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Controlled In-Cell Generation of Active Palladium(0) Species for Bioorthogonal Decaging

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Abstract: Owing to their bioorthogonality, transition metals have become very popular in the development of biocompatible bond-cleavage reactions. However, many approaches require design and synthesis of complex ligands or formulation of nanoparticles which often perform poorly in living cells. This work reports on a method for the generation of an active palladium species that triggers bond-cleaving reactions inside living cells. We utilized the water-soluble Na_2PdCl_4 as a simple source of Pd(II) which can be intracellularly reduced by sodium ascorbate to the active Pd(0) species. Once generated, Pd(0) triggers the cleavage of allyl ether and carbamate caging groups leading to the release of biologically active molecules. These findings do not only expand the toolbox of available bioorthogonal dissociative reactions but also provide an additional strategy for controlling the reactivity of Pd species involved in Pd-mediated bioorthogonal reactions.

Most of the bioorthogonal applications are focused on ligation reactions[1], however, bond-cleavage reactions[2] have recently emerged as useful bioorthogonal chemical transformations in research areas such as targeted payload delivery[3], activation of prodrugs[4] and studies on the gain-of-function of proteins[5]. Bond-cleavage reactions are often initiated by the addition of an exogenous small molecule[6] or a transition metal complex[7] as triggering agents. Some of the advantages of transition-metal-mediated processes are their catalytic performance and almost guaranteed layer of bioorthogonality. Among all transition metals which have been employed in bioorthogonal bond-cleavage reactions, palladium stands out as the most thoroughly studied metal trigger. Strategies based on biocompatible Pd-mediated reactions have been utilized for on-demand cleavage of functional groups such as allyl carbamates[8], propargyl carbamates[4b, 5a, 9], propargyl ethers[8c, 10], propargyl amines[11],[12] allenyl ethers[5d] and thioethers[3b, 13]. Whereas extracellular approaches have relied mostly on Pd-resins which could be implanted on the sites where the desired reaction is expected to take place[4b, 11–12], intracellular Pd-mediated cleavage reactions have been more challenging to achieve. This is mainly associated with poor cellular uptake, solubility or instability of many Pd catalytic systems under biological conditions and their interference with major cellular functions[14]. Nevertheless, commercially available Pd catalysts $Pd(dba)_2$ and $[Pd(allyl)Cl]_2$[5a, 5d] (Fig. 1a) or Pd complexes bearing purposely designed phosphine ligands[8c] (Fig. 1b) have been successfully used to promote bond-cleavage reactions inside living cells. To further expand on this, we envisioned a strategy which would overcome the limitations of available methods. Herein, we demonstrate that the biological activity of small molecules containing allyl ether and carbamate masking groups can be efficiently restored using catalytically active Pd(0) species formed by reduction of a water soluble Pd(II) salt with sodium ascorbate in living cells. (Fig. 1c) This simple, yet effective strategy allows to control the oxidation state of the Pd catalyst and the corresponding Pd-mediated bioorthogonal cleavage reaction in a spatiotemporal manner.

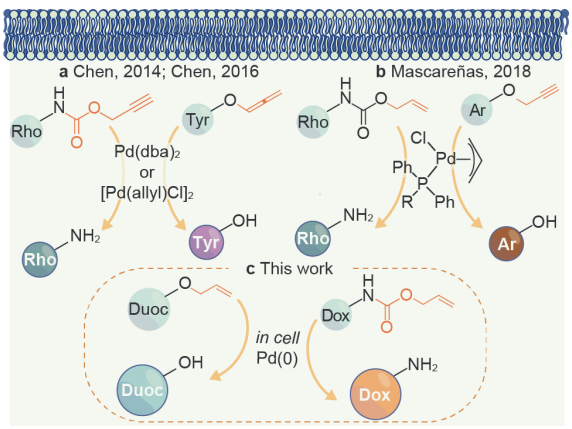


Figure 1. Metal-mediated bioorthogonal bond-cleavage reactions. Previously reported approaches for intracellular Pd-assisted bond-cleavage reactions

using **a**, commercially available Pd catalysts[5a, 5d] or **b**, Pd complexes bearing discrete phosphine ligands[8c]. **c**, Bioorthogonal cleavage of allyl ethers and carbamates by intracellularly generated Pd(0) species described in this work. Rho: Rhodamine; Tyr: Tyrosine; Ar: Aryl; Duoc: Duocarmycin; Dox: Doxorubicin.

