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Bright insights into palladium-triggered local chemotherapy†

Thomas L. Bray,a Mark Salji,bc Alessandro Brombin,bd Ana M. Pérez-López,da Belén Rubio-Ruiz,b Laura C. A. Galbraith,bc E. Elizabeth Patton,ad Hing Y. Leungb*c and Asier Unciti-Brocetaa

The incorporation of transition metal catalysts to the bioorthogonal toolbox has opened the possibility of producing supra-stoichiometric amounts of xenobiotics in living systems in a non-enzymatic fashion. For medical use, such metals could be embedded in implantable devices (i.e. heterogeneous catalyst) to “synthesize” drugs in desired locations (e.g. in a tumour) with high specificity and for extended periods of time, overcoming the useful life limitations of current local therapy modalities directed to specific organ sites (e.g. brachytherapy, controlled release systems). To translate this approach into a bona fide therapeutic option, it is essential to develop clinically-accessible implantation procedures and to understand and validate the activation process in relevant preclinical models. Herein we report the development of a novel Pd-activatable precursor of the red-fluorescent drug doxorubicin and Pd devices of optimized size and activity. Screening in state-of-the-art cancer models provided fundamental insights into the insertion protocols, safety and stability of the devices and into the prodrug distribution profile before and after activation.

Introduction

Advances in cancer diagnosis have led to major improvements in the early detection of clinically-localized prostate cancers.1 Because of the adverse effects of prostatectomy and external beam radiotherapy (impotence, incontinence, etc.), in most patients this low-to-moderate risk form of prostate cancer has been traditionally managed through active surveillance programs, which has resulted in an increment in the risk of dying from prostate malignancies.2,3 Focal therapies were introduced to facilitate localized tumour treatment with preservation of healthy prostate tissue, thus reducing the morbidity associated with whole-gland treatments without jeopardizing cancer control.1,4 Although current technologies mediating local treatment of disease (e.g. brachytherapy, external-beam focal radiotherapy, drug-eluting devices) can successfully improve a patient’s quality of life, they have important limitations that either restrict their widespread clinical use (high cost of brachytherapy and external-beam focal radiotherapy) or the duration of their therapeutic effect (radioisotope’s short half-life and limited drug cargo capacity of brachytherapy and controlled-release implants, respectively).

Our capacity to visualize and modulate physiological and pathological processes in the biological milieu has expanded enormously in the last 15 years through the development of a rich diversity of bioorthogonal tools and processes.5–9 Among them, the use of abiotic transition metals (ruthenium,10–14 palladium,15–21 copper,22–24 gold25–27) has emerged to facilitate the catalytic modification or manufacture of biomolecules and xenobiotics in living systems, contributing to the advent of a new prodrug modality that is not activated by a metabolic event but through a bioorthogonal bond cleavage reaction.8 Exploration of this novel prodrug paradigm in combination with heterogeneous Pd catalysis has stimulated the development of masking strategies that efficiently suppress the bioactivity of different classes of therapeutic agents while enabling selective drug activation – via Pd-mediated N- or O-dealkylation – both in vitro and in vivo.28–31 It is proposed that, by surgical implantation of Pd-containing inserts in a tumour followed by systemic administration of Pd-activatable therapeutic precursors, bioorthogonal drug release could take place exclusively in the disease site and minimise adverse effects in distant organs and tissues. Since the triggering mechanism is a catalytic process, multiple doses of one or more prodrugs could be intratumourally activated by the same implant to extend and/or
customise the therapeutic intervention, thereby facilitating an adaptive form of local chemotherapy that can respond to the severity and progression of the disease. Although studies in mammal models supporting the effectiveness of such an approach to treat tumours locally have not yet been reported, recent studies with Pd-based nanostructures have shown their capacity to generate tumour-controlling levels of drugs in xenografts in mice. However, the development of cancer-targeting nanotechnologies remains a significant challenge by itself.

From a translational perspective, the use of activating devices that can be surgically implanted in or near the tumour using well-established clinical procedures would ensure that the release of cytotoxic agents occurs exclusively at the disease site. Essential questions such as appropriate implantation protocols, stability and safety of the catalyst in vivo and optimal biodistribution of the prodrug before and after activation need to be first addressed to translate this novel therapeutic modality into a medically-viable alternative to brachtherapy and drug eluting devices. In our path towards this goal, herein we report the development of Pd devices of optimal size for ultrasound-guided intratumoural insertion and Pd-activatable prodrugs of doxorubicin: a strongly fluorescent cytotoxic drug that allowed us to monitor biodistribution in prostate cancer explants. Screening in state-of-the-art cancer models provided fundamental insights into the safety and catalytic properties of the activating device, and into the prodrug activation process.

