Enzymatic activation of caged tetrazines for cell-specific bioconjugation

Caroline H. Knittel[1], Stormi R. Chadwick[1], Cedrik Kuehling[1], Neal K. Devaraj[1]*

[1]Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, United States.

ABSTRACT

Tetrazine ligation techniques have evolved into a versatile tool set for bioconjugation. Despite their numerous applications, highly reactive tetrazines can suffer from long-term instability and off-target reactivity, and achieving cell-specific bioconjugation remains challenging. Tetrazine caging techniques intend to overcome these issues by achieving spatial and temporal control over tetrazine activation from stable dihydrotetrazine derivatives. Here we explore enzyme-initiated tetrazine uncaging, allowing for controlled release of reactive tetrazines by enzymes overexpressed in specific cell types. Using a modular synthetic strategy, we connected four distinct enzyme-cleavable motifs to caged tetrazine derivatives using a self-immolative linker unit. We validated cell-specific release using penicillin G amidase (PGA), which we genetically encoded into HEK293Ts. Cells expressing PGA were able to activate tetrazines which subsequently reacted with a dienophile-protected doxorubicin, lowering viability. In contrast, treatment of mammalian cells lacking PGA showed no reduction in cell viability. Additionally, we generated enzyme-cleavable conjugates that are hydrolyzed by enzymes commonly exploited for drug delivery in human cells, including esterases, cathepsin B, and alkaline phosphatases. We demonstrate cell-type specific activation of tetrazines based on the varying expression levels of alkaline phosphatases in different cell lines. Specifically, we compared tetrazine activation in the osteosarcoma cell line SAOS-2, known for its exceptionally high ALP expression levels, to cells that express lower levels of ALP, the colorectal adenocarcinoma cell line HT-29 and the stromal cell line HS-5. Overall, we find that tetrazine release from enzyme-cleavable tetrazine derivatives is dependent on the enzyme activity present in specific cells and therefore constitutes a promising technique for cell-type specific bioconjugation.

INTRODUCTION

Tetrazine bioconjugation is a widely used technique for applications in chemical biology that require chemical ligation under bioorthogonal conditions such as live-cell imaging.[1–3] Recently, tetrazine cycloadditions have gained attention for their potential use in medicine, as reactions can be coupled to the release of cytotoxic drugs[4,5] and proteolysis-targeting chimeras (PROTACs)[6–8].[9] Notably, a tetrazine-activated prodrug is currently undergoing clinical trials for treating advanced tumors, with interim results indicating increased patient tolerance.[10] Tetrazines rapidly react with strained dienophiles, such as *trans*-cyclooctenes (TCO),

cyclopropenes, and cycloalkynes through inverse electron-demand Diels Alder (IEDDA) ligations under catalyst-free conditions that are compatible with biological environments.[11,12] Nevertheless, drawbacks of tetrazine ligations include the hydrolytic instability of reactive tetrazines and their susceptibility towards strong nucleophiles.[13–15] As bioorthogonal applications are currently more widely pursued in medicine, there is increasing interest in targeted delivery of tetrazines and spatiotemporal control over their reaction with dienophiles.[16] Since tetrazines react rapidly with their ligation partners, it is extremely challenging to limit the cycloaddition reaction to a particular cell type, compartment, or tissue. Thus, the excellent reactivity of tetrazines with dienophiles can lead to undesirable off-target reactions and reduced specificity for drug-delivery applications. Improved spatial control of tetrazine activation could have a potential advantage for cell-type-specific drug release or for the delivery of larger therapeutics by assembling ligation partners intracellularly.[6]

As a step towards overcoming these challenges, a limited number of methods have been reported to generate stimuli-responsive tetrazines that require activation before cycloaddition with a dienophile.[17] The majority of these approaches leverage oxidative stimuli to activate tetrazines from dihydrotetrazines, which are reduced precursors of tetrazines with enhanced hydrolytic and nucleophilic stability. For instance, our lab showed that electrochemistry can be used to activate metal surface-bound dihydrotetrazines (**1**, Fig. 1A) to enable spatial patterning of independently addressable microelectrodes.[18] In a series of elegant studies, the Fox laboratory has achieved spatiotemporal control of tetrazine ligations through direct oxidation of unprotected dihydrotetrazines (**3**, Fig. 1B) using light and photocatalysts,[19,20] as well as oxidative enzymes.[21] Some limitations of these previous approaches are the instability of dihydrotetrazines towards oxygen and tedious activation procedures using multiple exogenous stimuli. Since tetrazine activation from dihydrotetrazines can only be triggered by oxidation, the diversity of stimuli that can be used to control tetrazine ligation reactions is limited. In addition to activating tetrazines from protected dihydrotetrazines, the use of photocleavable protecting groups has been explored to cage dienophiles such as cyclopropenes.[22] Dienophiles do not require oxidation as an additional step in order to be activated. Nevertheless, these approaches demonstrated slow reaction kinetics between the uncaged 3,3-substituted cyclopropenes and tetrazines, compared to more reactive dienophiles such as TCO.

Recently we developed a simplified method that uncages protected tetrazines using visible light (Fig. 1C).[23,24] This photoreaction leads to loss of the photocleavable protecting group and also triggers spontaneous oxidation yielding reactive tetrazines without the need for additional photocatalysts. Although visible light constitutes a minimally invasive stimuli and is well-suitable for live-cell imaging, limited deep tissue penetration as well as scattering often prohibits translational applications.[25] More importantly, light is unable to distinguish between different cell types or subcellular locations. We had previously suggested that our general approach of protecting dihydrotetrazines using chemical cages could be extended to protecting groups responsive to alternative stimuli, including enzymatic reaction.[23] As such, we decided to explore if we could generate tetrazine protecting groups that could be cleaved by endogenous enzymes overexpressed in specific cell or tissue types. Here we show that self-immolative linkers connecting enzyme-cleavable motifs to caged tetrazine derivatives form versatile chemical cages that can be deprotected in the presence of different enzymatic stimuli

(Fig. 1D). The use of self-immolative modules opens the substrate scope due to the easy functionalization of the linker with numerous chemical entities such as amides, esters, phosphates, and peptides. In our proof-of-concept experiments, we demonstrate that enzyme-triggered tetrazine uncaging can lead to cell-type-specific bioconjugation applications, namely the release of cytotoxic drugs in cell lines that overexpress specific enzymes.

