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Tetrazine-triggered release of carboxylic-acid containing molecules for activation of an anti-inflammatory drug

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Abstract: In addition to its usage for the study of biomolecules in living systems, bioorthogonal chemistry has emerged as a promising strategy to enable protein or drug activation in a spatially and temporally controlled manner. This study demonstrates the application of the bioorthogonal inverse electron-demand Diels-Alder (IEDDA) reaction to cleave *trans*-cyclooctene (TCO) and vinyl protecting groups from carboxylic acid-containing molecules. The TCO tetrazine-mediated decaging reaction proceeded under biocompatible conditions with fast reaction kinetics (< 2 minutes). Anti-inflammatory activity of ketoprofen was successfully reinstated after decaging of the nontoxic TCO-prodrug in live macrophages. Overall, this work expands the scope of functional groups and the application of decaging reactions to a new class of drugs.

Early research in the field of bioorthogonal chemistry focused on ligation reactions such as the Staudinger reaction,[1] copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC),[2,3] palladium-catalysed cross-couplings,[4] ruthenium-catalysed olefin metatheses,[5] strain-promoted azide-alkyne cycloaddition (SPAAC),[6] tetrazole photoinduced 1,3-dipolar cycloadditions,[7,8] and inverse electron-demand Diels-Alder (IEDDA) tetrazine ligation.[9,10] Of these, the IEDDA reaction between *trans*-cyclooctene (TCO) and a tetrazine is one of the more selective and fastest reported bioorthogonal reactions to date.[11] Since it was first introduced by Fox *et al.*,[9] this reaction has been used in innumerous biological applications such as cell and *in vivo* pretargeting imaging.[12–14] Recently, bioorthogonal cleavage reactions have emerged as promising strategies to control the activation of caged proteins, fluorophores, and small molecule drugs in living systems.[15] The TCO-tetrazine IEDDA ligation reaction can be re-engineered into a cleavage reaction by placing a leaving group at the allylic position of TCO. After the initial cycloaddition and elimination of nitrogen, the 4,5-dihydropyridazine now contains an appropriately placed substituent that eliminates upon tautomerisation.[10] The group of Robillard reported the first use of the TCO-tetrazine reaction for bioorthogonal decaging to release amine-containing drugs (Figure 1a), in which they demonstrate the release of doxorubicin from a TCO carbamate prodrug *in vitro*.[16] They then apply this 'click-to-release' strategy to successfully trigger the release of doxorubicin (Dox) and monomethyl auristatin E (MMAE) from an antibody-drug conjugate (ADC).[17,18] Mejia-Oneto and co-workers also reported targeted *in vivo* activation of a Dox-TCO carbamate prodrug by injecting an alginate hydrogel modified with tetrazines near the tumour site.[19] A limitation of the click-to-release strategy is the need for the delivery, and therefore optimization of the pharmacokinetic properties, of both the prodrug and the tetrazine.[20,21] However, the previously mentioned approaches are demonstrative of the potential of bioorthogonal decaging reactions for targeted drug-activation *in vivo*.

