemcitabine (1, in red) in (a) BxPC-3 and (b) Mia PaCa-2 cells. Cell viability was measured at day 4 using PrestoBlue reagent. Error bars: ± SD from \(n = 3\).
life of <6 h (Figure 3e). These results are in accordance with previous observations\textsuperscript{8,37,38} that have reported the efficient cleavage of various propargylated compounds by palladium species in biocompatible conditions. A potential mechanism for this process and for the reported formation of nontoxic 1-hydroxyacetone\textsuperscript{8,39} (6) as a reaction byproduct is proposed in Figure 3g.

**Pd\textsuperscript{0}-Mediated Prodrug Activation in Cell Culture.**

Bioorthogonal in situ generation of gemcitabine (1) from the carbamate prodrugs was first investigated in standard cell culture conditions with BxPC-3 cells using Pd\textsuperscript{0}-resins as the extracellular activating device. Prodrugs 5a,p,b and Pd\textsuperscript{0}-resins were incubated independently (negative controls) or in combination (BOOM activation assay) and unmodified gemcitabine (1) used as the positive control. While neither the prodrugs nor the Pd\textsuperscript{0}-resins exhibited cytotoxicity, a combination of 5a and 5p with Pd\textsuperscript{0}-resins displayed a strong toxigenic effect (Figure 4), confirming the in situ bioorthogonal synthesis of cytotoxic gemcitabine (1). In the context of the present study, “toxigenic effect” describes the gemcitabine levels generated by each prodrug/Pd\textsuperscript{0}-resin combination in cell culture which in turn result in a cytotoxic phenotype. This effect is indirectly quantified by determining the antiproliferative activity caused by each prodrug/Pd\textsuperscript{0}-resin combination, which is directly proportional to the concentration of cytotoxic drug generated, and qualitatively proved by the verification of its mode of action. Consistent with the anticipated resistance of the N-Cbz functional group to palladium-mediated oxidative cleavage, cells treated with prodrug 5b and Pd\textsuperscript{0}-resins did not produce any negative effect on cell viability.
Dose–response studies were subsequently carried out to compare the treatment of 5a and 5p/Pd0-resin combinations at different concentrations in two cell lines: BxPC-3 and Mia PaCa-2 cells. As shown in Figure 5a,b, reduction of cell viability was observed with compounds 5a and 5p only when incubated with Pd0-resins. Analysis of the toxicogen effect displayed by each prodrug/catalyst combination clearly ranked the N-Poc-protected prodrug 5p as the most effective gemcitabine-generating precursor in the presence of Pd0-resins, exhibiting analogous cytotoxic activity to that of the unmodified drug. Importantly, neither the palladium source nor the prodrugs exhibited antiproliferative activity when separately incubated at any of the concentrations tested, thus supporting the bioorthogonality of the strategy. It is important to note that any of the concentrations tested, thus supporting the exhibited antiproliferative activity when separately incubated at Importantly, neither the palladium source nor the prodrugs generating precursor in the presence of Pd0-resins, exhibiting 5p Alloc group of prodrug results show that the palladium-mediated cleavage of the display similar sensitivity to unmodified drug.

Figure 5. Bioorthogonally activated toxigenic effect in cancer cell culture: (a) BxPC-3 and (b) Mia PaCa-2 cells. Dose–response study: 0.1% (v/v) DMSO (untreated cell control, in gray); 0.67 mg/mL Pd0-resins (negative control, in black); gemcitabine (positive control, in red); prodrug 5a,p (negative control, in blue); and 0.67 mg/mL of Pd0-resins + prodrug 5a,p (BOOM activation, in green). Cell viability was measured at day 5 using PrestoBlue reagent. Error bars: ± SD from n = 3.

