Tetrazine-Triggered Release of Carboxylic-Acid-Containing Molecules for Activation of an Anti-inflammatory Drug

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In addition to its use 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 a bioorthogonal inverse electron-demand Diels–Alder (iEDDA) reaction to cleave trans-cyclooctene (TCO) and vinyl protecting groups from carboxylic acid-containing molecules. The tetrazine-mediated decaging reaction proceeded under biocompatible conditions with fast reaction kinetics (<2 min). The 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-catalysed 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, 7] 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 bioorthogonal reactions to date.[11] Since it was first introduced by Fox et al.,[12] this reaction has been used in numerous biological applications such as cell and in vivo pre-targeting 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 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.[16] Robillard’s group reported the first use of the TCO–tetrazine reaction for bioorthogonal decaging to release amine-containing drugs (Figure 1A), in which they demonstrated the release of doxorubicin (Dox) from a TCO carbamate prodrug in vitro.[16] They then applied this “click-to-release” strategy to successfully trigger the release of 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 delivery, and therefore optimisation of the pharmacokinetic properties, of both the prodrug and the tetrazine.[20, 21] However, the previously mentioned approaches demonstrate the potential of bioorthogonal decaging reactions for targeted drug activation in vivo.

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 using 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 might not be vital for the function of the drug; this means that 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, nonsteroidal anti-inflammatory drugs (NSAIDs) are an important class of drugs that contain a carboxylic acid group essential for their function. In our work, we expanded bioorthogonal cleavage reactions to the carboxylic acid functional group. As table TCO-protected NSAID was successfully decaged in the presence of tetrazine within 2 min (Figure 1E), thus reinstating the anti-inflammatory activity in living macrophages.

Initially, following on from previous work in our group on the vinyl ether handle on alcohols, we investigated the protection of carboxylic acids with a vinyl group. Computational studies on the reaction between vinyl acetate and different tetrazines (1–5; Figure 2A) predicted that the first cycloaddition step was rate-determining (Figure S20 in the Supporting Information), and that all tetrazines should have similar reactivity except dimethyltetrazine (5), which was predicted to be the least reactive (Tables S1 and S3). The kinetics of the cycloaddition were experimentally determined with these tetrazines and the test substrate vinyl propionate 6 (Figures S1 and 2B). The fastest rate occurred with tetrazine 4, a monosubstituted 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, whereas a small, non-bulky group increases the rate of the elimination step.

Next, the stability of tetrazines 1–5 in 50 % DMSO/H₂O was assessed by monitoring the UV absorbance at 530 nm (Figure S2). Tetrazine 1 showed moderate stability (t₁₀₀ = 15.8 h), and tetrazine 2 was the most unstable (t₁₀₀ = 5.7 h). Tetrazines 3–5 proved highly stable (≃85–90 % intact after 24 h, Figure S2). The biological stability of tetrazine 4 was then assessed, and it proved to be stable in cell culture medium, phosphate-buffered saline (PBS, pH 7.4),

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and 10% plasma for at least 24 h (Figure S3). Therefore, we decided to use tetrazine 4 in further studies.

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. Although 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] Using palladium coupling,[29] we converted ketoprofen into the vinyl ester 9, which proved stable in PBS (pH 7.4; Figure S4). However, limited stability (as assessed by HPLC) was observed in 10% plasma (t1/2 = 12 min) and cell culture medium (t1/2 = 4 h) probably because the vinyl group does not sterically protect the ester group from nucleophilic attack and subsequent hydrolysis. This fact, along with the slow rate of reaction (ca. 20% of free drug observed after 24 h with 30 equiv of tetrazine, 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 for ketoprofen. A more reactive alkene was necessary to make this a rapid, useful bioorthogonal cleavage reaction. Quantum mechanical calculations (Table S2) suggested that the initial cycloaddition between TCO esters and different tetrazines is much faster than with vinyl esters, thus causing the rate-limiting step to depend on the tetrazine substituent. Hence, whereas the initial cycloaddition step is rate-limiting for 5 (Figure S22), for 1, the allylic elimination step (decaging) is the rate-limiting step (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 (Table S2). Note, our calculations reproduced the experimentally observed trend of axial-TCO being slightly more reactive than its equatorial isomer (Figures S21 and S22). Concerning the isomerisation 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 tautomerisation (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 the synthetic feasibility and stability of the ester bond. The cis-protected ketoprofen drug 11 was synthesised in 75% yield (see the Supporting Information) and, unlike the vinyl ester, proved to be stable in cell medium, PBS (pH 7.4), and 10% plasma (only 5% free drug after 24 h; Figure S6). We propose that this increase in stability is due to the increased steric hindrance at the ester bond caused by 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) is 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 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 isomerising to the cis form (Figure 3A). This highlights a common problem with synthesising TCO-functionalised molecules. The isomerisation of cis to trans under 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 centre. Indeed, using thionyl chloride as an activating agent, we observed by HPLC the formation of eight diastereomers (Figure 3B) from the cis and trans isomers of TCO and the chiral centre of the protected ketoprofen. Using chiral HPLC, we were able to separate each diastereomer, analyse them by NMR spectroscopy and characterise the four trans isomers as enantiomeric pairs of either the axial 12ax or equatorial 12eq isomer (Figure 3C). As the axial TCO isomer has previously been shown to have different reaction rates from the equatorial isomer, each enantiomeric pair of axial and equatorial isomers was combined. We demonstrated a method of separating isomers of TCO and successfully overcame the isomerisation problem commonly experienced in synthesis with TCO, even in the challenging case of having an additional chiral centre on the drug. Now, although a low yield might be obtained, it is possible to subject TCO to reaction conditions that readily cause isomerisation and still obtain the pure trans isomer at the end.