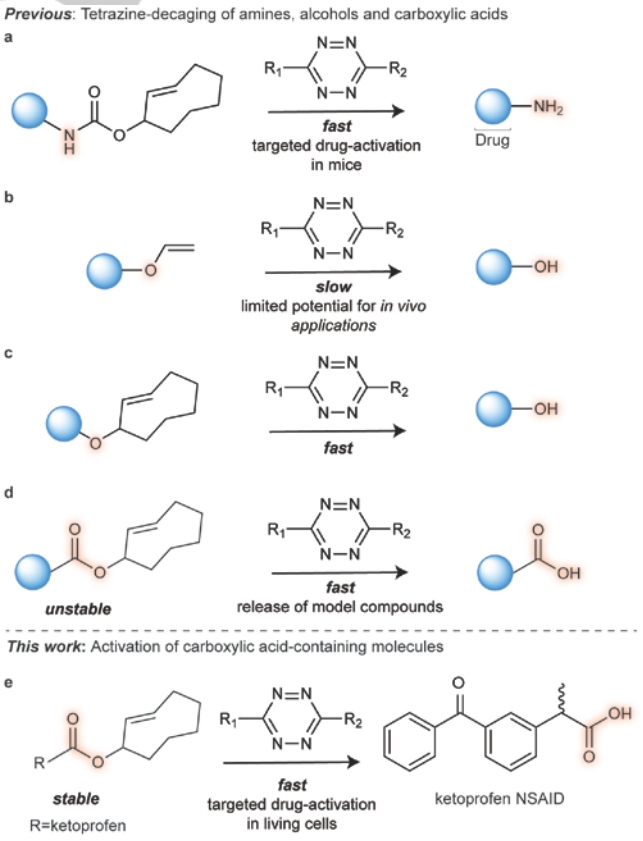

Figure 1. Bioorthogonal decaging reactions. **a.** Reported TCO carbamate linker for amine release, successfully applied *in vivo* for doxorubicin and MMAE activation. **b.** Reported vinyl decaging for alcohol release, limited by its slow reaction rate. **c.** Reported TCO ether linker for the rapid release of alcohols. **d.**

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Reported TCO ester for the release of carboxylic acids (model compounds benzoic acid and . **e.** Activation of anti-inflammatory drug ketoprofen in live cells from a stable TCO ester prodrug.

Bioorthogonal chemistry has also been applied for the release of alcohols. Our group[22] and the groups of Bradley[23] and Devaraj[24] independently reported the use of the vinyl ether protecting group which could be cleaved by reaction with tetrazines to release alcohols (Figure 1b). This is, however, significantly slower than the TCO reaction for the release of amines. In addition, Robillard recently reported bioorthogonal cleavage of ethers, carbonates and esters from TCO to release alcohols (Figure 1c) or carboxylic acids (Figure 1d), respectively. However, the reported TCO-protected carboxylic acids proved highly unstable (~90% fragmentation in 50% mouse serum at 37°C). In addition, the ether linker was only successfully used to deprotect tyrosine and control cell growth in tyrosine-free medium.[25]

Previous examples of drug release have so far been limited to the release of amine- (Figure 1a) or alcohol-containing (Figure 1b,c) anticancer drugs. These groups, although often found in small molecule drugs, are not always present and may not be vital for the function of the drug, meaning chemical modification at this site to form a prodrug does not lead to reduced activity. For this reason, it is important to expand the scope of bioorthogonal decaging reactions to include other functional groups. For example, non-steroidal anti-inflammatory drugs (NSAIDs) are an important class of drugs that contain a carboxylic acid group essential for their function.[26] Herein, we expanded the application of bioorthogonal cleavage reactions to the carboxylic acid functional group. A stable TCO-protected NSAID was successfully decaged in the presence of tetrazine within 2 minutes (Figure 1e) enabling the reinstatement of anti-inflammatory activity in living macrophages.

Initially, following on from previous work in our group on the vinyl ether handle on alcohols,[22] we investigated the protection of carboxylic acids with the vinyl group (Figure 2). Computational studies on the reaction between vinyl acetate and different tetrazines **1**–**5** (Figure 2a) predicted that the first cycloaddition step was rate determining (SI, Figure S20), and that all tetrazines should have similar reactivity, with the exception of dimethyltetrazine **5** which was predicted to be the least reactive (SI, Tables S1, S3). The kinetics of the cycloaddition were experimentally determined with these tetrazines and the test substrate vinyl propionate **6** (SI, Figure S1). The fastest rate occurred with tetrazine **4**, a mono-substituted tetrazine bearing a moderately electron-withdrawing group (benzoic acid, Figure 2b). It has previously been shown that tetrazines bearing strong electron withdrawing substituents have faster rates for cycloadditions while a small, non-bulky group increases the rate of the elimination step.[27] Next, the stability of tetrazines **1**–**5** in 50% DMSO/H_2O was assessed by monitoring the UV absorbance at 530 nm (SI, Figure S2). Tetrazine **1** showed moderate stability ($t_{1/2}$ = 15.8 h) and tetrazine **2** was the most unstable ($t_{1/2}$ = 5.7 h). Tetrazines **3**–**5** proved highly stable (~85 – 90% intact after 24 h, SI, Figure S2). The biological stability of tetrazine **4** was then assessed and it proved to be stable in cell culture media, phosphate-buffered saline (PBS) pH 7.4, and 10% plasma for at least 24 h (SI, Figure S3). Therefore, we decided to use tetrazine **4** in further studies.