Real-time monitoring of BxPC-3 cell proliferation using an IncuCyte ZOOM microscope was performed to compare the effect of each prodrug/catalyst combination upon the kinetics of tumor cell growth. Time-lapse imaging of the negative controls 5a and 5p showed a standard growth curve up to 100% cell confluence level by day 4–5 (light and dark blue curves, respectively; Figure 6), while gemcitabine treatment efficiently suppressed cell proliferation within 24 h. In accordance with the cell viability studies, the toxigenic effect mediated by either prodrug 5a or 5p incubated with Pd0-resins (light and dark green curves, respectively; Figure 6) were patently different (see Supporting Information for a motion picture of the IncuCyte experiment). The prodrug 5p/catalyst combination inhibited cell proliferation with the same efficacy and kinetic response as unmodified gemcitabine (1), proving that cytotoxic levels of the drug were rapidly generated. Although treatment with the 5a/catalyst combination markedly delayed BxPC-3 cell growth relative to the negative controls, its antiproliferative effect was significantly inferior to either the 5p/Pd0-resins combination or unmodified gemcitabine (1), indicating quantitatively lower levels of active drug being generated.

Study of Cytotoxic Mode of Action. Double-stranded breaks caused by DNA damage induce phosphorylation of the variant histone of the H2A protein family, γ-H2AX. Phosphorylated γ-H2AX is responsible for recruiting and localizing the DNA repair mechanism. Gemcitabine damages DNA through partial DNA chain termination and by stalling of replication forks. In order to analyze the toxicogen effect caused by the prodrug/catalyst combination at the molecular level, immunofluorescence studies were carried out to probe for phosphorylated γ-H2AX as a marker of DNA damage in Mia PaCa-2 cells (Figure 7). While the negative controls (Pd0-resins or prodrug 5p separately incubated; Figure 7a,b) did not show the presence of phosphorylated γ-H2AX in the cell nuclei, cells treated with either gemcitabine (Figure 7c) or the Pd0-resins + 5p combination (Figure 7d) expressed significant levels of phospho-γ-H2AX. This study further illustrates that the cytotoxic activity generated from the prodrug/catalyst combination and unmodified gemcitabine is equivalent.

Pd0-Mediated Probe Activation in Zebrafish. We have recently shown that catalytically functional Pd0-resins can be implanted in the yolk sac of zebrafish embryos without inducing toxicity or affecting the embryo development. To compare the in vivo bioorthogonality and palladium sensitivity

Figure 6. Real-time cell confluence study in pancreatic cancer BxPC-3 cells. The cell population was monitored for 120 h using an IncuCyte ZOOM system in an incubator (5% CO2 and 37 °C). Drug/prodrug concentration: 30 nM. Error bars: ± SD from n = 3.

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strategy would allow generating locally increased concentrations of a functionally active small molecule by palladium heterogeneous catalysis in vivo.

## CONCLUSIONS

The chemical protection of gemcitabine’s 4-amino group as a carbamate led to a significant reduction of the drug’s cytotoxic activity. Among the carbamate derivatives investigated, the N-Poc group exhibited the highest sensitivity to palladium-mediated cleavage at 37 °C in both buffer solution and cell culture. This is noteworthy since, to date, our group and others have favored the use of N-Alloc groups over N-Poc to implement coupling and decoupling strategies for BOOM studies. Cell viability, time-lapse microscopy, and DNA damage assays confirmed the bioorthogonal generation of cytotoxic gemcitabine from the combined treatment of extracellular Pd³⁻-resins and the N-Poc-protected precursor in pancreatic cancer cell culture. Unlike the N-Alloc- and N-Cbz-protected derivatives, the bis-N,N’-Poc-rhodamine 110 became locally activated by a Pd³⁻-resin implanted in the yolk sac of zebrafish embryos, further reinforcing its nomination as the carbamate group of choice for developing bioorthogonal studies based on heterogeneous palladium catalysis. Parallel independent studies from Chen et al. (intracellular protein activation by palladium-mediated homogeneous catalysis), published just before the submission of this article, reported analogous findings with the N-Poc group. Finally, the simplicity of the masking strategy together with the biocompatibility and efficacy of the palladium-mediated deprotection process underlines the potential of this method to modulate the pharmacodynamics and pharmacokinetics of amino-containing drugs.

