Structurally Redesigned Bioorthogonal Reagents for Mitochondria-Specific Prodrug Activation

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ABSTRACT: The development of abiotic chemical reactions that can be performed in an organelle-specific manner can provide new opportunities in drug delivery and cell and chemical biology. However, due to the complexity of the cellular environment, this remains a significant challenge. Here, we introduce structurally redesigned bioorthogonal tetrazine reagents that spontaneously accumulate in mitochondria of live mammalian cells. The attributes leading to their efficient accumulation in the organelle were optimized to include the right combination of lipophilicity and positive delocalized charge. The best performing mitochondrialotropic tetrazines enable subcellular chemical release of TCO-caged compounds as we show using fluorogenic substrates and mitochondrial uncoupler niclosamide. Our work demonstrates that a shrewd redesign of common bioorthogonal reagents can lead to their transformation into organelle-specific probes, opening the possibility to activate prodrugs and manipulate biological processes at the subcellular level by using purely chemical tools.

KEYWORDS: bioorthogonal reactions, click-to-release, mitochondria, prodrug activation, tetrazines

INTRODUCTION

Our ability to perform abiotic chemical reactions under stringent biological conditions has advanced significantly within the last decades. The ever growing repertoire of bioconjugation reactions allows for precise modification of biomolecules to enable detailed studies of their structure and function.1−7 We have entered the era in which organic chemists can perform chemical reactions on complex biological systems and even inside living organisms.8−10 However, the majority of biochemical processes in cells actually take place within distinct subcellular compartments. Our capability to perform chemical reactions in living cells with such precision is still in its infancy. Only a handful of recent examples indicate that this can be achieved.11−16 Within a cellular context, the reagents must surmount several barriers to reach the desired location and ideally accumulate within the specific compartment. This can be especially problematic because many factors influence the intracellular distribution of small molecules inside the cell.17,18

The development of chemical reactions allowing for manipulation of molecules within a specific cellular organelle thus represents a significant challenge. However, the ability to chemically trigger, for example, the release of therapeutic molecules with organellar precision could provide us with new valuable strategies to achieve better therapeutic outcomes.19

Mitochondria are vital cellular organelles providing the energy for cells in the form of ATP. Beyond energy production, mitochondria are involved in numerous other biological...
processes. These include the generation of reactive oxygen species (ROS), the modulation of oxidation−reduction (redox) status, cell signaling, metabolism, and the initiation of apoptosis. Due to the multifunctional role of mitochondria, the imbalance in any of these functions can result in severe disorders. Indeed, mitochondrial dysfunction has been linked to neurodegenerative diseases, obesity, diabetes, and cancer. New approaches to combat these mitochondriopathies are thus highly desirable.

In this work, we show that by embedding specific features into the structure of 1,2,4,5-tetrazines, it is possible to achieve their spontaneous accumulation in mitochondria of live cells where they undergo selective reaction with trans-cyclooctene-modified molecules (Figure 1). After optimization using fluorogenic substrate, we demonstrate the utility of the approach by organelle-specific reactivation of the protonophoric activity of a mitochondrial uncoupler, which ultimately leads to cell death. This work thus demonstrates that it is possible to achieve localization of bioorthogonal reagents to a specific intracellular site, and in this way, to activate prodrugs within a subcellular compartment using abiotic chemical transformations.

**RESULTS AND DISCUSSION**

**Design and Selection of Mitochondriotropic Tetrazines**

It is known that certain molecules are able to accumulate in mitochondria. It is the large negative membrane potential ($\Delta \psi$ approximately $–180$ mV) which is believed to drive the enrichment of lipophilic molecules containing a delocalized positive charge in this organelle. In contrast to previous attempts to deliver various cargoes to mitochondria by, for example, conjugation to targeting moieties, we sought to find 1,2,4,5-tetrazine derivatives having the inherent ability to accumulate in this organelle. With this goal in mind, we first synthesized a series of tetrazines bearing various heterocyclic substituents, into which we introduced the desired positive
Experimentally determined log \( P \) values for the 12 tetrazines. Compound 1e has the highest log \( P \) value followed by 1i > 1d > 1c > 1b and then the rest of the tetrazines. Experimentally determined log \( P \) values for these tetrazines follow the same order (Table S2). Among these derivatives, compounds 1c and 1d are structurally different containing the anilinium moiety. Compounds 1b, 1e, and 1i, which best accumulate in mitochondria, all contain a delocalized positive charge embedded within the hydrophobic isoquinolinium moiety. However, the presence of quinolinium or isoquinoline moiety alone is not sufficient for mitochondrial accumulation (see compounds 1a, 1f, 1g, and 1h).