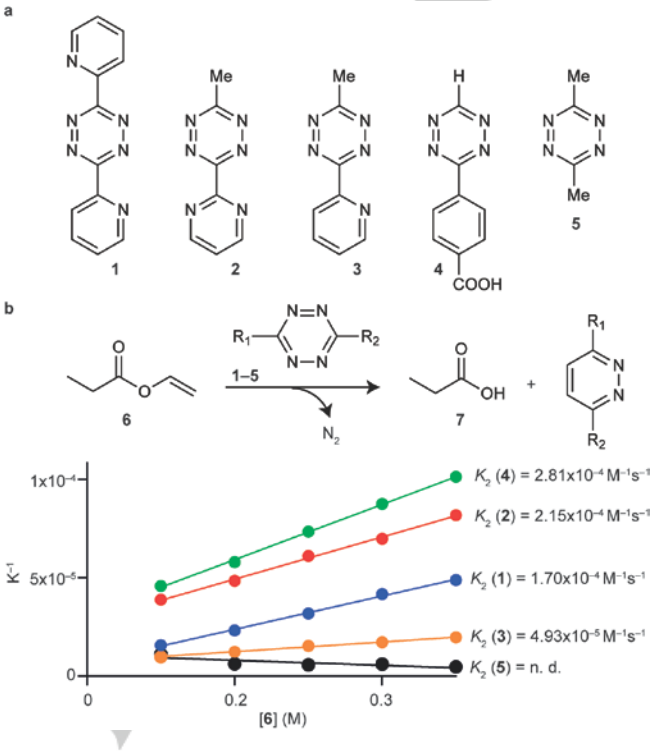

Figure 2. Decaging of vinyl esters. **a.** Tetrazines used in this study. **b.** Kinetics of the cycloaddition step for the reaction of vinyl propionate with different tetrazines determined by following the decay of the UV absorbance of the tetrazine at 530 nm. n.d. = not determined.

Ketoprofen **8** is a NSAID with a chiral centre. Although it is used as a racemate, the anti-inflammatory activity of ketoprofen mainly resides with the *S*-enantiomer. While the *R*-enantiomer is approximately 100 to 1000 times less potent than the *S*-enantiomer as a cyclooxygenase inhibitor, research has shown that the *R*-enantiomer is still important in that it contributes to the analgesic effect of ketoprofen.[28] We started by converting ketoprofen into the vinyl ester **9** using palladium coupling[29] which proved stable in PBS pH 7.4 (SI, Figure S4). However, limited stability (assessed by High-Performance Liquid Chromatography (HPLC)) was observed in 10% plasma ($t_{1/2}$ = 12 min) and cell culture media ($t_{1/2}$ = 4 h) likely because the vinyl group does not provide steric protection of the ester group from nucleophilic attack and subsequent hydrolysis. This fact, along with the slow rate of reaction (approx. 20% of free drug observed after 24 h with 30 equiv. of tetrazine, SI, Figure S5) resulted in the vinyl handle being abandoned, as its use for *in vivo* applications would be rather limited.