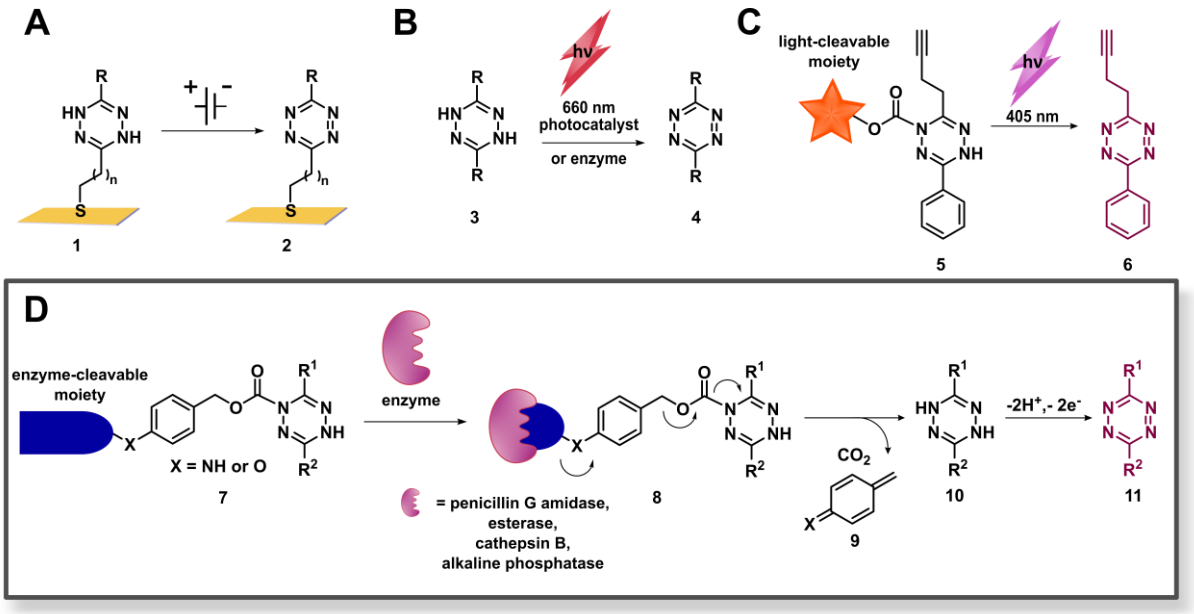

*Figure 1: Comparative graphical illustration of stimuli-responsive activation of tetrazines A) In previous work, our lab applied electrochemistry for the formation of tetrazines (**2**) on metal surfaces*[18]*. B) Several reports by the Fox group have used a combination of light, photocatalyst, and enzymes to activate tetrazines.*[19–21] *C) In recent work we utilized visible light for the uncaging of protected tetrazines (**5**).*[23,24] *(**D**) In this work we generated several enzyme-cleavable tetrazine conjugates (**7**). Enzymatic cleavage of the protecting group releases the aromatic spacer (**9**), carbon dioxide, and a dihydrotetrazine intermediate (**10**) which spontaneously oxidizes to a reactive tetrazine (**11**).*

RESULTS AND DISCUSSION

Tetrazine formation from penicillin G amidase-cleavable conjugates.

While previous approaches have successfully demonstrated the light-driven formation of tetrazines in living cells with spatiotemporal control,[17,19,20,23] they lack the ability to distinguish between specific cell types. Our objective was to achieve cell selectivity in the uncaging of tetrazines without relying on external stimuli such as light or electrochemistry. We were inspired by clinical antibody-drug conjugates (ADCs) that utilize self-immolative linker units functionalized with enzyme-recognition motifs.[26] The enzyme-recognition group is connected to the antibody via an aromatic linker, a crucial component that undergoes self-immolation after enzymatic hydrolytic cleavage, facilitating controlled drug release in cancer therapy.[27,28] Adapting this approach, we decided to employ a self-immolative spacer to conjugate various chemical moieties to caged tetrazines (Fig. 1D, **8**). We anticipated that these protecting groups

would be cleaved by enzymes that are overexpressed in specific cell types releasing the spacer, carbon-dioxide and a dihydrotetrazine intermediate (**10**). Subsequently, the dihydrotetrazine would undergo spontaneous air oxidization to form a reactive tetrazine (**11**).

Since we were aiming to achieve cell-selective uncaging of active tetrazines without relying on external stimuli, we initially focused on identifying dihydrotetrazines capable of spontaneously oxidizing in air to stable and reactive tetrazines using aqueous reaction conditions. We observed that dihydrotetrazines functionalized with alkyl and phenyl groups spontaneously undergo oxidation in aqueous solution without the need for catalysts or added oxidants (SI, Fig. S1). For instance, the 3-butynyl-6-phenyl-functionalized dihydrotetrazine (Fig. 1, **6**) was almost fully converted to the corresponding tetrazine within 1 h under slightly basic (pH = 8) as well as acidic buffer (pH = 5) conditions. Moreover, we found that derivatization of the phenyl and alkyl moieties with amino and alkyne functional groups did not impede the oxidation process, suggesting versatility for future conjugation of the caged tetrazines to various molecules such as fluorophores, drug fragments, or targeting ligands.

As previous work had demonstrated that 3-butynyl-6-phenyl-tetrazine is sufficiently reactive for ligation with TCO under live cell conditions,[23] we selected **6** as a model for our subsequent experiments investigating the enzymatic uncaging of tetrazines. We synthesized a caged tetrazine conjugate (**14**, Fig 2A) consisting of a 3-butynyl-6-phenyl-tetrazine derivative connected to a phenylacetyl group by a self-immolative aminobenzyl alcohol moiety. As a caging group, we selected the phenylacetyl group as it is susceptible to hydrolysis in the presence of bacterial penicillin G amidase (PGA) with excellent substrate specificity.[29] We chose to initially explore PGA-susceptible linkers given previous studies demonstrating the lack of PGA activity in mammalian cells. Moreover, certain human cell lines can be genetically modified to encode active PGA capable of uncaging phenylacetyl functionalized substrates.[30,31] The two-step synthesis of **14** was conveniently performed in one pot using triphosgene for the carbamoylation reaction between **12** and the aromatic protecting group. With **14** in hand, we investigated tetrazine uncaging by enzymatic hydrolysis of the phenylacetamide motif using PGA from *E. coli* (Fig. 2B). To better mimic conditions present in living cells, we performed the reaction in Dulbecco's Modified Eagle's Medium (DMEM) and observed complete uncaging and formation of tetrazine **6** within 45 min (Fig. 2C). As expected, formation of dihydrotetrazine **12** was initially observed as an intermediate after hydrolysis of the protecting group, followed by spontaneous oxidation and generation of tetrazine **6**. When we performed the reaction in DMEM after degassing with Argon, we observed no oxidation of dihydrotetrazine **12** to tetrazine **6** within 45 min (SI, Fig. S2), confirming that air is crucial for the tetrazine oxidation. We tested the stability of **6**, after the uncaging and oxidation reaction, and could not detect any hydrolyzed or reduced products within a 24-hour timeframe using LC-MS (SI, Fig. S3). We also explored the tetrazine substrate scope of our two-step conjugate formation strategy and synthesized two additional conjugates (**15** and **16**) with different derivatizations of the aliphatic and aromatic functional groups at the 3- and 6-positions of the tetrazine derivative. While PGA-uncaging and tetrazine formation from conjugate **15** took approximately 60 min (SI Fig. S4), conjugate **16** was converted to tetrazine **6** within 45 min (SI, Fig. S5) suggesting that asymmetric 3-alkyl-6-phenyl substituted dihydrotetrazines are more prone to spontaneous oxidation in aqueous solution than symmetric diphenyl dihydrotetrazines.