Results and discussion
Design and synthesis of bioorthogonal prodrugs of doxorubicin
Doxorubicin, 1, is an anthracycline anticancer antibiotic used alone or in combination in the treatment of a wide range of hematological and solid malignancies. After entering cells by diffusion, this potent cytotoxic drug engages with various cytoplasmic and nuclear targets to synergistically disrupt cellular function in cancer cells and induce apoptosis (Scheme 1). 1 has a multitude of moderate to severe side effects that negatively impact on its therapeutic window, limiting its maximum tolerated dose and frequency of administration (typically administered as a single intravenous infusion in days 1 and 2 of 21 day cycles). The most serious adverse effect of 1 is cardiotoxicity, typically observed by an early onset of cardiomyopathy that may progress to myocardial infarction. As a result, much of the research pertaining to 1 and their anthracycline analogues focuses on suppressing the cardiotoxic effect of this class of cytotoxic drugs. A variety of prodrug strategies are currently under investigation to improve tumour targetability and have shown that blockade of the primary amino group of the daunosamine moiety of 1 significantly reduces the bioactivity of the resulting derivative. Based on this evidence, the masking of the NH₂ group as a carbamate group was investigated to develop novel biochemically-stable, Pd-sensitive prodrugs of 1 (Scheme 1).

Four carbamate derivatives of 1 were prepared as shown in Scheme 1 (see full experimental protocols in the ESI†). As the propargyloxy carbonyl (Poc) has been previously shown to be relatively resistant to metabolic activation and sensitive to Pd catalysis, derivative 2a was developed as a positive control, whereas the Cbz-protected analogue 2b (which is expected to be even more biochemically stable than Poc but unreactive to Pd catalysis under physiological conditions) was used as a negative control. With the aim of increasing steric hindrance to further protect the carbamate bond from unspecific enzymatic cleavage, novel compounds containing a propargyloxycarbonyl (Poc) group were developed to enhance the metabolic stability of the derivatives whilst providing sensitivity to Pd catalysis. The para and ortho isomers 2c and 2d, which are designed to undergo 1,6- or 1,4-elimination upon O-deprotection (ESI, Fig. S1†), were prepared to test which position provides superior protection and, consequently, a greater therapeutic window relative to the active drug (EC₅₀ (2c,d)/EC₅₀ (1)).

Cytotoxicity study: drug vs. prodrug
The efficacy of the deactivation strategy was evaluated by treating human prostate cancer DU145 and glioma U87 cells...
increment of its size. compared to the masking group of DU145 and U87, respectively). These results indicate that the antiproliferative activity of more than two orders of magnitude relative to unmodified drug 1 (approx. 350- and 150-fold in DU145 and U87, respectively). These results indicate that the masking group of 2d provides superior protection to the carbamate bond against unspecific biological cleavage, even compared to the para analog 2c and without the need of further increment of its size.

Table 1  Calculated EC50 values (μM) for 1 and 2a–d treatment in DU145 and U87 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Reagent</th>
<th>EC50 (DU145)</th>
<th>EC50 (U87)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EC50</td>
<td>0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>2a</td>
<td>EC50</td>
<td>0.83</td>
<td>41.5</td>
</tr>
<tr>
<td>2b</td>
<td>EC50</td>
<td>2.69</td>
<td>134.5</td>
</tr>
<tr>
<td>2c</td>
<td>EC50</td>
<td>5.54</td>
<td>277</td>
</tr>
<tr>
<td>2d</td>
<td>EC50</td>
<td>7.13</td>
<td>356.5</td>
</tr>
</tbody>
</table>

Zebrafish cardiotoxicity study

As mentioned above, the induction of cardiotoxicity is the most severe side effect of 1. Given the greater therapeutic window afforded by 2d, an in vivo cardiotoxicity assay was designed to study its effect on the heart of developing zebrafish in comparison to 1. Dechorionated zebrafish embryos (1 dpf) were treated for 4 days with 1 or 2d at a range of concentrations (50–200 μM) and then imaged (brightfield and fluorescent microscopy). Embryos in E3 medium alone or with DMSO were used as negative controls. To determine the enlargement of the heart (estimate of pericardial edema), the ratio between the cardiac area and the total body of the treated fish was measured and compared with the untreated controls. As shown in Fig. 1, treatment of 1 at 50 μM induced significant increment of the cardiac area, with 100% lethality observed in all populations treated at higher concentrations. In contrast, 2d did not induce lethality or cardiac edema even up to 200 μM; well above the theoretical blood concentration achieved with the maximum therapeutic dosage of clinical dosing regimens. Since spectrophotometry analysis of the optical properties of 1 and 2d showed similar red fluorescent properties (ESI, Fig. S3†), drug/prodrug distribution patterns were analyzed by fluorescent microscopy (Ex/Em = 520/560 nm). Image analysis showed that significant levels of 1 accumulated across the body of the zebrafish, including the heart area, while 2d did not (ESI, Fig. S4†). This observation is consistent with 2d’s lack of capacity to engage with the in vivo molecular targets of 1.