Next, we decided to investigate TCO as a caging group to render ketoprofen inactive. A more reactive alkene was necessary in order to make this a rapid, useful bioorthogonal cleavage reaction. Quantum mechanical calculations (SI, Table S2) suggested that the initial cycloaddition between TCO esters

and different tetrazines is much faster than with vinyl esters, causing the rate-limiting step to depend on the tetrazine substituents. Hence, while the initial cycloaddition step is rate-limiting for dimethyl-tetrazine **5** (SI, Figure S22), for dipyridyl-tetrazine **1** the allylic elimination step (decaging) is the rate-limiting step (SI, Figure S21). Irrespective of which step determines the reaction rate, all reactions involving TCO acetate were calculated to be much faster than those with vinyl acetate (SI, Table S2). Of note, our calculations reproduced the experimentally observed trend of axial-TCO being slightly more reactive than its equatorial isomer (SI, Figures S21, S22). Concerning the isomerization of the dihydropyridazine intermediate necessary for carboxylate release (i.e. decaging), the proposed water-mediated shift (in which one water molecule adds to one of the imine moieties and is subsequently β-eliminated) appears to be more favourable than direct water-assisted imine-enamine tautomerization (SI, Figure S23).

We started by assessing the stability of the TCO ester bond. For this, we used *cis*-cycloocten-1-ol **10** to test both synthetic feasibility and stability of the ester bond. The *cis*-protected ketoprofen drug **11** was synthesised in 75% yield (SI) and, unlike the vinyl ester, proved to be stable in cell media, PBS pH 7.4, and 10% plasma (only 5% free drug after 24 h) (SI, Figure S6). We propose that this increase in stability is due to the increased steric hindrance at the ester bond due to the TCO handle compared with the vinyl handle. It appears that significant steric hindrance is required on both sides of the ester bond and the ester proves unstable if either the protecting group (vinyl handle) or active molecule (in the case of the TCO-esters reported by Robillard)[25] are not sterically bulky. In the case of **12**, a stereocentre α to the ester bond provides steric protection on one side as does TCO on the other.

With these results in hand, we decided to further evaluate the TCO ester for bioorthogonal IEDDA decaging. Repeating the synthesis with *trans*-cycloocten-1-ol resulted in the desired product **12** with approximately 50% of the TCO isomerizing to the *cis* form (Figure 3a). This highlights a common problem with synthesising TCO-functionalised molecules. The isomerization of *cis* to *trans* using UV light is very low yielding and is not always suitable for the final step in syntheses that need a large amount of valuable drug. However, TCO's highly reactive double bond is not compatible with several reaction conditions, such as the halide ions used in the formation of the acyl chloride intermediate. In addition to the modes of chirality on the *cis* and *trans* isomers of the TCO protecting group, ketoprofen also has a chiral center. Indeed, using thionyl chloride as an activating agent we observed the formation of 8 diastereomers by HPLC (Figure 3b) from the *cis* and *trans* isomers of TCO and the chiral center of the protected ketoprofen. Using chiral HPLC we were able to separate each diastereomer, analyse them by NMR and characterize the four *trans* isomers as enantiomeric pairs of either the axial $\mathbf{12_{ax}}$ or equatorial $\mathbf{12_{eq}}$ isomer (Figure 3c). Since the axial TCO-isomer has previously been shown to have different reaction rates than the equatorial isomer, each enantiomeric pair of axial and equatorial isomers were combined. We demonstrated a method of separating isomers of TCO and successfully overcame the isomerization problem commonly experienced in synthesis using TCO, even in the challenging case of having an additional chiral center on the drug. Now, although a low yield may be obtained, it is possible to subject TCO to reaction conditions that readily cause isomerization and still obtain the pure *trans* isomer at the end.