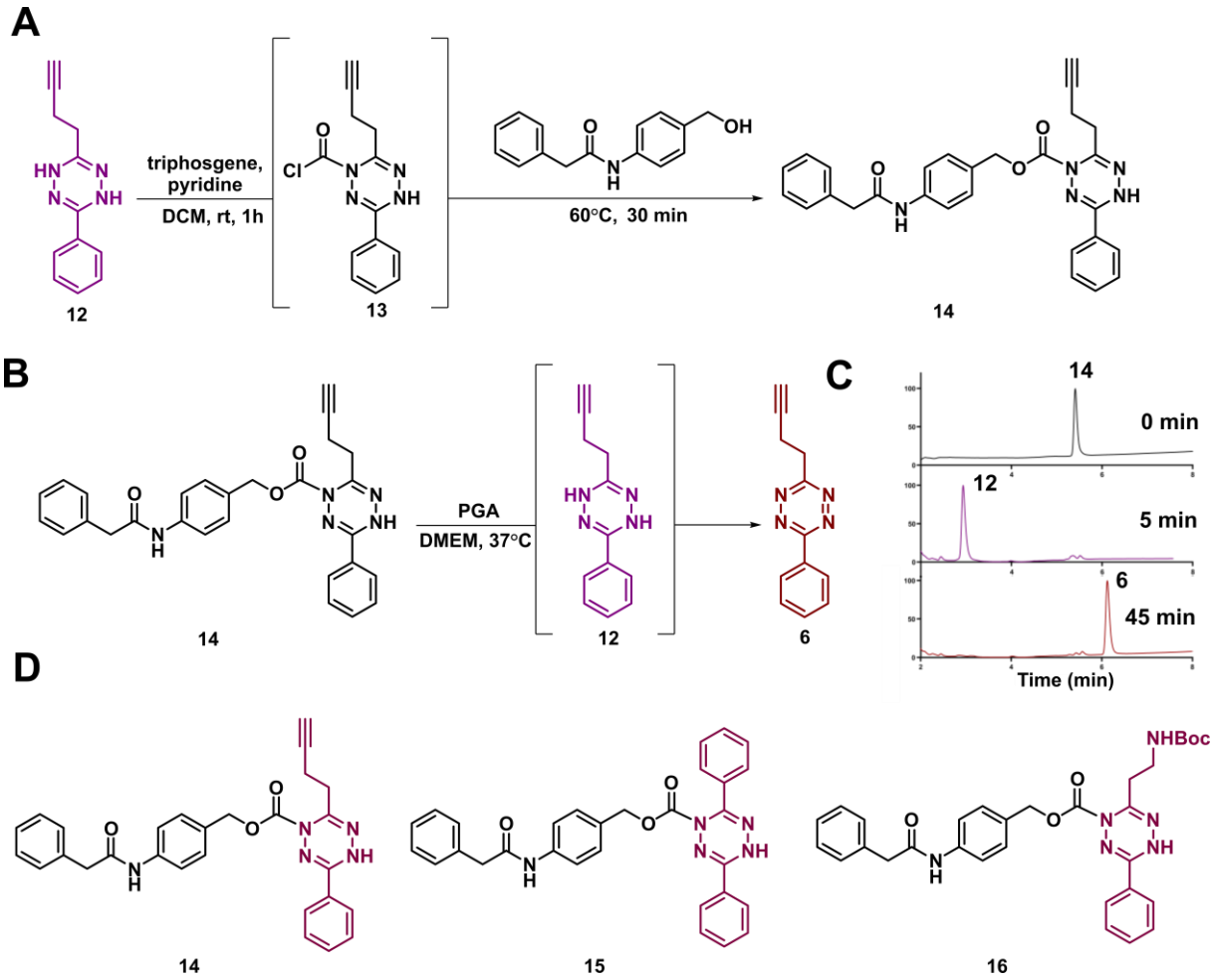

*Figure 2: Synthesis and enzymatic uncaging of PGA-cleavable tetrazine conjugates. A) One-pot synthesis of a phenylacetamide-functionalized 1,2,4,5-tetrazine derivative **14**. B) Enzymatic uncaging of tetrazine **6** using PGA in DMEM via dihydrotetrazine **12** as an intermediate. C) Monitoring of the reaction via HPLC-UV (at the 243 nm isosbestic point of dihydrotetrazine **12** and tetrazine **6**) demonstrates full conversion to **6** after 45 min. D) Substrate scope of phenylacetamide-functionalized 1,2,4,5-tetrazine derivatives **14**, **15**, and **16** tested in this work.*

Enzymatic tetrazine uncaging using PGA for the bioorthogonal release of doxorubicin

Having established a system for releasing tetrazines from PGA-cleavable conjugates, we next determined if enzyme release of tetrazine **6** from **14** could lead to reaction with a dienophile-caged drug such as doxorubicin. Doxorubicin is a potent cytotoxic drug used in cancer therapy, but its clinical application is limited due to its high cardiotoxicity.[32] Caged doxorubicin prodrugs, however, have demonstrated reduced cardiotoxicity compared to the unmodified drug. Notably, TCO-functionalized doxorubicin (TCO-DOX) exhibits enhanced cell permeability and reacts with tetrazines to release doxorubicin in a traceless elimination reaction.[4,33] Several reports have highlighted the promising potential of TCO-caged doxorubicin prodrugs for targeted therapies with low off-target toxicity.[16,34] Recent studies have reported on human clinical trials examining TCO-derivatized doxorubicin activated by tetrazine ligation.[35]

We initially investigated DOX-uncaging using PGA and PGA-cleavable conjugate **14** by adding TCO-DOX (25 µM) and **14** (50 µM) to a stirred solution of the PGA enzyme (70 nM) in DMEM and observed initial release of DOX after 15 min *via* LC-MS. After 2h we observed only trace amounts TCO-DOX via HPLC-UV with near quantitative conversion to uncaged DOX. (SI, Fig. S6). In the absence of PGA, no tetrazine uncaging occurred and likewise no release of DOX was observed. These results confirmed that conjugate **14** releases an activated tetrazine (**6**) that can react by cycloaddition with a dienophile-caged drug (Fig. 3A).

Satisfied with our initial testing, we then determined whether tetrazine-uncaging from **14** was compatible with live-cell applications. Adapting a previous method reported by the Spitale group,[31] we hypothesized that a human cell line engineered to express PGA would trigger tetrazine release when exposed to **14** (Fig. 3B). In contrast, an untransduced human cell line would serve as a negative control for tetrazine uncaging since human proteases do not hydrolyze the phenylacetamide moiety (Fig. 3C). To assay tetrazine release, we co-incubated **14** with TCO-DOX reasoning that successful tetrazine uncaging would result in subsequent cycloaddition with TCO-DOX, leading to the liberation of cytotoxic DOX and consequent induction of cell death. We established a stable human cell line expressing PGA *via* lentiviral transduction and treated the cells with DOX, TCO-DOX, and the PGA conjugate respectively (Fig. 3D). Subsequent cell proliferation assays indicated that treatment of the cells with 0.9 µM DOX resulted in cell death, while treatment with 0.9 µM TCO-DOX or PGA-cleavable conjugate **14** alone, showed no significant change in viability. When we treated the cells with a combination of 0.9 µM conjugate **14** and 0.9 µM TCO-DOX (**17**), we observed cell death almost to the same extent as DOX alone (**18**), and similar to a positive control using a combination of tetrazine **6** and TCO-DOX (**17**). When we repeated the experiment using human cells not expressing PGA, the combination of 0.9 µM conjugate **14** and 0.9 µM TCO-DOX did not impact the cell viability while treatment with TCO-DOX and tetrazine **6** showed cell death to a similar extent as DOX alone (Fig. 3E). Thus, we were able to demonstrate cell-specific activation of tetrazines and bioorthogonal ligation using an enzymatically cleavable protecting group.