The initial interest in the development of a new allyl-Pd bioorthogonal bond-cleavage pair was based on our previous work on the tetrazine-mediated decaging of vinyl ethers.[15] While the release of alcohols from vinyl ether containing molecules proceeded under biocompatible conditions[15], the kinetics of this decaging reaction are much slower than those obtained from strained alkenes[4a]. We anticipated that some drawbacks of the vinyl ether moiety could be resolved using a different masking group. Allyl ether was previously reported as a Ru-[16] or Pt-labile[17] caging group in living cells, however, decaging of allyl ethers mediated by Pd under biocompatible conditions has not been reported yet.

In order to find optimal reaction conditions, various types of Pd catalysts were screened for the deallylation of a model 2-(allyloxy)naphthalene **2** under aqueous conditions (**Fig. 2a** and **Table S1**). Among these, Na_2PdCl_4 in combination with a TPPTS ligand and morpholine provided the best result (**Fig. 2a**, Entry 4 and **Table S1**). Other sources of Pd in the absence of ligands and presence of external nucleophiles (see **Table S1** for all tested conditions) only gave low yields of the isolated alcohol **1**. Since reduction of Pd(II) to Pd(0) is facilitated in the presence of phosphine ligands[7], these observations are in accordance with previously published works[18], where the combination of a Pd(0) catalyst and an external nucleophile was necessary to ensure a successful decaging reaction[19]. On the other hand, a simple Pd(II)→Pd(0) reduction of Na_2PdCl_4 mediated by sodium ascorbate[20] (**Fig. 2a**, Entry 9 and **Table S1**) did not prove to be efficient in this type of bond-cleavage reaction. This is probably due to the observed formation and aggregation of catalytically inactive Pd black[21] under the reaction conditions and in the absence of a stabilizer[22]. The optimized decaging conditions were then used for the deallylation of various small molecules containing allyl ethers **2**, **4**, **6**, **8**, **10** and even an allyl ester **12** (**Fig. 2b**).