## EXPERIMENTAL PROCEDURES

### Synthetic Procedures. General Methods.

Chemicals and solvents were purchased from Fisher Scientific, Sigma-Aldrich, or VWR International Ltd. Gemcitabine HCl was purchased from Shandong Boyuan Pharmaceutical Co. Ltd. NMR spectra were recorded at ambient temperature on a 500 MHz Bruker Avance III spectrometer. Chemical shifts are reported in parts per million (ppm) relative to the solvent peak. Chemicals and solvents used in the biological studies were purchased from Fisher Scientific and used as received.

### General Methods.

**Pd⁰-functionalized resins** were prepared from NovaSyn TG amino resin HL (0.39 mmol NH₂/g) as previously described.

**Synthesis of Carbonate-Protected Derivatives 2a,p,b.** Gemcitabine HCl (150 mg, 0.5 mmol) was dissolved in dry DMF (3 mL) with DBU (194 μL, 1.30 mmol) under N₂ atmosphere. The mixture was then cooled to 4 °C in an ice bath. Allyl, propargyl, or benzyl chloride (0.75 mmol) was added dropwise to the mixture. The mixture was stirred overnight and allowed to warm up to room temperature (r.t.). The solution was then removed in vacuo, the crude dissolved with 25% isopropanol (IPA) in CHCl₃ (20 mL), and washed with H₂O (20 mL). The aqueous layer was washed with 25% IPA in CHCl₃ (3 × 20 mL) and the combined organic layers dried over MgSO₄, filtered, and concentrated in vacuo.

**4-Amino-1-(2-deoxy-2,2-difluoro-5-O-[allyloxycarbonyl]-β-D-erythro-pentofuranosyl)pyrimidin-2(1H)-one (2a).** The crude was purified by column chromatography using 8% MeOH in DCM (Rf...
Figure 8. Pd-mediated carbamate cleavage of rhodamine precursors in zebrafish. (a) BOOM conversion of nonfluorescent lipophilic compounds 8a,p,b into highly fluorescent hydrophilic rhodamine 110. (b) 3-dpf zebrafish embryos (n = 5) containing a nonfunctionalized resin (indicated with white arrows) after incubation with 5 μM of compounds 8a (top), 8p (middle), and 8b (bottom) for 24 h at 31 °C. (c) 3-dpf zebrafish embryos (n = 5) containing a Pd0-resin (indicated with purple arrows) after incubation with 5 μM of compounds 8a (top), 8p (middle), and 8b (bottom) for 24 h at 31 °C. Fish were imaged by phase contrast and fluorescent microscopy (ex., 470/40; em., 525/50). (d) Analysis of fluorescence intensity/pixel across a horizontal line of approximately 300 μm drawn in the yolk sac of the zebrafish embryos and encompassing both the resin and the area surrounding it. Note: the red and blue lines represent the areas of fluorescence intensity profile measured over the inactive resin (b, middle right panel) and the Pd0-resin (c, middle left panel), respectively.

0.29, 10% MeOH in DCM), which yielded a light green solid (90 mg, 52% yield). 1H NMR (500 MHz, DMSO) δ 7.50 (d, J = 7.5, 1H), 7.42 (d, J = 5.7, 2H), 6.44 (d, J = 6.3, 1H), 6.17 (s, 1H), 5.99–5.89 (m, 1H), 5.79 (d, J = 7.5, 1H), 5.38–5.25 (m, 2H), 4.63 (dt, J = 5.5, 1.3, 2H), 4.47–4.37 (m, 2H), 4.18 (s, 1H), 4.07–4.00 (m, 1H). 13C NMR (126 MHz, DMSO) δ 165.71 (C), 154.80 (C), 154.07 (C), 141.15 (CH), 132.02 (CH), 122.57 (t, JCF = 264.6, C), 118.56 (CH2), 94.87 (CH), 83.89 (CH), 77.22 (CH), 70.09 (t, JCF = 25.2, CH), 68.16 (CH2), 66.31 (CH). LRMS (ESI) m/z 346.0 [M - H]+. HRMS (FAB) m/z [M - H]− calc for C13H12O6N3F2. 346.0856; found, 346.0859.