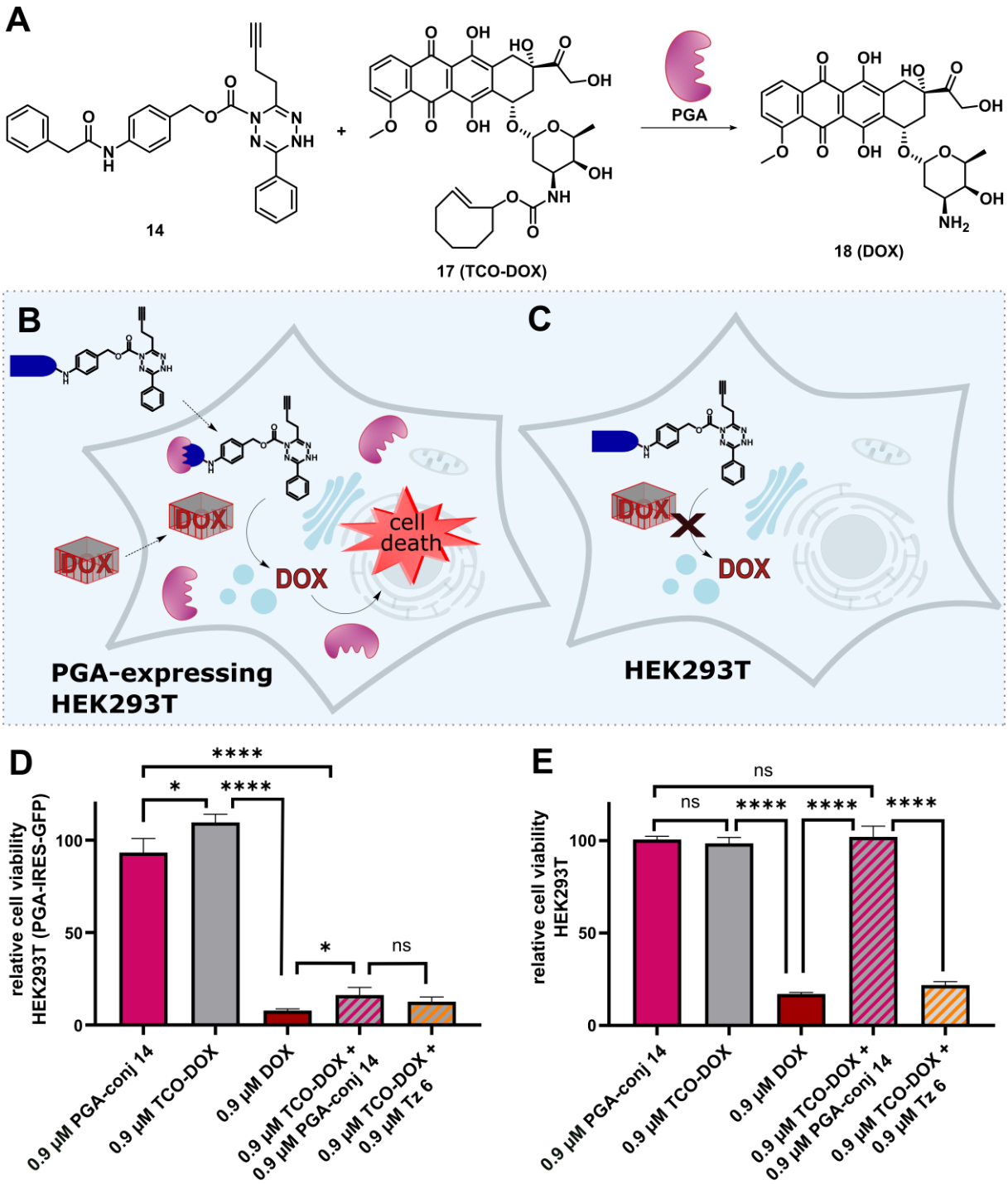

*Figure 3: Doxorubicin-release from a PGA-cleavable tetrazine conjugate **14**. A) Adding a mixture of PGA-cleavable conjugate **14** and TCO-DOX to a stirring solution of PGA in DMEM (pH = 7.4) results in uncaging of doxorubicin. B) Graphical illustration of doxorubicin release in PGA-expressing HEK293T cells from TCO-DOX and **14** causing cell death. C) The cell viability of HEK293T cells that do not express the enzyme stays unaffected. C) Cell proliferation assay (XTT) of PGA-expressing HEK293T cells demonstrate that treatment of the cells with conjugate **14** and TCO-DOX alone does not result in cell death, while treatment with a combination of **14** and TCO-DOX leads to a significant decrease in cell viability after 48 h, to a similar degree as treatment with DOX alone and the positive control using a combination of TCO-DOX and tetrazine **6**. D) Cell proliferation assay (XTT) of HEK293T cells that were not transduced with PGA. Treatment with a combination of **14** and TCO-DOX does not decrease the cell viability after 48 h while treatment with DOX alone and the positive control using a combination of*

*TCO-DOX and tetrazine **6** leads to significant cell death. Viability assays were performed in three biological replicates and are shown as means ± SD. Statistically significant differences between points of PGA-expressing HEK293T (D) or wild-type HEK293T (E) are indicated: *P < 0.05, ***P < 0.001, ****P < 0.0001. ns, not significant.*

Development of tetrazine conjugates cleavable by enzymes produced by human cells

Having demonstrated the feasibility of cell-specific enzymatic drug release from caged tetrazines using a transduced bacterial enzyme, we next directed our efforts toward exploring tetrazine protecting groups that would be susceptible to cleavage by enzymes produced in human cells. One advantage of our synthetic strategy is its modularity, as many different enzyme-cleavable moieties could be substituted using the same linker chemistry. For instance, we synthesized a conjugate (**19**, Fig. 5A) carrying an acetylated self-immolative spacer that is cleavable by esterases. Given the ubiquity of esterases in cells and the widespread use of esterase-cleavable prodrugs,[36] this conjugate could be used to release tetrazines in a wide variety of cells. To address enzymes known to be overexpressed in certain disease-specific cells, we synthesized a conjugate (**20**) containing a valine-alanine peptide motif that is hydrolyzed by cathepsin B (CatB), a lysosomal cysteine protease upregulated in various fatal tumors such as those resulting from breast and metastatic kidney cancers.[37,38] Furthermore, CatB is an enzyme that has been widely exploited for triggering intracellular drug release. For instance, more than 40 clinically used ADCs contain CatB-cleavable linkers including the valine-alanine motif.[39,40] Finally, we synthesized phosphorylated conjugate **21**, which is cleavable by alkaline phosphatases (ALP). Alkaline phosphatases are known to be overexpressed in cells from aggressive cancers such as osteosarcomas and hepatocarcinomas.[41,42] In contrast, low ALP expression is present in the colorectal adenocarcinoma cell line HT-29,[43] as well as stromal cells such as HS-5.[42] We successfully demonstrated enzyme-directed tetrazine uncaging from all three caged conjugates (SI, Fig. S7, S8, and S9) using commercially available mammalian enzymes. Conjugate **19** reacted with porcine liver esterase, **20** with cathepsin B from bovine spleen, and **21** with alkaline phosphatase from bovine intestine.