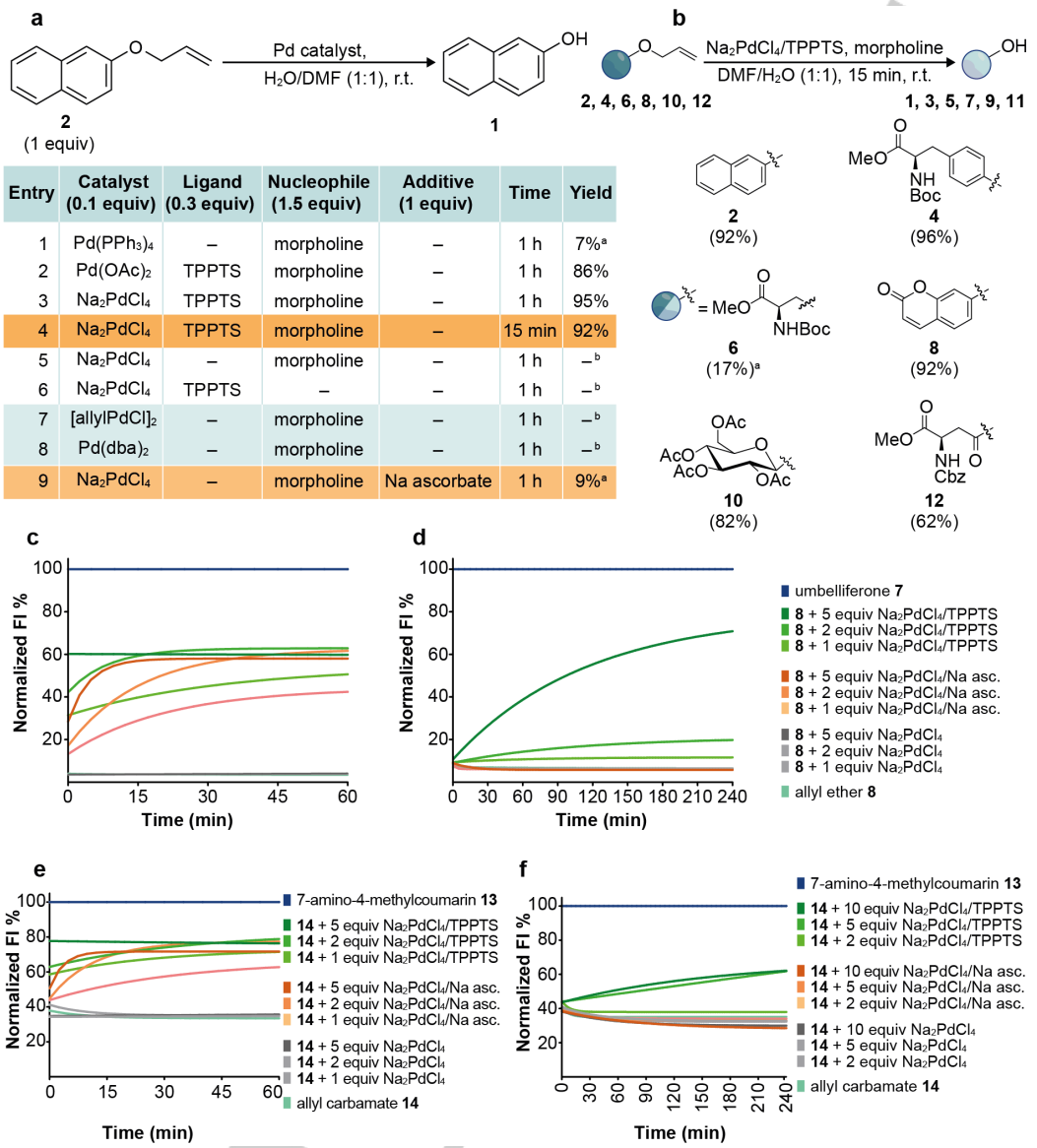
Next, we studied this Pd-triggered bond-cleavage reaction by fluorescence recovery experiments. Formation of the allyl ether bond effectively quenched the fluorescence of umbelliferone **7** which allowed monitoring of the cleavage reaction in response to a Pd trigger (**Fig. 2c–f**). We observed that the presence of morpholine did not significantly affect the Pd(0)-mediated decaging of allyl ether **8** when the decaging reaction was performed in PBS buffer pH 7.4 at 37 ºC (see SI, **Fig. S1**). This suggests that under tested conditions, no external nucleophile is needed and perhaps water or TPPTS can act as a nucleophile in the release of the desired alcohol **7** from the Pd(II)-allyl complex[18a]. In fact, this could be beneficial in more complex biological settings, e.g., in cell culture media which will contain various nucleophiles that could promote this type of cleavage reactions. Although the Pd(0)/TPPTS complex performed well in these decaging experiments, it was previously shown that such complex could not cross cell membranes and thus was not available for intracellular reactions[5a, 23]. Therefore, we sought for simpler or ligandless sources of Pd(0) species which would facilitate its cellular entry.

Earlier works showed that active Pd(0) catalyst can be obtained by Pd(II)→Pd(0) reduction by sodium ascorbate[5a, 8c, 23–24] in form of nanoparticles with different sizes, shapes and catalytic activities[20]. In contrast with the catalyst optimization (**Fig. 2a** and **Table S1**), Pd(0) generated by reduction of Na_2PdCl_4 Pd(II) salt with sodium ascorbate promoted the cleavage of allyl ether **8** in PBS buffer at 37 ºC almost as efficiently as the Pd(0)/TPPTS complex with about 60% fluorescence recovery observed (**Figs 2c** and **S2**). The non-quantitative yield of the recovered fluorescent signal was associated with partial fluorescence quenching of the released fluorophore, probably caused by microprecipitated metallic Pd (**Fig. S6**). In the absence of sodium ascorbate, decaging of **8** was not observed, suggesting that reduced Pd(0) is the active species in this cleavage reaction. Poisoning experiments with Hg, CS_2 and EDTA further support our hypothesis that the uncaging is indeed mediated by Pd(0), and kinetics experiments suggest the reaction has an heterogeneous (nanoparticles) contribution (SI, **Figs S16–18**). Additionally, reduction of Pd(II) to Pd(0) does not occur in the presence of other bioadditives, assuming that the reducing capability is exclusive to sodium ascorbate also in biological media (**Table S3**). However, when carried out in cell culture medium, it was only the Pd(0)/TPPTS complex that efficiently promoted the decaging of **8** and higher amount of the catalyst was required to obtain acceptable levels of decaging (**Figs 2d** and **S3**). The reason for this may be the deactivation of the formed Pd(0) catalyst by the additional compounds present in the cell culture medium.