4-Amino-1-(2-deoxy-2,2-difluoro-5-O-[propargyloxy-carbonyl]-β-D-erythro-pentofuranosyl)pyrimidin-2(1H)-one (2p). The crude was purified by column chromatography using 6% MeOH in DCM (Rf 0.05, 4% MeOH in DCM), which yielded a white solid (70 mg, 53% yield). 1H NMR (500 MHz, DMSO) δ 7.49 (d, J = 7.5, 1H), 7.44–7.33 (m, 7H), 6.44 (d, J = 6.2, 1H), 6.17 (s, 1H), 5.76 (d, J = 7.5, 1H), 5.17 (s, 2H), 4.52–4.35 (m, 2H), 4.18 (s, 1H), 4.03 (t, J = 6.1, 1H).

13C NMR (126 MHz, DMSO) δ 165.60 (C), 154.45 (C), 154.20 (C), 141.15 (CH), 135.04 (C), 128.50 (CH2), 128.42 (CH2), 128.17 (CH2), 122.57 (t, JCF = 258.3, C), 94.83 (CH), 83.86 (CH), 77.18 (CH), 70.10 (t, JCF = 23.4, CH), 69.22 (CH2), 66.39 (CH2). LRMS (ESI) m/z 398.0 [M + H]+. HRMS (FAB) m/z [M - H]− calc for C13H14O6N3F2. 396.1013; found, 396.1016.

Synthesis of Carbamate-Protected Derivatives 5a,p,b. Synthesis of Silylated Derivative 3. Gemicatbine HCl (500 mg, 1.67 mmol) was dissolved in dry DMF (5 mL) with imidazole (398 mg, 5.85 mmol) and TBS-Cl (301 mg, 2.0 mmol) stirred at r.t. overnight. The mixture was then concentrated in vacuo, redissolved in EtOAc (50 mL), and washed with H2O (50 mL). The aqueous layer was washed twice with EtOAc and, subsequently, the organic layers combined, washed with brine (150 mL), and dried over MgSO4. The crude was purified by column chromatography (eluent 8% MeOH in DCM) to yield intermediate 3 as a white solid 552 mg (88%). 1H NMR (500 MHz, DMSO) δ 7.63 (d, J = 7.5, 1H), 7.38 (s, 2H), 6.31 (d, J = 5.1, 1H), 6.14 (t, J = 7.7, 1H), 5.76 (d, J = 7.5, 1H), 4.19–4.06 (m, 1H), 3.95 (d, J = 11.8, 1H), 3.90–3.77 (m, 2H), 0.90 (s, 9H), 0.09 (d, J = 2.1, 6H).

13C NMR (126 MHz, DMSO) δ 165.58 (C), 154.56 (C), 139.91 (CH), 122.95 (t, JCF = 258.3, C), 94.51 (CH), 83.45 (t, JCF = 31.5, CH), 79.76 (CH), 68.23 (t, JCF = 22.7, CH), 60.72 (CH2), 25.71 (CH2), 17.99 (C), −5.52(CH3)3, −5.63(CH2)2-MS (ESI) m/z 378.0 [M + H]+.
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**Synthesis of Intermediate 4a.** Compound 3 (100 mg, 0.27 mmol) was dissolved in dry THF (2.5 mL) with pyridine (61 μL, 0.75 mmol) and the mixture cooled to 4 °C in an ice bath. Alkyll chlorofomate (55 μL, 0.52 mmol) was added dropwise to the mixture and stirred for 1 h. The solvent was removed in vacuo and purified via flash chromatography (eluent: 3% MeOH in DCM) to yield compound 4a as a white solid.

4-Allylcarboxyaminolino)-1-(2-deoxy-2,2-difluoro-5-O-[tert-butyldimethylsilyl]-β-erythro-pentofuranosylpyrimidin-2(1H)-one (4d). Eighty-three milligrams, 69% yield (R<sub>95</sub>, 0.53, 10% MeOH in DCM).