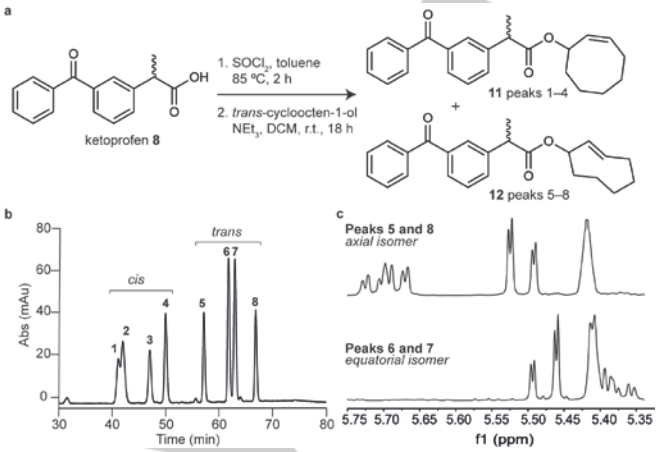

Figure 3. Studies of TCO-ketoprofen ester. **a.** Synthesis of TCO-protected ketoprofen resulted in 84% yield of the product as a mixture of *cis* and *trans*-isomers (NMR ratio 51:49 *cis*:*trans*). **b.** Separation of the 8 product diastereomers using HPLC. **c.** Alkene region from ^{1}H NMR spectra of *trans*-products.

Reaction of TCO ketoprofen (axial and equatorial isomers) with tetrazine **4** (Figure 4a, SI, Figure S7) was then studied by HPLC over time. Considering the fast kinetics observed for the decaging, an excess of free TCO was added to quench the reaction at various time points. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis of the quenched solution showed similar reaction profiles and decaging yields for both isomers, therefore further tetrazines were tested with only the axial isomer. The reaction of TCO-ketoprofen was then studied with tetrazines **1**,**3** and **5** (tetrazine **2** was not chosen due to its high instability compared to other tetrazines, SI, Figure S2). For tetrazines **1**, **3** and **4**, TCO-ketoprofen is consumed within 30 seconds and after 2 minutes the change in the amount of ketoprofen is negligible (Figure 4b and SI, Figure S8) which is in good agreement with the low activation barriers predicted computationally. The observed accumulation of dihydropyridazine intermediate(s) A/B (Figure 4b) demonstrates our prediction (for tetrazine **1**, SI, Figure S21) that elimination of the carboxylate after IEDDA is the rate limiting step. In agreement with computational predictions, tetrazine **5** showed a different reaction profile (SI, Figure S8). In this case, no significant amount long-lived intermediate was observed, indicating that the elimination is much quicker and therefore, for this tetrazine, it is the cycloaddition step which is rate limiting. This is also confirmed by the much slower disappearance of TCO-ketoprofen and the corresponding formation of ketoprofen (not complete after 2 min). It is also important to note that the three tetrazines with the same rate determining step all show comparable decaging yields (~25%). Interestingly, Tetrazine **5**, which showed no reaction with vinyl ketoprofen, gives a decaging yield double that of the other tetrazines (54%, Figure 4c). This highlights the fact that different tetrazines are optimal for different decaging reactions. Next, the effect of water content and pH on

decaging yield were assessed (Figure 4d and SI, Figures S9-12). Tetrazine **3** was chosen as a representative example of tetrazines **1**,**3** and **4**. It was shown that the reaction yield increased from 26% (0% water) to 33% (75% water), however no increase in yield was observed by the addition of 1% formic acid. Conversely, tetrazine **5** showed no increase in yield upon increasing water concentration. However, the yield was increased to 65% by the addition of 1% formic acid (Figure 4d). This study highlights the importance of optimising the tetrazine for the decaging reaction as changing the tetrazine substituents can alter the rate-limiting step of the reaction, resulting in different kinetics and yields of decaging.