Having established the Pd(0)-mediated decaging for allyl ethers, we next attempted to extend this methodology to the cleavage of allyl carbamates. In PBS buffer at 37 ºC, about 40% increase in the fluorescence signal was observed upon the treatment of the allyl carbamate caged 7-amino-4-methylcoumarin **14** with either the Pd(0)/TPPTS complex or the combination of Na_2PdCl_4 and sodium ascorbate (**Figs 2e** and **S4**). Similarly to the experiments with allyl ether **8**, no uncaging reaction was observed in the presence of Pd(II) and again, only the Pd(0)/TPPTS complex was effective in the cleavage of allyl carbamate bond in cell culture medium (**Figs 2f** and **S5**). Fluorescence recovery experiments have demonstrated that both Pd(0)/TPPTS complex and Pd(0) species generated by sodium ascorbate-mediated Pd(II)→Pd(0) reduction can be used for the cleavage of allyl ether and carbamate bonds under biocompatible conditions. Notably, these experiments also confirmed the stability of both caging groups in cell culture medium ruling out spontaneous deallylation reactions.

Our final goal was to apply the developed strategy for Pd-mediated bond-cleavage to the release of biologically active small molecules in living cells (**Fig. 3**). Duocarmycin **15**[25] and doxorubicin **17**[26] were chosen as examples of cytotoxic drugs containing functional groups to be uncaged by our method. We showed that the masking of the aromatic hydroxyl of **15** and amino group of **17** leads to about 19- and 72-fold (respectively) decrease in the cytotoxic activity in SKBR3 breast cancer cells (SI, **Fig. S9**). These results correspond to previous reports[3b, 9, 15] and provide therapeutic windows suitable for use in the prodrug activation therapy concept. Using LC-MS analysis, it was confirmed that both prodrugs **16** and **18** can be efficiently decaged via bond-

cleavage reactions mediated by Pd(0) catalysts under biocompatible conditions at 37 ºC in PBS buffer (SI, **Figs S7,8**).



Entry	Catalyst (0.1 equiv)	Ligand (0.3 equiv)	Nucleophile (1.5 equiv)	Additive (1 equiv)	Time	Yield
1	$Pd(PPh_3)_4$	–	morpholine	–	1 h	7%[a]
2	$Pd(OAc)_2$	TPPTS	morpholine	–	1 h	86%
3	Na_2PdCl_4	TPPTS	morpholine	–	1 h	95%
4	Na_2PdCl_4	TPPTS	morpholine	–	15 min	92%
5	Na_2PdCl_4	–	morpholine	–	1 h	–[b]
6	Na_2PdCl_4	TPPTS	–	–	1 h	–[b]
7	$[allylPdCl]_2$	–	morpholine	–	1 h	–[b]
8	$Pd(dba)_2$	–	morpholine	–	1 h	–[b]
9	Na_2PdCl_4	–	morpholine	Na ascorbate	1 h	9%[a]

Figure 2. Optimization of the biocompatible Pd-mediated decaging of allylated small molecules. **a**, Overview of reaction conditions tested for the Pd-mediated deallylation on a model substrate **2** (see **Table S1** for all tested conditions). **b**, Scope of deallylated small molecules with isolated yields obtained under optimized decaging reaction conditions. Fluorescence recovery experiments following the Pd-mediated decaging of allyl ether **8** in **c**, PBS buffer or **d**, in cell culture medium and of allyl carbamate **14** in **e**, PBS buffer or **f**, in cell culture medium. For full experimental details see SI. [a]Without the full conversion of the starting material. [b]No reaction observed.

We then proceeded with experiments with prodrug activation in living cells. The inspiration for generating active Pd(0) species inside of living cells was found in works which have reported production of Pd nanoparticles in bacteria[27]. We hypothesized that a similar approach employing sodium ascorbate-promoted reduction of Na_2PdCl_4 could be used to generate active Pd(0) species in living cells. Various metabolites and biomolecules present in cells could potentially act as capping agents or stabilizers providing catalytically active Pd(0) species. Concentrations of Pd catalysts and sodium ascorbate used in these experiments were optimized based on performed cytotoxicity assays (SI, **Fig. S10**). Incubation with prodrug **16** or **18** and Na_2PdCl_4 did not significantly decrease the viability of SKBR3 breast cancer cells (**Fig. 3a,b**). However, preincubation with prodrug and Pd(II), followed by incubation with sodium ascorbate recovered the cell killing activity for both prodrugs (**Fig. 3a,b**). As anticipated, cytotoxic activity of the uncaged drugs as well as the unwanted toxic effects of the bond-cleavage reaction triggers increased in a dose dependent manner. Such significant cell-killing effect was not observed using Pd(0)/TPPTS complex which confirms the poor cellular uptake of the bulky and charged Pd(0)/TPPTS complex (**Figs 3c,d** and **S19**). Nonetheless, when the prodrug is administered together with Pd(0)/TPPTS, bond-cleavage reaction takes place extracellularly and the liberated