We decided to pursue additional live-cell studies using CatB-cleavable conjugate **20,** given its already mentioned well-validated use as an ADC linker component.[39,44] Initially we performed an enzyme activity assay on HeLa and HEK293Ts, and confirmed positive CatB expression as well as CatB inhibition by inhibitor CA074 in both cell lines (SI, Fig. S10). When we submitted HeLa cells to treatment with either CatB-cleavable conjugate **20** or TCO-DOX alone, we observed no significant decrease in cell viability after 48 hours (Fig. 4B), consistent with our previous observations in PGA-expressing human cells treated with conjugate **14**. However, concurrent 48 h treatment with 0.9 µM **20** and 0.9 µM TCO-DOX led to a significant decrease in cell viability to the same extent as treatment with the TCO-DOX/tetrazine **6** positive control. In contrast, when we preincubated the cells with a cell-permeable CatB inhibitor (CA074-Me, 12.5µM),[45] cell viability was rescued by 4.5 fold, supporting that CatB activity is responsible for tetrazine uncaging. HEK293T cells tolerated a lower concentration of inhibitor CA074-Me (3 µM) compared to HeLa cells. However, cell viability still increased by 2-fold when HEK293T

cells were treated simultaneously with CA074-Me inhibitor, CatB conjugate **20**, and TCO-DOX, compared to treatment with **20** and TCO-DOX alone (SI, Fig. S11).

Finally, we tested if ALP-cleavable conjugate **21** can release a cytotoxic drug in ALP-overexpressing cell lines such as the osteosarcoma cell line SAOS-2. Osteosarcoma is a malignant form of bone cancer that is most commonly diagnosed in teenagers and young adults.[46] While doxorubicin is a state-of-the-art drug in the treatment of osteosarcoma,[47] dosages of the cytotoxic drug are limited due to increased cardiotoxicity.[48] Initially, we validated increased ALP levels in SAOS-2 cells by performing an ALP activity assay on cell lysates from SAOS-2 and comparing them to ALP-levels in lysates from HS-2 and HT-29 cells (SI, Fig. S12). We observed ALP-activity in SAOS-2 lysates to be 18-fold higher than in HS-2 lysates, and 25-fold higher than in HT-29 lysates. When we treated SAOS-2 cells with a combination of 1.5 µM ALP conjugate **21** and 1.5 µM TCO-DOX (Fig. 4C), we observed substantial cell death after 72 h, comparable to the observed cell death in the doxorubicin and TCO-DOX/tetrazine **6** positive controls. Remarkably, when HS-2 (Fig. 4D) and HT-29 cells (Fig. 4E) were treated with the same treatment conditions, cell proliferation assays revealed improved cell viability compared to SAOS-2. Cell viability in HS-5 cells improved by 4-fold during concurrent treatment with TCO-DOX and **21** when compared to the TCO-DOX/tetrazine **6** positive control. HT-29 cells retained almost full cell viability and had a 3-fold improvement compared to the positive control. These observations are in alignment with our previous ALP-activity assessment in these cell lines. Ultimately, these results indicate that we were able to achieve cell-type-specificity in the release of tetrazines by generating protected tetrazines responsive to enzymes with varying expression levels between cell lines.