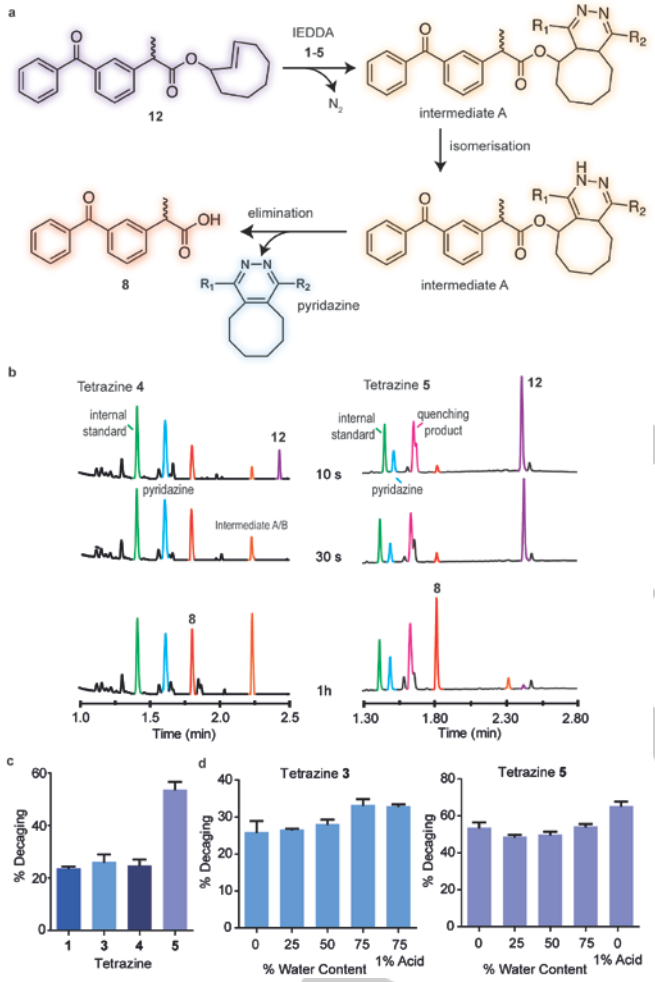

Figure 4. Studies of TCO-ketoprofen ester. **a.** Mechanism of tetrazine-triggered TCO ester decaging reaction. **b.** LC–MS trace for the reaction of TCO-ketoprofen $\mathbf{12}_{ax}$ with tetrazines **4** and **5** that demonstrates the ketoprofen release from TCO-ester prodrug after 30 seconds (tetrazine **4**). **c**. Decaging yield of the reaction of TCO-KTP $\mathbf{12}_{ax}$ with tetrazines **1**, **3**, **4** and **5** assessed by LC-MS. Concentration of the released drug was determined by HPLC/UV using a calibration curve prepared with ascending concentrations of ketoprofen. **d.** Effect of water content and the addition of 1% formic acid on the decaging yield for reaction with tetrazines **3** and **5**, which both have a different rate determining step.

The promising stability and decaging results prompted us to further evaluate the application of this strategy in live cell studies. Using the macrophage cell line RAW264.7 (ATCC® T1B-71), we started by establishing the non-toxic concentrations of each compound (SI, Figure S13, S14). Although tetrazine **5** results in a higher decaging yield by LC-MS, this tetrazine proved toxic to macrophages even at low concentrations (~70% viability at 5 μM). Furthermore, the volatility of this tetrazine made it impractical for use in cell experiments. Tetrazine **4** was chosen for further studies as it proved to be non-toxic at high concentrations (~90% viability at 148 μM). Surprisingly, the anti-inflammatory effect of tetrazines and their reactivity with nitric oxide was observed as previously described (SI, Figure S15).[30] However, a concentration of tetrazine (50 μM) was chosen such that no anti-inflammatory activity was observed.