drug can then enter cells where it exerts its cytotoxic activity (SI, **Fig. S11**). Noteworthy, in control experiments, simultaneous addition of the prodrug, Na_2PdCl_4 and sodium ascorbate did not exhibit any significant levels of cell cytotoxicity (SI, **Fig. S13**) indicating that the active Pd(0) species required for the release of cytotoxic drugs is effectively generated in the intracellular environment. These observations, together with the evidence that sodium ascorbate promotes the reduction of Pd(II) to the active Pd(0), suggest that spatiotemporal control can be achieved using this Pd-mediated bioorthogonal bond-cleavage strategy.

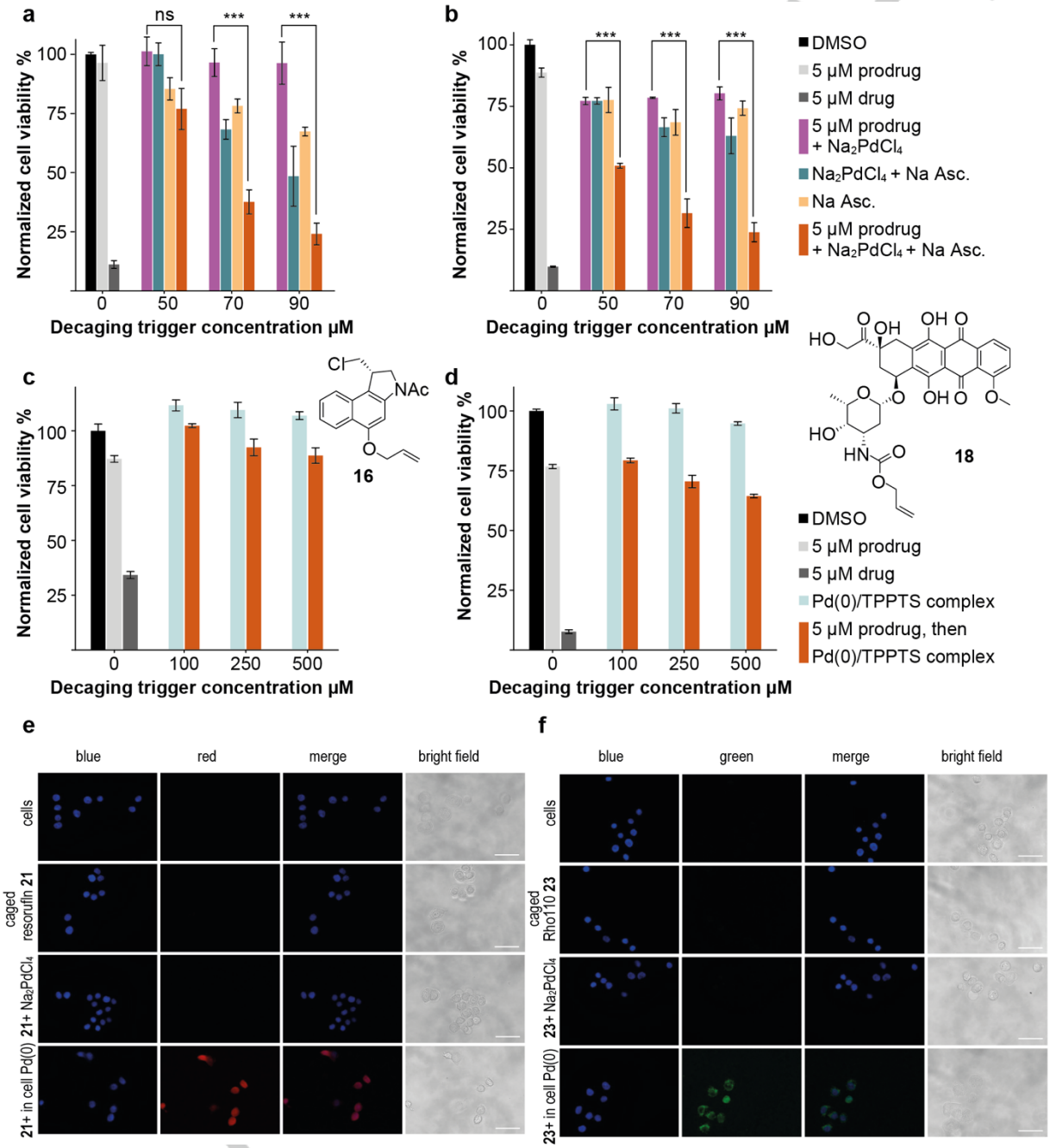


Figure 3. Pd-mediated decaging experiments in SKBR3 cells. Decaging of prodrugs **a**, **16** and **b**, **18** by in cell generated Pd(0) species compared to their decaging by Pd(0)/TPPTS complex **c**, and **d**. Error bars represent SEM. ns ($P > 0.05$), *** ($P \leq 0.001$). Decaging of caged fluorophores **e**, **21** and **f**, **23** followed by fluorescence microscopy. Scale bars represent 100 µm. For full experimental details see SI.

We also investigated another prodrug of doxorubicin, a propargyl carbamate **19**, and observed that the Pd-mediated decaging of **19** is less efficient under these conditions (SI, **Fig. S12**). This suggests that the depropargylation reaction requires different Pd species[28]. Finally, fluorescence microscopy imaging experiments were performed to provide more evidence in the favor of the presented method for in cell generation of the active Pd(0) species (**Fig. 3e,f**). Caging with either allyl ether or carbamate

group resulted in fluorescence quenching of resorufin allyl ether **21** and bis-*N*,*N*′-allyloxycarbonyl-Rhodamine 110 **23**. As expected, no fluorescent signal was recovered in the presence of Na_2PdCl_4, however, upon addition of sodium ascorbate 'turn-on' of the fluorescence signal was detected inside of SKBR3 cells in both cases (**Fig. 3e,f**). Furthermore, experiments to support the mechanism for extracellular decaging employing the Pd(0)/TPPTS complex