**Synthesis of Intermediates 4p,b.** Compound 3 (100 mg, 0.27 mmol) was dissolved in dry DMF (2.5 mL) with pyridine (61 μL, 0.75 mmol) and the mixture cooled to 4 °C in an ice bath. Propargyl (63 μL, 0.65 mmol) or benzyl chlorofomate (56 μL, 0.39 mmol) was added dropwise to the mixture. The mixture was stirred at r.t. overnight (for 4b) or for 48 h (for 4p). The solvents were removed and the mixture resuspended with ETOAc (30 mL) and washed with H2O (30 mL). The aqueous layer was washed twice more with ETOAc and the combined organic layers dried over MgSO4, solids filtered off, and concentrated in vacuo. The crude was purified via flash chromatography (eluent: 3% MeOH in DCM) to yield compounds 4p,b as white solids.

4-(Propargyloxycarbonylamino)-1-(2-deoxy-2,2-difluoro-5-O-[tert-butyldimethylsilyl]-β-erythro-pentofuranosylpyrimidin-2(1H)-one (4p). Fifty-three milligrams, 48% yield (R<sub>71.0, 0.6, 10% MeOH in DCM).

**Synthesis of Nonfluorescent Probes 8a.p.** Probes 8a and 8p were prepared from rhodamine 110 chloride, 7, as previously described.1,4

**Synthesis of Nonfluorescent Probe 8b.** Under an nitrogen atmosphere, rhodamine 110 chloride (150 mg, 0.41 mmol) was dissolved in dry DMF (4 mL). Separately, benzyl chlorofomate (350 μL, 2.45 mmol) and pyridine (298 μL, 3.69 mmol) were dissolved in DMF (1 mL) and added dropwise to the mixture. The reaction mixture was stirred at r.t. for 48 h, solvents removed in vacuo, and the resulting crude resuspended in 25% isopropanol in CHCl3 (30 mL) and washed with H2O. The aqueous layer was then washed 5 times with 25% isopropanol in CHCl3 (30 mL). The combined organic layers were dried over anhydrous MgSO4 and the solids filtered off and concentrated in vacuo. The crude was purified via flash chromatography (hexane/ethyl acetate:1) to yield bis-N'-benzylcarboxyramino

**Synthesis of Carbamate-Protected Derivatives 5a,p,b.** Compounds 4a,p,b (48 mg, 0.11 mmol, 31 mg, 0.07 mmol respectively) and TBAF solution (1.5 equiv for 4b and 2.5 equiv for 4p) were dissolved in anhydrous THF (3 mL) and stirred at room temperature for 3 h (for 5b) or overnight (for 5a,p). The resulting mixture was concentrated in vacuo and purified via flash chromatography (eluent: 6% MeOH in DCM) to yield compounds 5a,p,b as white solids.

**Synthesis of Intermediate 5a.** Thirty-two milligrams (92% yield). R<sub>0.27</sub> (10% MeOH in DCM).

**Biological Studies.** General Methods. Cell lines were grown in cell culture media supplemented with serum (10% FBS) and l-glutamine (2 mM) and incubated in a tissue culture incubator at 37 °C and 5% CO2. Human pancreas adenocarcinoma BxPC-3 cells (a kind gift from Dr. Mark Durbux) were cultured in Roswell Park Memorial Institute (RPMI) media. Human pancreatic carcinoma Mia PaCa-2 cells (a kind gift from Dr. Simon Wilkinson) were cultured in Dulbecco’s modified Eagle’s media (DMEM). Cell Viability Studies. Cells were seeded in a 96 well plate format at the appropriate cell concentration (2,500 cells/well for BxPC-3 cells and 1,000 cells/well for MiaPaCa-2 cells) and incubated for 48 h before treatment. Each well was then replaced with fresh media containing compound 1 or 2a,p,b or 3a,p,b and incubated for 4 days. Untreated cells were incubated with DMSO (0.1% v/v). PrestoBlue
cell viability reagent (10% v/v) was added to each well and the plate incubated for 1 h. Fluorescence emission was detected using a PerkinElmer VICTOR2 multilabel reader (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.