The reaction of TCO-ketoprofen (12; axial and equatorial isomers) with tetrazine 4 (Figures 4A and 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 re-
assessed by 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 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 have different rate-determining steps.

action 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 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 have different rate-determining steps.

observed accumulation of dihydropyridazine intermediate(s) A/B (Figure 4B) demonstrates our prediction (for tetrazine 1, Figure S21) that elimination of the carboxylate after iEDDA is the rate-limiting step. In agreement with computational predictions, tetrazine 5 had a different reaction profile (Figure S8). In this case, no significant amount of long-lived intermediate was observed; this indicated that the elimination is much quicker and therefore, for this tetrazine, it is the cycloaddition step that is rate-limiting. This is also confirmed by the much slower disappearance of TCO-ketoprofen and the corresponding formation of ketoprofen (incomplete 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 (Figures 4D and S9–S12). Tetrazine 3 was chosen as a representative example of tetrazines 1, 3 and 4. It was shown that the reaction yield increased from 26% (no water) to 33% (75% water); however, no increase in yield was observed when 1% formic acid was added. 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.

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 established the nontoxic concentrations of each compound (Figures S13 and 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 nontoxic 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 (Figure S15). 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, see Figure S16 for optimisation of concentration), and the anti-inflammatory effect of the bioorthogonal pair (TCO-ester 12 and tetrazine 4) was assessed by using the Griess assay (Figure S5A). By monitoring the levels of nitric oxide (NO), we verified that when 12 was treated with 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

Figure 4. Studies of TCO-ketoprofen ester. A) Mechanism of tetrazine-triggered TCO ester decaging. B) LC-MS trace for the reaction of TCO-ketoprofen 12ax with tetrazines 4 and 5, which demonstrates the release of ketoprofen from the TCO ester prodrug after 30 s (tetrazine 4). C) Decaging yield of the reaction of TCO-KTP 12eq with tetrazines 1, 3, 4 and 5 assessed by LC-MS. The concentration of the released drug was determined by HPLC/UV by 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 have different rate-determining steps.
itself failed to reduce the NO levels, whereas 12 has a moderate effect on reducing NO levels (Figure 5B). This is likely to be due to the poor membrane permeability of ketoprofen when compared to the caged drug. When ketoprofen was protected as the TCO-ester, its cell permeability greatly improved, leading to a higher concentration of free drug in the cell, as assessed by HPLC (Figure 5C). Briefly, this study involved incubating cells for 24 h with either the prodrug or free drug. Subsequent HPLC analysis of the extracellular medium revealed almost no prodrug, although 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 that from the bioorthogonal pair (Figure S17). Although 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 medium for 24 h at 37°C. Therefore, this observation might be due to the hydrolytic enzymes inside the cell, as was confirmed by a reaction carried out with TCO-ketoprofen and esterase from porcine liver (see the synthesis section). After 4 h, a small amount of ketoprofen had already been released, and the amount increased over the next 20 h to a yield of 71% for the axial isomer and 14% for the equatorial. The release of carboxylic acids from ester prodrugs through the action of 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 might 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.

The levels of inflammation were then assessed by using an enzyme-linked immunosorbent assay (ELISA, R&D Systems) to monitor the levels of prostaglandin E₂ (PGE₂; 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 PGE₂ in the samples and PGE₂-alkaline phosphatase molecules. The alkaline phosphatase produces a chromogenic signal upon the addition of p-nitrophenyl phosphate. Therefore, the concentration of PGE₂ present in a sample is inversely proportional to the absorbance produced by the bound enzyme (Figure S18).

Cells with only LPS showed the highest level of PGE₂ (4060 pg mL⁻¹), which confirmed that inflammation was successfully stimulated. A similarly high concentration (4010 pg mL⁻¹) was observed with the tetrazine, thus 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 PGE₂ (215 pg mL⁻¹). The anti-inflammatory effect of ketoprofen can be seen by using this assay as ELISA has a higher sensitivity than the Griess assay (detection limit 0.5 μM). TCO-ketoprofen also showed lower levels of PGE₂ than the LPS control (12ₚ₈:...
686 pg mL\(^{-1}\), \(12_{\text{eq}}: 486 \text{ pg mL}^{-1}\)). The bioorthogonal pair resulted in a statistically significant reduction in PGE\(_2\) concentration compared to the prodrug alone (\(12_{\text{eq}}: 4: 193 \text{ pg mL}^{-1}, p < 0.001\); \(12_{\text{eq}}: 4: 200 \text{ pg mL}^{-1}, p < 0.001\). 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 decaying in live macrophages.

In summary we have described the bioorthogonal tetrazine-triggered release of carboxylic acid-containing molecules and demonstrated their 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 might also find use for protein activation where carboxylic acid side chains can be caged/decaged.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

- anti-inflammatory drugs
- bioorthogonal decaging
- inverse electron-demand Diels–Alder reaction
- tetrazine
- trans-cyclooctene

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