Inflammation was induced on macrophages by using lipopolysaccharide (LPS, SI, Figure S16 for optimisation of concentration) and the anti-inflammatory effect of the bioorthogonal pair (TCO-ester **12** and tetrazine **4**) was assessed using the Griess assay (Figure 5a). By monitoring the levels of nitric oxide (NO), we verified that when TCO-ketoprofen **12** was reacted with tetrazine **4** on LPS-stimulated macrophages a significantly enhanced anti-inflammatory effect was observed after 11 h (Figure 5b) ($p < 0.001$ for equatorial, $p < 0.01$ for axial). This reduction in inflammation corresponds to the successful cleavage of the TCO-ester bond from the caged drug leading to the release of ketoprofen. During our studies, we also observed that ketoprofen itself failed to reduce the NO levels, while TCO-ketoprofen **12** has a moderate effect on reducing NO levels (Figure 5b). This is likely due to the poor membrane permeability of the ketoprofen when compared to the caged drug. When protected as the TCO-ester, the cell permeability of ketoprofen was greatly improved, leading to a higher concentration of free drug in the cell as assessed by HPLC (Figure 5c). Briefly, this study involved incubation of cells for 24 h with either the prodrug or free drug. Subsequent HPLC analysis of the extracellular media revealed that almost no prodrug was detected while a significant amount of ketoprofen was still observed (Figure 5c).

After 24 h, the level of NO from the prodrug alone was the same as the bioorthogonal pair (SI, Figure S17). While we expected the caged drug to show very little anti-inflammatory activity, this result suggested that activation might also happen without the tetrazine trigger. It is worth mentioning once again that the ester bond was shown to be fully stable in complete cell culture media for 24 h at 37 ºC. Therefore, this observation may be due to the hydrolytic enzymes inside the cell, which was confirmed by a reaction carried out with TCO-ketoprofen and esterase from porcine liver (SI, synthesis section). After 4 h, a small amount of ketoprofen was already released, and the amount increased over the next 20 hours to a yield of 71% for the axial isomer and 14% for the equatorial. The release of carboxylic acids from ester prodrugs via intracellular esterases has been widely reported.[31–33] Although enzyme-mediated hydrolytic activation of TCO-ketoprofen was observed, the nearly spontaneous release of the active drug after tetrazine reaction suggests that this approach may play an important role in biological applications. For example, we anticipate that an ADC could be used to target the ester prodrug to extracellular receptors expressed on macrophages which would allow the fast and local delivery of ketoprofen at sites of inflammation.

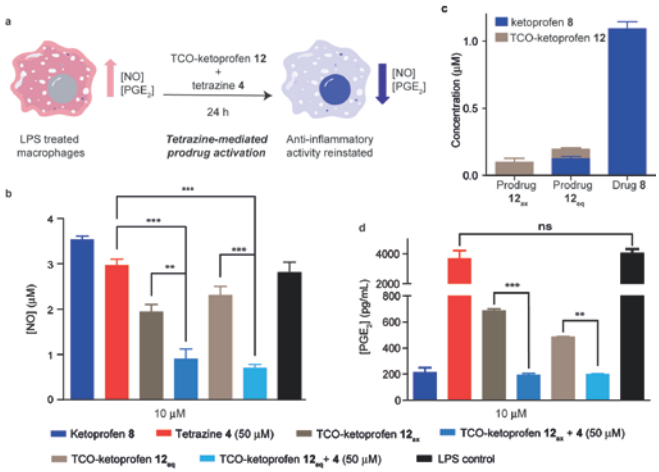

Figure 5. Live cell decaging experiments **a.** Tetrazine-mediated decaging of TCO-ketoprofen ester in live macrophages. Initial inflammation triggered by LPS causes an increase in the concentration of nitric oxide and prostaglandin E_2 **b**. Griess assay used to determine the concentration of nitric oxide. Decaging of TCO-ketoprofen prodrug resulted in decreased NO concentrations. **c.** Concentration of TCO-ketoprofen **12** and ketoprofen **8** present in the media after 24 h after incubation of either $\mathbf{12_{ax}}$, $\mathbf{12_{eq}}$ or **8**. The high presence of ketoprofen that remains in the media confirms the relatively poor membrane permeability of ketoprofen **8**. Results are expressed as an average of three independent experiments. **d.** ELISA used to determine the concentration of PGE_2. Decaging of TCO-ketoprofen prodrug resulted in decreased concentrations of PGE_2.