**Pd0-Mediated Deprotection of 2a,p,b in Biocompatible Conditions.** Compounds 3a, 5p, and 5b (100 μM) were dissolved in PBS (1 mL) with 1 mg of Pd0-resin and shaken at 1400 rpm and 37 °C in a Thermomixer. Reaction crudes were monitored at 0, 6, 24, and 48 h by analytical HPLC (Agilent) using an UV detector at 280 nm to avoid the detection of PBS salts. Eluent A, water and formic acid (0.1%); eluent B, acetonitrile and formic acid (0.1%); A/B = 95:5 to 5:95 in 3 min, isocratic 1 min, 5:95 to 95:5 in 1 min, and isocratic 1 min.

**Pd0-Mediated Dealkylation of 5a,p,b in Cell Culture.** BxPC-3 and MiaPaCa-2 cells were plated as described above. Each well was then replaced with fresh media containing Pd0-resins (0.67 mg/mL) with DMSO (0.1% v/v); 2a,p,b (3, 10, 30, 100, and 300 nM) with DMSO (0.1% v/v); gemcitabine (3, 10, 30, 100, and 300 nM) with DMSO (0.1% v/v); or a combination of 0.67 mg/mL of Pd0-resins + 2a,p,b (3, 10, 30, 100, and 300 nM) with DMSO (0.1% v/v). Untreated cells were incubated with DMSO (0.1% v/v).

**Cell Viability Assay.** Cells were incubated with drugs for 5 days. PrestoBlue cell viability reagent (10% v/v) was added to each well and the plate incubated for 60 min. Fluorescence emission was detected and results normalized as described above.

**Time-Lapse Imaging Study.** BxPC-3 cell growth studies were carried out at a single dose of gemcitabine (1) or 2a,p (30 nM). Each well was imaged every 3 h over 5 d under standard incubation conditions using an IncuCyte ZOOM microscope (placed inside the incubator). Image-based analysis of cell confluence and Supporting Information movie 1 were produced using the IncuCyte software.

**DNA Damage Study.** MiaPaCa-2 cells were seeded in an 8-well chamber slide with a density of 2,000 cells/well and incubated for 48 h prior to treatment. Each well was then replaced with fresh media containing 0.1% DMSO (v/v) and the following treatments: Pd0-resins (0.67 mg/mL); 2a,p,b (3, 10, 30, 100, and 300 nM) with DMSO (0.1% v/v); gemcitabine (3, 10, 30, 100, and 300 nM) with DMSO (0.1% v/v); or a combination of 0.67 mg/mL of Pd0-resins + 2a,p,b (3, 10, 30, 100, and 300 nM) with DMSO (0.1% v/v). Untreated wells were incubated with DMSO (0.1% v/v).

**PrestoBlue cell viability reagent** (10% v/v) was added to each well and the plate incubated for 60 min. Fluorescence emission was detected using ImageJ software. A line was drawn horizontally in comparison to that with the inactive resin + 2a,p,b (30 nM). Each well was imaged every 3 h using an Olympus FV1000 microscope and merged using the ImageJ software. A 3D fold change in fluorescence with DMSO (0.1% v/v); or a combination of 0.67 mg/mL of Pd0-resins + 2a,p,b (3, 10, 30, 100, and 300 nM) with DMSO (0.1% v/v) was added to each well and the plate incubated for 60 min. Fluorescence emission was detected and results normalized as described above.

**ABSTRACT**

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**ABBREVIATIONS USED**

BOOM, bioorthogonal organometallic; Alloc, allyloxycarbonyl; Boc, tert-butyloxycarbonyl; Cbz, carboxybenzyl; TBS-Cl, tert-butylmethlysilyl chloride; TBAF, tetrabutylammonium fluoride; dpf, days postfertilization; TLC, thin layer chromatography; DBU, 1,8-diazabicycloundec-7-ene; DMF, dimethylformamide; DCM, dichloromethane; r.t., room temperature; CD3OD, deuterated methanol; RPMI, Roswell Park Memorial Institute; DMEM, Dulbecco’s modified Eagle’s media; DMSO, dimethyl sulfoxide

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