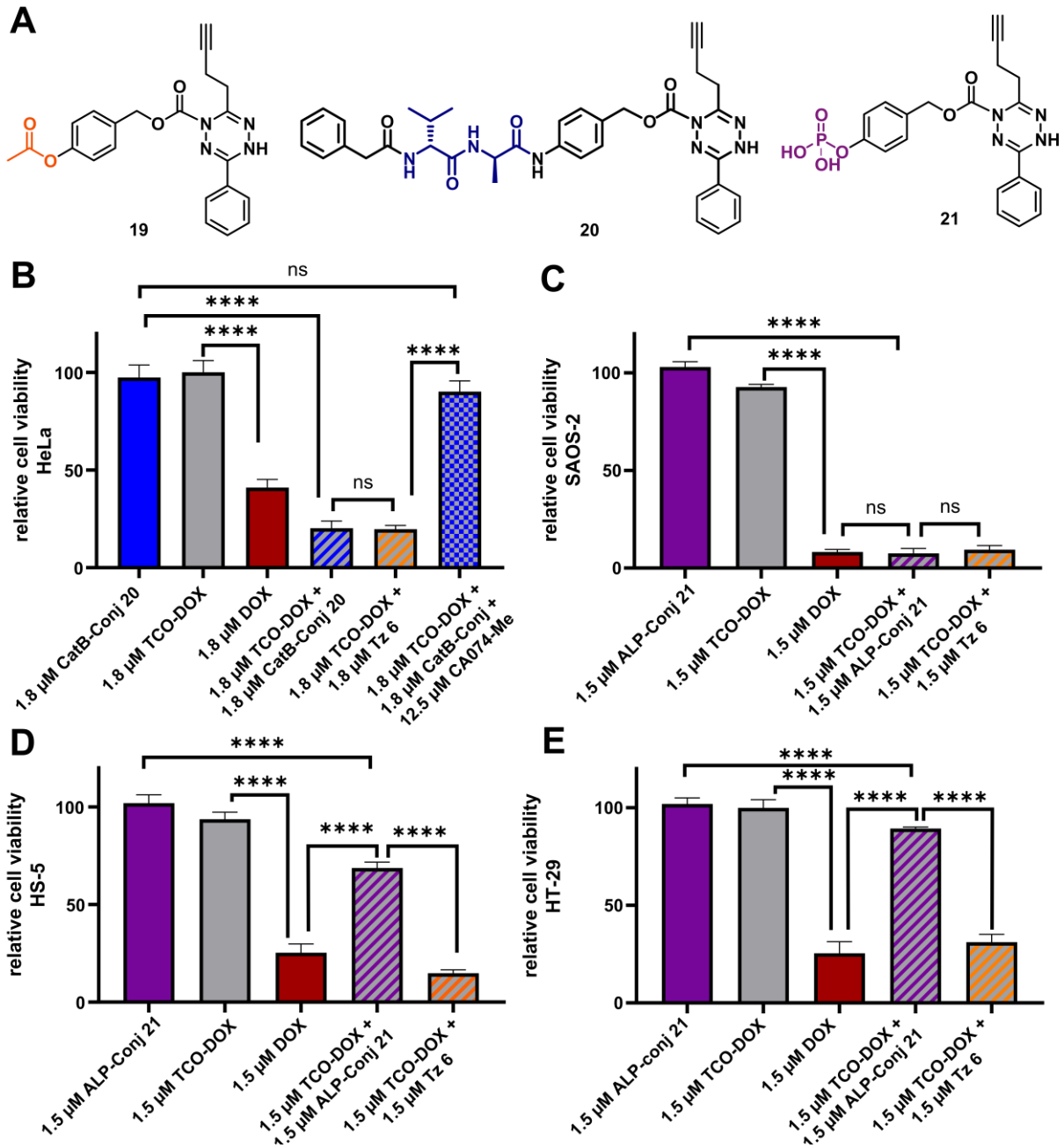

*Figure 4: Tetrazine conjugates cleavable by enzymes produced by human cells. A) Substrate scope tested for tetrazine-release in this work. B) Cell proliferation assay (XTT) of HeLa demonstrates that treatment with CatB-cleavable conjugate **20** and TCO-DOX alone does not result in cell death, while treatment with a combination of **20** and TCO-DOX leads to a significant decrease in cell viability after 48 h to the same extent as treatment with the TCO-DOX/tetrazine **6** control. Addition of CatB inhibitor CA074-Me to cells treated with **20** and TCO-DOX raises the cell viability by 4.5 fold. C) Cell proliferation assay (XTT) of ALP-overexpressing SAOS-2 shows significant decrease in cell viability upon treatment with ALP-cleavable conjugate **21** and TCO-DOX to the same extent as the DOX and TCO-DOX/tetrazine **6** control, whereas simultaneous treatment of low ALP-expressing cells HS-5 (D) and HT-29 (E) leads a 4- (D) and 3-fold (E) higher cell viability compared to the TCO-DOX/tetrazine **6** control. Viability assays were performed in six biological replicates and are shown as means ± SD. Statistically significant differences between points of (B), (C), (D), and (E) are indicated: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, not significant.*

CONCLUSIONS

In summary, we have demonstrated a straightforward approach for releasing reactive tetrazines in living cells in response to endogenous enzymes. Employing self-immolative linker chemistries, we connected enzyme-cleavable protecting groups to caged tetrazine derivatives enhancing cell specificity. We successfully implemented our methodology for drug release, particularly focusing on the uncaging of the potent cytotoxic drug doxorubicin. Through four enzyme-cleavable conjugates, we achieved controlled doxorubicin uncaging, suggesting a promising bioorthogonal strategy for confronting the off-target toxicity challenges associated with unmodified doxorubicin. Our process offers the potential for higher cell specificity by using cages that are responsive to enzymes overexpressed in certain cell types. Furthermore, we think that our work significantly contributes to the evolving field of stimuli responsive tetrazine activation. The ability to selectively uncage tetrazines in response to cleavage by endogenous enzymes provides a platform for various potential applications ranging from drug delivery to cell-specific imaging. We foresee caged tetrazines being used for the release of cytotoxic drugs,[49] improved coformulation of protected tetrazines with dienophiles, triggering bioorthogonal polymerization,[50] and intracellular drug assembly.[6] Overall, we believe caged bioorthogonal reagents have high potential for advancing targeted therapies and will have numerous applications in broadening the utility of tetrazine ligation chemistry.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health (R35GM141939),
C. H. K. acknowledges the financial support from the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG, KN 1447/1-1)

NOTES

N. K. D. and C. H. K. are listed as inventors on a provisional patent application related to the work published in this manuscript.

AUTHOR INFORMATION

Corresponding Author

Neal K. Devaraj - Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, United States; https://orcid.org/0000-0002-8033-9973; Email: ndevaraj@ucsd.edu

Authors

Caroline H Knittel - Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, UnitedStates; https://orcid.org/0000-0001-7968-7455

Stormi R Chadwick - Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, United States; https://orcid.org/0000-0003-0407-9740

Cedrik Kuehling - Max Planck Institute of Molecular Physiology, Otto-Hahn-Str. 11, 44227 Dortmund, Germany https://orcid.org/0009-0007-0088-3690

REFERENCES

(1) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. Tetrazine-Based Cycloadditions: Application to Pretargeted Live Cell Imaging. *Bioconjug. Chem.* **2008**, *19* (12), 2297–2299. https://doi.org/10.1021/bc8004446.

(2) Blackman, M. L.; Royzen, M.; Fox, J. M. Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels−Alder Reactivity. *J. Am. Chem. Soc.* **2008**, *130* (41), 13518–13519. https://doi.org/10.1021/ja8053805.

(3) Wu, H.; Devaraj, N. K. Advances in Tetrazine Bioorthogonal Chemistry Driven by the Synthesis of Novel Tetrazines and Dienophiles. *Acc. Chem. Res.* **2018**, *51* (5), 1249–1259. https://doi.org/10.1021/acs.accounts.8b00062.

(4) Versteegen, R. M.; Rossin, R.; ten Hoeve, W.; Janssen, H. M.; Robillard, M. S. Click to Release: Instantaneous Doxorubicin Elimination upon Tetrazine Ligation. *Angew. Chem. Int. Ed.* **2013**, *52* (52), 14112–14116. https://doi.org/10.1002/anie.201305969.

(5) Li, H.; Conde, J.; Guerreiro, A.; Bernardes, G. J. L. Tetrazine Carbon Nanotubes for Pretargeted In Vivo "Click-to-Release" Bioorthogonal Tumour Imaging. *Angew. Chem. Int. Ed.* **2020**, *59* (37), 16023–16032. https://doi.org/10.1002/anie.202008012.

(6) Lebraud, H.; Wright, D. J.; Johnson, C. N.; Heightman, T. D. Protein Degradation by In-Cell Self-Assembly of Proteolysis Targeting Chimeras. *ACS Cent. Sci.* **2016**, *2* (12), 927–934. https://doi.org/10.1021/acscentsci.6b00280.

(7) Chang, M.; Gao, F.; Pontigon, D.; Gnawali, G.; Xu, H.; Wang, W. Bioorthogonal PROTAC Prodrugs Enabled by On-Target Activation. *J. Am. Chem. Soc.* **2023**, *145* (25), 14155–14163. https://doi.org/10.1021/jacs.3c05159.

(8) Bi, T.; Liang, P.; Zhou, Y.; Wang, H.; Huang, R.; Sun, Q.; Shen, H.; Yang, S.; Ren, W.; Liu, Z. Rational Design of Bioorthogonally Activatable PROTAC for Tumor-Targeted Protein Degradation. *J. Med. Chem.* **2023**, *66* (21), 14843–14852. https://doi.org/10.1021/acs.jmedchem.3c01423.

(9) Carlson, J. C. T.; Mikula, H.; Weissleder, R. Unraveling Tetrazine-Triggered Bioorthogonal Elimination Enables Chemical Tools for Ultrafast Release and Universal Cleavage. *J. Am. Chem. Soc.* **2018**, *140* (10), 3603–3612. https://doi.org/10.1021/jacs.7b11217.

(10) Chawla, S. P.; Batty, K.; Aleckovic, M.; Bhadri, V.; Bui, N.; Guminski, A. D.; Mejia Oneto, J. M.; Srinivasan, S.; Strauss, J. F.; Subbiah, V.; Weiss, M. C.; Wilson, R.; Yee, N. A.; Zakharian, M.; Kwatra, V. Interim Phase 1 Results for SQ3370 in Advanced Solid Tumors. *J. Clin. Oncol.* **2022**, *40*, 3085–3085. https://doi.org/10.1200/JCO.2022.40.16.

(11) Yang, J.; Šečkutė, J.; Cole, C. M.; Devaraj, N. K. Live-Cell Imaging of Cyclopropene Tags with Fluorogenic Tetrazine Cycloadditions. *Angew. Chem. Int. Ed.* **2012**, *51* (30), 7476–7479. https://doi.org/10.1002/anie.201202122.