To investigate if the accumulation of 1i is membrane potential driven, we performed the targeting experiments after pretreating the cells with carbonyl cyanide m-chlorophenyl hydrzone (CCCP), a known uncoupler of mitochondrial membrane potential (MMP). Depolarization of MMP by CCCP has led to a significant reduction of the fluorescent signal in the cells. Similarly, we did not observe any fluorescent signal in cells with intact MMP incubated with the noncharged tetrazine analogue 1i* (Figures 3A, S15, and S16). Collectively, these results imply that the overall lipophilicity and a delocalized positive charge is the correct combination for efficient accumulation of the compounds in the organelle, which is driven by the MMP.

Mitochondria-Specific Release Reaction in Live Cells

In addition to the increasing popularity of tetrazines as bioconjugation reagents, we were especially intrigued by their use as trigger molecules in the so-called click-to-release reaction. This type of chemistry is based on the reaction of specific dienophiles with tetrazines, which leads to the release of the dienophile-caged cargo in a sequence of tautomerization/elimination steps. This method has been successfully applied for uncaging nucleic acids and biologically active compounds or therapeutics from antibody–drug conjugates. Therefore, we thought that our mitochondriotropic tetrazines would provide us with the unique possibility to release TCO-caged molecules specifically within mitochondria.
The releasing ability of 1,2,4,5-tetrazines depends on several factors such as pH and substitution pattern. Because our cationic tetrazines comprise a structurally unprecedented motif, we first evaluated their releasing efficacy. Toward this goal, we employed our recently developed TCO-caged resorufin dye conjugate (TCO-Reso). Our initial experiments performed in a well plate format revealed that tetrazines bearing a single methyl substituent release almost 50% of the resorufin dye from TCO-Reso within the first hour. In contrast, tetrazines having an aryl substituent at this position displayed a rather slow gradual release of the dye over 12 h (Figure S21B). Correlation between reaction rates and the release efficiency of the tetrazines can be found in the Supporting Information (Figure S11).

Next, we used the TCO-Reso to probe the release chemistry in live cells. U2OS cells were incubated with 1 μM tetrazines 1a−l, washed, and loaded with 5 μM TCO-Reso. After 16 h, the cells were analyzed by flow cytometry. Tetrazines capable of quick mitochondrial accumulation (1b, 1e, and 1i) led to a marked release of the resorufin dye from TCO-Reso within the first hour. In contrast, tetrazines having an aryl substituent at this position displayed a rather slow gradual release of the dye over 12 h (Figure S21B). Correlation between reaction rates and the release efficiency of the tetrazines can be found in the Supporting Information (Figure S11).

Despite the higher release efficiency observed in cells loaded first with the TCO-Reso construct, closer inspection by confocal microscopy revealed that a significant portion of the resorufin signal was not localized inside the mitochondrial compartment (Figure 3B). The lack of mitochondrial enrichment signal in the TCO-Reso construct is most likely the reason for the observed inferior specificity.

With the aim to further improve the organelle-specificity of the reaction, we decided to add a mitochondria-localizing triphenylphosphonium (TPP) moiety to the TCO-Reso construct (TCO-TPP-Reso, Supporting Information). As expected, attachment of the TPP moiety to the TCO-caged resorufin has led to significant improvement in mitochondrial localization, as we confirmed after labeling the compound inside cells with coumarin tetrazine probe (Coum-Tz) (Figure 3C and 3D). This fluorogenic tetrazine derivative