The levels of inflammation were then assessed using an enzyme-linked immunosorbent assay (ELISA, R&D Systems) to monitor the levels of prostaglandin E_2 (PGE_2) (Figure 5d), which has been shown to be overexpressed in this cell line when inflammation is stimulated by LPS.[34] Briefly, this assay uses a monoclonal antibody that competitively binds both PGE_2 in the samples and PGE_2-alkaline phosphatase molecules. The alkaline phosphatase produces a chromogenic signal upon the addition of *p*-nitrophenyl phosphate. Therefore, the concentration of PGE_2 present in a sample is inversely proportional to the absorbance produced by the bound enzyme (SI, Figure S18). Cells with only LPS showed the highest level of PGE_2 (4060 pg/mL), which confirmed that inflammation was successfully stimulated. A similarly high concentration (4010 pg/mL) was observed with the tetrazine, confirming that the tetrazine alone does not have an anti-inflammatory effect. Despite the poor cell permeability of ketoprofen, cells incubated with ketoprofen showed the lowest level of PGE_2 (215 pg/mL). The anti-inflammatory effect of ketoprofen can be seen using this assay as the ELISA has a higher sensitivity than the Griess assay (detection limit 0.5 μM). TCO-ketoprofen **12** also showed lower levels of PGE_2 than the LPS control ($\mathbf{12_{ax}}$: 686 pg/mL, $\mathbf{12_{eq}}$: 486 pg/mL). The bioorthogonal pair resulted in a statistically significant reduction in PGE_2 concentration compared to the prodrug alone ($\mathbf{12_{ax}}$ + **4**: 193 pg/mL, $\mathbf{12_{eq}}$ + **4**: 200 pg/mL) ($p < 0.001$ for axial, $p < 0.01$ for equatorial). It was observed that the concentration of PGE_2 was the same for the bioorthogonal pair as for free ketoprofen, therefore confirming that the anti-inflammatory activity was successfully reinstated up decaging in live macrophages.

In summary we describe the bioorthogonal tetrazine-triggered release of carboxylic acid-containing molecules and demonstrate the application on a new class of anti-inflammatory drugs. In doing so, we have expanded the bioorthogonal drug activation strategy to encompass a wider range of drugs and diseases. Finally, this approach may also find utility for protein activation where carboxylic acid side-chains can be caged/decaged.

Acknowledgements

We thank the EPSRC (PhD studentship to S.D.), Herchel-Smith Fund (MPhil studentship to L.Q.), FCT Portugal (FCT Investigator to G.J.L.B., IF/00624/2015), European Commission (Marie Sklodowska-Curie Fellowship to B.L.O., GA no. 702574) and D.G.I. MINECO/FEDER (CTQ2015-70524-R and RYC-2013-14706 grants to G.J.O and a fellowship to C.D.N.). We also thank UR (Beronia cluster) for computer support. G.J.L.B. is a Royal Society University Research Fellow (UF110046 and URF\R\180019) and the recipient of a European Research Council Starting Grant (TagIt, GA no. 676832).

Conflict of interest

The authors declare no conflict of interest.

Keywords: bioorthogonal decaging • *trans*-cyclooctene • tetrazine • targeting • anti-Inflammatory drugs

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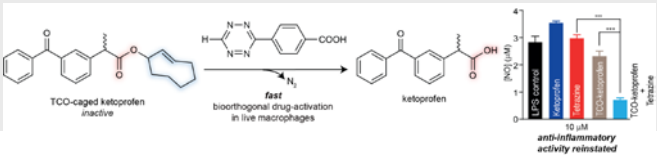

Activate on demand: The inverse electron-demand Diels-Alder decaging reaction between *trans*-cyclooctene and tetrazines is used for the release of carboxylic acids. Anti-inflammatory activity is reinstated after tetrazine-triggered deprotection of a TCO-ester prodrug in live macrophages.

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Tetrazine-triggered release of carboxylic-acid containing molecules for activation of an anti-inflammatory drug