Prodrug-into-drug conversion studies

Pd-devices were prepared from amino-functionalized Tentagel® HL resins of 110 μm in average diameter (Rapp
Polymere GmbH) as previously described.26 Content in Pd metal was determined to be 4.47% w/w by inductively coupled plasma optical emission spectrometry (ICP-OES). The conversion of inactive 2a-d into cytotoxic 1 was first tested by performing a Pd-mediated activation assay in cancer cell culture at a single concentration, using cell viability measurements as a functional readout of the experiment. DU145 cells were treated with prodrugs 2a-d (0.3 μM) and Pd-devices (0.6 mg mL⁻¹) and cell viability determined at day 5 using the PrestoBlue® reagent. Cells treated with only Pd-devices (0.6 mg mL⁻¹) or 2a-d (0.3 μM) were used as negative controls and unmodified 1 (0.3 μM) as positive control.

As expected, Cbz-protected 2b displayed no signs of toxicity either on its own or in the presence of the catalyst, which confirms its resistance to both metabolic and Pd-mediated activation (ESI, Fig. S5†). On the contrary, prodrugs 2a–2d elicited no harm on their own but significant antiproliferative effects in the presence of the catalytic devices, thus proving the selective in situ generation of 1 in response to the presence of Pd. However, the assay revealed a statistically-significant gap in cytotoxicity between 1 and the prodrug/Pd-devices combinations, particularly for prodrugs 2c and 2d. By simply mixing the prodrugs and the Pd-devices in a test tube, it was visibly noticed that the devices had a remarkable capacity to sequester 2d from the solution, an effect that was not seen for 1 (Fig. 2A). The polymeric component of these devices is made of TentaGel®, a copolymer consisting of a low crosslinked polystyrene (PS) matrix on which polyethylene glycol (PEG) is grafted.24 Based on the strong “trapping” effect observed for 2d, we reasoned that the lipophilic polystyrene core of the devices were likely responsible for capturing the prodrug, partially deterring its effective interaction with the catalyst and, in turn, resulting in a moderate reduction of the reaction rate and subsequent liberation of 1 into the cell culture.

Aiming to optimize the functionality of the devices, we prepared, characterized and tested a range of Pd-devices using polymer resins of reduced size and greater PEG/PS ratio (=increased hydrophilicity). Pd-functionalized devices with sizes of 10, 20, 30 and 75 μm were synthesized using commercially available TentaGel® resins (Rapp Polymere GmbH) following the same protocol used for the preparation of 110 μm Pd-devices.26 ICP-OES analysis demonstrated the presence of the metal in each of the devices, although lower Pd content was observed in the smaller ones (Fig. 2B). The catalytic capacity of the devices was tested by 24 h treatment with nonfluorescent bis-N,N’-(propargyloxycarbonyl)rhodamine 110 (3) in cell culture conditions (PBS + 10% serum, pH = 7.4, 37 °C). This assay is based on the fluorescent release of rhodamine 110 (4)}
Fig. 2C, dose active drug at the intended site of treatment. As shown in circulation, whereby they would be uncaged and released as an could potentially serve to pull the prodrug molecules out of 

In principle, the superior a device's biocompatibility and functional stability of the Pd-devices. 

Fig. S8 of the ESI†, an optimal situation for the therapeutic goal of the strategy.

Tumour growth was not affected by the presence of the devices. The catalytic capacity of Pd-devices in serum-free media at physiological conditions (pH = 7.4, isosotopic, 37 °C) and analyzed by UPLC. Remarkably, the 30 mm Pd-devices showed full cathodic performance of 21 day-in-tumor Pd-devices was found optimal to perform this procedure with ease, whereas partial blockage of the producing signal from the chromatogram in  Fig. S8 of the ESI†, it showed efficient synthesis and release of another drug at the intended site of treatment.

Apart from the presence of the tumour, animals remained healthy and free from signs of toxicity. Tumours were removed and sliced in half to study the distribution of the Pd-devices (ESI, Fig. S11†). In an optimal situation for the therapeutic goal of the strategy, 

Based on the safety, catalytic performance and convenient size of the 30 mm Pd-devices, they were then chosen for the next phase of the investigation. 