were performed and fluorescence of both fluorophores was recovered in this instance as well (SI, **Figs S14,15**). ICP-MS analysis of the cellular extracts revealed the intracellular amount of Pd (ca. 230 ppm) after incubation of Na_2PdCl_2 following several washing steps and lytic treatment (**Fig. S19**). Altogether, our findings demonstrate that it is the Pd(0) species generated inside of living SKBR3 cells that is responsible for the restoration of the biological activity of caged allyl ethers and carbamates.

In summary, presented results show that intracellular Pd(II)→Pd(0) reduction provides spatiotemporal control over Pd(0)-catalyzed bioorthogonal chemical reactions. Our simple yet elegant method utilizes the commercially available and water soluble Na_2PdCl_4 which is reduced to the active Pd(0) species by the action of sodium ascorbate inside of living cells. We show that the active catalyst effectively triggers the cleavage of allyl ether and carbamate caging groups, leading to the release of parent small molecules such as fluorophores or cytotoxic drugs. The present study is a proof-of-concept that opens up several possibilities to perform not only bond-cleavage but also other transition metal-assisted bioorthogonal reactions[29]. It is yet not clear how different cell lines, cell growth medium composition and additives influence the production and reactivity of the formed Pd(0) species. Further investigations will determine what type of catalytically active Pd(0) species is generated inside of living cells and new applications of this strategy towards other types of biocompatible reactions are currently underway in our laboratory.

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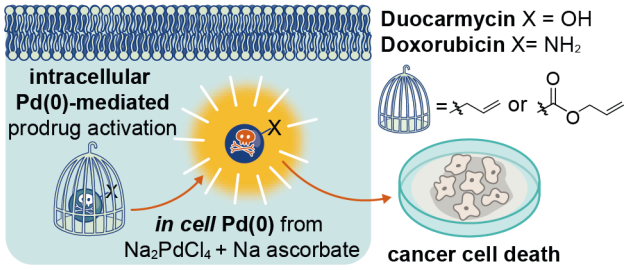

A simple, cell-permeable source of Pd(II) is intracellularly reduced to the catalytically active Pd(0) through the addition of sodium ascorbate. Controlled *in cell* generation of Pd(0) enables Pd-mediated bioorthogonal intracellular activation of caged cytotoxic drugs and fluorophores in cancer cells.

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