(12) Selvaraj, R.; Fox, J. M. Trans-Cyclooctene--a Stable, Voracious Dienophile for Bioorthogonal Labeling. *Curr. Opin. Chem. Biol.* **2013**, *17* (5), 753–760. https://doi.org/10.1016/j.cbpa.2013.07.031.

(13) Svatunek, D.; Wilkovitsch, M.; Hartmann, L.; Houk, K. N.; Mikula, H. Uncovering the Key Role of Distortion in Bioorthogonal Tetrazine Tools That Defy the Reactivity/Stability Trade-Off. *J. Am. Chem. Soc.* **2022**, *144* (18), 8171–8177. https://doi.org/10.1021/jacs.2c01056.

(14) Kämpchen, T.; Massa, W.; Overheu, W.; Schmidt, R.; Seitz, G. Zur Kenntnis von Reaktionen des 1,2,4,5-Tetrazin-3,6-dicarbonsäure-dimethylesters mit Nucleophilen. *Chem. Ber.* **1982**, *115* (2), 683–694. https://doi.org/10.1002/cber.19821150228.

(15) Tu, J.; Svatunek, D.; Parvez, S.; Liu, A. C.; Levandowski, B. J.; Eckvahl, H. J.; Peterson, R. T.; Houk, K. N.; Franzini, R. M. Stable, Reactive, and Orthogonal Tetrazines: Dispersion Forces Promote the Cycloaddition with Isonitriles. *Angew. Chem. Int. Ed.* **2019**, *58* (27), 9043–9048. https://doi.org/10.1002/anie.201903877.

(16) Wu, K.; Yee, N. A.; Srinivasan, S.; Mahmoodi, A.; Zakharian, M.; Oneto, J. M. M.; Royzen, M. Click Activated Protodrugs against Cancer Increase the Therapeutic Potential of Chemotherapy through Local Capture and Activation. *Chem. Sci.* **2021**, *12* (4), 1259–1271. https://doi.org/10.1039/D0SC06099B.
(17) Knittel, C. H.; Devaraj, N. K. Bioconjugation Strategies for Revealing the Roles of Lipids in Living Cells. *Acc. Chem. Res.* **2022**, *55* (21), 3099–3109. https://doi.org/10.1021/acs.accounts.2c00511.
(18) Ehret, F.; Wu, H.; Alexander, S. C.; Devaraj, N. K. Electrochemical Control of Rapid Bioorthogonal Tetrazine Ligations for Selective Functionalization of Microelectrodes. *J. Am. Chem. Soc.* **2015**, *137* (28), 8876–8879. https://doi.org/10.1021/jacs.5b03371.
(19) Jemas, A.; Xie, Y.; Pigga, J. E.; Caplan, J. L.; am Ende, C. W.; Fox, J. M. Catalytic Activation of Bioorthogonal Chemistry with Light (CABL) Enables Rapid, Spatiotemporally Controlled Labeling and No-Wash, Subcellular 3D-Patterning in Live Cells Using Long Wavelength Light. *J. Am. Chem. Soc.* **2022**, *144* (4), 1647–1662. https://doi.org/10.1021/jacs.1c10390.
(20) Wang, C.; Zhang, H.; Zhang, T.; Zou, X.; Wang, H.; Rosenberger, J. E.; Vannam, R.; Trout, W. S.; Grimm, J. B.; Lavis, L. D.; Thorpe, C.; Jia, X.; Li, Z.; Fox, J. M. Enabling In Vivo Photocatalytic Activation of Rapid Bioorthogonal Chemistry by Repurposing Silicon-Rhodamine Fluorophores as Cytocompatible Far-Red Photocatalysts. *J. Am. Chem. Soc.* **2021**, *143* (28), 10793–10803. https://doi.org/10.1021/jacs.1c05547.
(21) Zhang, H.; Trout, W. S.; Liu, S.; Andrade, G. A.; Hudson, D. A.; Scinto, S. L.; Dicker, K. T.; Li, Y.; Lazouski, N.; Rosenthal, J.; Thorpe, C.; Jia, X.; Fox, J. M. Rapid Bioorthogonal Chemistry Turn-on through Enzymatic or Long Wavelength Photocatalytic Activation of Tetrazine Ligation. *J. Am. Chem. Soc.* **2016**, *138* (18), 5978–5983. https://doi.org/10.1021/jacs.6b02168.
(22) Kumar, P.; Jiang, T.; Li, S.; Zainul, O.; Laughlin, S. T. Caged Cyclopropenes for Controlling Bioorthogonal Reactivity. *Org. Biomol. Chem.* **2018**, *16* (22), 4081–4085. https://doi.org/10.1039/C8OB01076E.
(23) Liu, L.; Zhang, D.; Johnson, M.; Devaraj, N. K. Light-Activated Tetrazines Enable Precision Live-Cell Bioorthogonal Chemistry. *Nat. Chem.* **2022**, 1–8. https://doi.org/10.1038/s41557-022-00963-8.
(24) Liu, L.; Zhang, D.; Johnson, M.; Devaraj, N. K. Light-Activated Tetrazines Enable Live-Cell Spatiotemporal Control of Bioorthogonal Reactions. bioRxiv December 2, 2020, p 2020.12.01.405423. https://doi.org/10.1101/2020.12.01.405423.
(25) Hsieh, Z.-H.; Fan, C.-H.; Ho, Y.-J.; Li, M.-L.; Yeh, C.-K. Improvement of Light Penetration in Biological Tissue Using an Ultrasound-Induced Heating Tunnel. *Sci. Rep.* **2020**, *10* (1), 17406. https://doi.org/10.1038/s41598-020-73878-4.
(26) Bargh, J. D.; Isidro-Llobet, A.; Parker, J. S.; Spring, D. R. Cleavable Linkers in Antibody-Drug Conjugates. *Chem. Soc. Rev.* **2019**, *48* (16), 4361–4374. https://doi.org/10.1039/c8cs00676h.
(27) Chia, C. S. B. A Patent Review on FDA-Approved Antibody-Drug Conjugates, Their Linkers and Drug Payloads. *ChemMedChem* **2022**, *17* (11). https://doi.org/10.1002/cmdc.202200032.
(28) Joubert, N.; Beck, A.; Dumontet, C.; Denevault-Sabourin, C. Antibody–Drug Conjugates: The Last Decade. *Pharmaceuticals* **2020**, *13* (9), 245. https://doi.org/10.3390/ph13090245.
(29) Kadereit, D.; Waldmann, H. Enzymatic Protecting Group Techniques. *Chem. Rev.* **2001**, *101* (11), 3367–3396. https://doi.org/10.1021/cr010146w.
(30) Barrett, R. M.; Liu, H.; Jin, H.; Goodman, R. H.; Cohen, M. S. Cell-Specific Profiling of Nascent Proteomes Using Orthogonal Enzyme-Mediated Puromycin Incorporation. *ACS Chem. Biol.* **2016**, *11* (6), 1532–1536. https://doi.org/10.1021/acschembio.5b01076.
(31) Nguyen, K.; Fazio, M.; Kubota, M.; Nainar, S.; Feng, C.; Li, X.; Atwood, S. X.; Bredy, T. W.; Spitale, R. C. Cell-Selective Bioorthogonal Metabolic Labeling of RNA. *J. Am. Chem. Soc.* **2017**, *139* (6), 2148–2151. https://doi.org/10.1021/jacs.6b11401.
(32) Schöffski, P.; Delord, J.-P.; Brain, E.; Robert, J.; Dumez, H.; Gasmi, J.; Trouet, A. First-in-Man Phase I Study Assessing the Safety and Pharmacokinetics of a 1-Hour Intravenous Infusion of