The catalytic capacity of Pd-devices was determined using 488 nm laser excitation (FL-1) and visualized by live-cell imaging confocal microscopy for 24 h after 21 days, the animals were sacrificed. Necropsy of animals and gross observation of major organs showed no macroscopic signs of toxicity. Tumours were removed and sliced in half to study the distribution of the Pd-devices (ESI, Fig. S11†). A standard 1 mm syringe to place such small devices with millimeter range could potentially serve to pull the producing molecules out of the prostate tumour of anaesthetized mice under real time monitoring with ultrasound imaging. Fig. 3B and Movie S1† show the highly echogenic Pd-devices being precisely inserted into a hypoechogenic area of the tumour. The mice were then sacrificed, necropsy of animals and gross observation of major organs showed no macroscopic signs of toxicity. Tumours were removed and sliced in half to study the distribution of the Pd-devices (ESI, Fig. S11†). A standard 1 mm syringe to place such small devices with millimeter range could potentially serve to pull the producing molecules out of the prostate tumour of anaesthetized mice under real time monitoring with ultrasound imaging. Fig. 3B and Movie S1† show the highly echogenic Pd-devices being precisely inserted into a hypoechogenic area of the tumour. The mice were then sacrificed, necropsy of animals and gross observation of major organs showed no macroscopic signs of toxicity. Tumours were removed and sliced in half to study the distribution of the Pd-devices (ESI, Fig. S11†). 

1 mm sections, soaked in media containing 1 mm of PBS) into a murine prostate tumour. (B) An ex vivo ultrasound image captions of the prostate tumour area of an anaesthetised mouse taken during the implantation procedure at 10 s (left) and 10.8 (right).
both 0- and 21 day-in-tumour Pd-devices, evidence that maintaining the devices in living tissue for extended periods of time does not have a negative impact in the underlying turnover rate of the devices. In contrast, in the absence of the activation devices, tumour explants treated with \( \text{3} \) showed minimal background signal (Fig. 4A, right). The good biocompatibility and catalytic stability of the catalyst in living tissue, which are herein shown for the first time for a bioorthogonal catalyst that has been hosted for weeks in a tumour in vivo, are optimal features that support the use of heterogeneous Pd devices for site-specific prodrug activation in precision medicine.

**Ex vivo prodrug-into-drug conversion study**

Next, the capacity of the devices to generate therapeutic (=cytotoxic) levels of \( \text{1} \) from an inactive dose of \( \text{2d} \) was tested in tumour explants bearing 21 day-in-tumour Pd-devices. Tissue samples were treated for 48 h in media containing 100 \( \mu \text{M} \) of \( \text{2d} \) in the presence of DAPI (a nuclei stain not permeable in viable cells). Treatment of Pd-free explants (in the presence of DAPI) with either \( \text{2d} \) (100 \( \mu \text{M} \)) or \( \text{1} \) (100 \( \mu \text{M} \)) were used as a negative and positive control, respectively. Cancer cell viability and prodrug/drug distribution was monitored by live-cell imaging