the Doxorubicin Prodrug DTS-201 Every 3 Weeks in Patients with Advanced or Metastatic Solid Tumours. *Eur. J. Cancer* **2017**, *86*, 240–247. https://doi.org/10.1016/j.ejca.2017.09.009.
(33) Lin, F.; Chen, L.; Zhang, H.; Ching, N. W. S.; Zeng, X.; Lin, J.; Chen, P. R. Bioorthogonal Prodrug–Antibody Conjugates for On-Target and On-Demand Chemotherapy. *CCS Chem.* **2019**, *1* (2), 226–236. https://doi.org/10.31635/ccschem.019.20180038.
(34) Srinivasan, S.; Yee, N. A.; Wu, K.; Zakharian, M.; Mahmoodi, A.; Royzen, M.; Mejía Oneto, J. M. SQ3370 Activates Cytotoxic Drug via Click Chemistry at Tumor and Elicits Sustained Responses in Injected and Non-Injected Lesions. *Adv. Ther.* **2021**, *4* (3). https://doi.org/10.1002/adtp.202000243.
(35) Srinivasan, S.; Yee, N. A.; Zakharian, M.; Alečković, M.; Mahmoodi, A.; Nguyen, T.-H.; Oneto, J. M. M. SQ3370, the First Clinical Click Chemistry-Activated Cancer Therapeutic, Shows Safety in Humans and Translatability across Species. bioRxiv March 29, 2023, p 2023.03.28.534654. https://doi.org/10.1101/2023.03.28.534654.
(36) Lavis, L. D. Ester Bonds in Prodrugs. *ACS Chem. Biol.* **2008**, *3* (4), 203–206. https://doi.org/10.1021/cb800065s.
(37) Bengsch, F.; Buck, A.; Günther, S. C.; Seiz, J. R.; Tacke, M.; Pfeifer, D.; von Elverfeldt, D.; Sevenich, L.; Hillebrand, L. E.; Kern, U.; Sameni, M.; Peters, C.; Sloane, B. F.; Reinheckel, T. Cell Type-Dependent Pathogenic Functions of Overexpressed Human Cathepsin B in Murine Breast Cancer Progression. *Oncogene* **2014**, *33* (36), 4474–4484. https://doi.org/10.1038/onc.2013.395.
(38) Gondi, C. S.; Rao, J. S. Cathepsin B as a Cancer Target. *Expert Opin. Ther. Targets* **2013**, *17* (3), 281–291. https://doi.org/10.1517/14728222.2013.740461.
(39) Su, Z.; Xiao, D.; Xie, F.; Liu, L.; Wang, Y.; Fan, S.; Zhou, X.; Li, S. Antibody–Drug Conjugates: Recent Advances in Linker Chemistry. *Acta Pharm. Sin. B* **2021**, *11* (12), 3889–3907. https://doi.org/10.1016/j.apsb.2021.03.042.
(40) Lee, A. Loncastuximab Tesirine: First Approval. *Drugs* **2021**, *81* (10), 1229–1233. https://doi.org/10.1007/s40265-021-01550-w.
(41) Zhou, J.; Du, X.; Berciu, C.; He, H.; Shi, J.; Nicastro, D.; Xu, B. Enzyme-Instructed Self-Assembly for Spatiotemporal Profiling of the Activities of Alkaline Phosphatases on Live Cells. *Chem* **2016**, *1* (2), 246–263. https://doi.org/10.1016/j.chempr.2016.07.003.
(42) Feng, Z.; Han, X.; Wang, H.; Tang, T.; Xu, B. Enzyme-Instructed Peptide Assemblies Selectively Inhibit Bone Tumors. *Chem* **2019**, *5* (9), 2442–2449. https://doi.org/10.1016/j.chempr.2019.06.020.
(43) Herz, F.; Schermer, A.; Halwer, M.; Bogart, L. H. Alkaline Phosphatase in HT-29, a Human Colon Cancer Cell Line: Influence of Sodium Butyrate and Hyperosmolality. *Arch. Biochem. Biophys.* **1981**, *210* (2), 581–591. https://doi.org/10.1016/0003-9861(81)90224-1.
(44) Zammarchi, F.; Corbett, S.; Adams, L.; Tyrer, P. C.; Kiakos, K.; Janghra, N.; Marafioti, T.; Britten, C. E.; Havenith, C. E. G.; Chivers, S.; D'Hooge, F.; Williams, D. G.; Tiberghien, A.; Howard, P. W.; Hartley, J. A.; van Berkel, P. H. ADCT-402, a PBD Dimer–Containing Antibody Drug Conjugate Targeting CD19-Expressing Malignancies. *Blood* **2018**, *131* (10), 1094–1105. https://doi.org/10.1182/blood-2017-10-813493.
(45) Yoon, M. C.; Christy, M. P.; Phan, V. V.; Gerwick, W. H.; Hook, G.; O'Donoghue, A. J.; Hook, V. Molecular Features of CA-074 pH-Dependent Inhibition of Cathepsin B. *Biochemistry* **2022**, *61* (4), 228–238. https://doi.org/10.1021/acs.biochem.1c00684.
(46) Gorlick, R. G.; Toretsky, J. A.; Marina, N.; Wolden, S. L.; Randall, R. L.; Gebhardt, M. C.; Teot, L. A.; Bernstein, M. Osteosarcoma. In *Holland-Frei Cancer Medicine. 6th edition*; BC Decker, 2003.
(47) Ji, P.; Yu, L.; Guo, W.-C.; Mei, H.-J.; Wang, X.-J.; Chen, H.; Fang, S.; Yang, J. Doxorubicin Inhibits Proliferation of Osteosarcoma Cells Through Upregulation of the Notch Signaling Pathway. *Oncol. Res.* **2014**, *22* (4), 185–191. https://doi.org/10.3727/096504015X14343704124340.
(48) Rathore, R.; Van Tine, B. A. Pathogenesis and Current Treatment of Osteosarcoma: Perspectives for Future Therapies. *J. Clin. Med.* **2021**, *10* (6), 1182. https://doi.org/10.3390/jcm10061182.

(49) Wang, Y.; Zhang, C.; Wu, H.; Feng, P. Activation and Delivery of Tetrazine-Responsive Bioorthogonal Prodrugs. *Molecules* **2020**, *25* (23), 5640. https://doi.org/10.3390/molecules25235640.
(50) Chavez, D. E.; Tappan, B. C.; Kuehl, V. A.; Schmalzer, A. M.; Leonard, P. W.; Wu, R.; Imler, G. H.; Parrish, D. A. [3+2] Click Chemistry Approach to Tetrazine Containing Polymers. *RSC Adv. 12* (44), 28490–28493. https://doi.org/10.1039/d2ra05339j.