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**Fig. 4** (A) Live-cell imaging study of the catalytic activity of tumour-implanted Pd-devices: 0 d vs. 21 d after implantation. Pd-bearing explants \((n = 2)\) were incubated with \( \text{3} \) (100 \( \mu \text{M} \)) and imaged by live-cell confocal microscopy (488 laser excitation) at 3, 9 and 24 h. The presence of the Pd-devices is indicated with white arrows. Note that Pd-devices are identified due to their capacity to quench green fluorescence. Images of the controls (3 alone (100 \( \mu \text{M} \)); 4 alone (100 \( \mu \text{M} \)); and DMSO) are shown at 24 h. Pictures were generated using ImageJ software. Scale bar = 30 \( \mu \text{m} \). (B) Live-cell imaging study of the Pd-mediated conversion of inactive \( \text{2d} \) into cytotoxic \( \text{1} \) in a human prostate tumour explant model. Images of a representative tumour explant \((n = 2)\) bearing 21 day-in-tumor Pd-devices after treatment with \( \text{2d} \) (100 \( \mu \text{M} \)) in the presence of DAPI for 24, 36 and 48 h (left panel). Tissue samples were imaged under laser excitation at 405 nm (for DAPI staining, in blue) and 543 nm (for \( \text{2d}/\text{1} \) distribution, in red). The presence of the Pd-devices, indicated with white arrows, is identified by a bright fluorescence signal in the red channel (as previously reported. Red fluorescence is not quenched by Pd). Images of the Pd-free controls \( \text{1} \) (100 \( \mu \text{M} \), mid panel) and \( \text{2d} \) (100 \( \mu \text{M} \), right panel) are shown at 48 h. Pictures were generated using ImageJ software. Scale bar = 30 \( \mu \text{m} \).
confocal microscopy using two laser excitations: 405 nm to image nuclei staining by DAPI (=dying/dead cells) and 543 nm to visualize 2d and/or 1 (the spectroscopic properties of both compounds are indistinguishable; see Fig. S3†). In the absence of Pd-devices (Fig. 4B, right panel), 48 h treatment with 2d did not elicit any sign of cell death in the cancer tissue. Analysis of the red fluorescent channel shows that 2d was not retained inside the cancer cells, which is consistent with its expected inability to bind with the intracellular targets of doxorubicin. A drastically different distribution profile was observed upon treatment with 1, with bright red fluorescence being accumulated in the cells (Fig. 4B, middle panel; and ESI, Fig. S12†). Remarkably, the distribution pattern of the red fluorescent signal from explants bearing Pd-devices and treated with 2d became identical to that of the explants directly treated with 1 after 48 h, indicating that 2d has been chemically converted into 1. It is also noteworthy that compound 2d rapidly entered the devices during the first hours of treatment, making the Pd-devices patently visible under the 543 nm laser excitation (Fig. 4B, see panels at 24 and 36 h). This observation agrees with the high capacity of the devices to “pull” the prodrug from the environment (Fig. 2A). Importantly, the 2d/Pd-devices treatment combination induced a strong cytotoxic effect (DAPI staining), equivalent to that of 1 treatment, demonstrating the in situ bioorthogonal generation of bioactive levels of drug. Ex vivo studies with the 0 day-in-tumour Pd-devices provided equivalent results (ESI, Fig. S12†).

Last, samples of an explant containing 21 day-in-tumour Pd-devices and previously treated with fluorogenic compound 3 were washed and subsequently incubated with either 1, prodrug 2d or DMSO for 48 h. Tissue samples were then fixed and analysed by immunohistochemical staining with caspase 3 (apoptosis biomarker) or γ-H2AX (biomarker for DNA double-strand breaks) antibodies (ESI, Fig. S13†). As expected, damaged cells (indicated by brown staining) were only observed in the Pd-containing samples treated with 1 and 2d. This test agrees with the inert nature of the devices and supports their capacity to activate multiple doses of prodrug.

Conclusions

In conclusion, fundamental insights into the validity, reliability and clinical feasibility of a novel approach to accurately target chemotherapy spatially within a tumour have been reported. This therapeutic approach, which is conceived for the treatment of localised cancers, consists of two components: (i) an inactive derivative of a cytotoxic drug designed to be specifically activated by palladium chemistry; and (ii) an inert implantable polymer-based device functionalised with Pd nanoparticles to catalyse drug conversion and release in a spatially controlled manner. As part of the investigation, a novel caged doxorubicin was developed by blocking the NH₂ group of the drug’s sugar moiety with a Pd-labile PBC group. The derivative having the propargyloxy moiety in ortho was found to offer superior reduction of bioactivity than the one in para and high sensitivity to Pd catalysis. Studies in zebrafish proved that the cardiotoxic effect induced by doxorubicin was abolished in the caged precursor; a clinically-relevant result that to the best of our knowledge is shown in this animal model for the first time for a doxorubicin prodrug. Pd-devices of optimised size and activity were also developed and tested in a state-of-the-art orthotopic murine model of human prostate cancer. The devices showed high echogenicity, enabling precise injection into the tumour using ultrasound scanning, an image-guided microsurgical technique widely used in the clinic. The study provided evidence of the non-perturbing effect of the devices to the mice and of the resilience and biocompatibility of the catalyst to the tumour microenvironment. Devices hosted in a tumour of mice for 21 d elicited catalytic release of a fluorogenic probe and a caged doxorubicin at an equivalent rate than freshly injected devices. Ex vivo time-lapse imaging enabled to monitor the distinct distribution profiles of the drug and the inactive precursor, and the capacity of the devices to “sequester” circulating prodrug molecules and generate cytotoxic levels of drug in the tumour explant. This investigation provides compelling evidence that heterogeneous palladium catalysts can be applied as bioorthogonal tools to enable local treatment of disease and reports a standardized preclinical methodology to perform and study local bioorthogonal release of bioactive substances in preclinical disease models.

Conflicts of interest

The authors declare that compounds 2c, d are protected under patent application PCT/GB2017/051379.

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Notes and references