Figure 4. (A) Efficient and mitochondria-specific release reaction can be achieved even at low tetrazine concentrations. Scale bar 10 μm. (B) Resorufin release in one-cell mouse embryos. Scale bar 10 μm. (C) Pluripotency assessment using anti-Oct3/4 antibody. Scale bar 50 μm. (D) Time-lapse of embryo development after incubation with 1i or DMSO as a control.
reacts with the TCO-tagged compound but does not release the cargo (Figure S7). With TCO-TPP-Reso introduced into cells first, followed by the addition of tetrazine \( \text{Ii} \), we observed a clear mitochondrial signal of the released resoru (Figure 3E). Time-lapse experiment revealed that in the case of \( \text{Ib, Ie, and Ii} \), the dye is released in the cells already after only 2 h of incubation. In contrast, the other tetrazines released much less of the compound and in some cases even did not release the dye at all (Figure S22). This highlights the importance of targeting also the second reagent to the organelle. Moreover, by targeting both reagents to mitochondria, we further lowered the tetrazine concentration to 100 nM and still observed efficient and specific resoru release inside the cells (Figure 4A).

To challenge the generality of the mitochondria-specific chemistry in a more complex biological system, we performed the release reaction in one-cell mouse embryos. The mouse embryos were first incubated with 5 \( \mu \text{M} \) TPP-TCO-Reso, washed, and incubated with 1 \( \mu \text{M} \) \( \text{Ii} \) for 2 h. Inspection of the cells under microscope confirmed a successful mitochondria-specific reaction (Figure 4B). Importantly, 1 \( \mu \text{M} \) concentration of \( \text{Ii} \) did not have any detrimental effects on the developmental potential of the embryos (Figures 4C and D and S23, Table S3; for toxicity of \( \text{Ib, Ie, and Ii} \) on different cell lines, see Table S4).

**Intramitochondrial Delivery and Activation of Niclosamide**

Delivery of certain type of drugs to mitochondria can have a beneficial therapeutic effect, especially in the treatment of cancer.\(^{56,57}\) To demonstrate the utility of our system for mitochondria-specific activation of a biologically active compound, we prepared TCO-caged, TPP-tagged niclosamide (TCO-TPP-niclosamide, Supporting Information). Niclosamide (trade name Niclotide) is an FDA approved drug used for the treatment of intestinal parasite infections.\(^{58}\) The compound acts as a mitochondrial uncoupler, and its mode of action involves the inhibition of oxidative phosphorylation and the modulation of ATP synthase activity.\(^{59,60}\) Niclosamide was the subject of several recent studies and was found to have potent anticancer\(^{61–64}\) and antiviral activity, including promising activity against the recent SARS-CoV-2.\(^{65,66,67}\) The protonophoric activity of niclosamide is linked to the weakly acidic phenolic hydroxyl group.\(^{61}\) Based on this information, we designed and synthesized TCO-TPP-niclosamide containing the TCO and TPP substituents attached to this hydroxyl group via a self-immolative carbamate linker (Figure 5A).

TCO-TPP-niclosamide showed excellent mitochondrial accumulation, as we confirmed by labeling the compound inside the cells using the Coum-Tz probe (Figure 5B and S24). HeLa cells pulsed with 5 \( \mu \text{M} \) solution of TCO-TPP-niclosamide for 1 h did not show any alteration in the membrane potential (TMRE positive staining), demonstrating that the protonophoric activity of this compound is switched off (Figure 5C). This activity could be efficiently restored back in the presence of 0.7 \( \mu \text{M} \) tetrazine \( \text{Ii} \) (Figure 5D). Importantly, cells treated under identical conditions with tetrazine \( \text{Ii} \) in the absence of TCO-TPP-niclosamide (Figure 5F) did not show membrane depolarization (positive TMRE signal).

The determined IC\(_{50}\) for TCO-TPP-niclosamide was 40 ± 6 \( \mu \text{M} \). After its mitochondria chemical activation, the IC\(_{50}\) dropped to 0.8 ± 0.3 \( \mu \text{M} \), which represents a 50-fold improvement in potency. For comparison, the IC\(_{50}\) of free niclosamide under the same conditions is >100 \( \mu \text{M} \). For comparison, we also performed the release experiment with the nontargeted TCO-niclosamide and nontargeted tetrazines \( \text{Ii* and Ii} \). The determined IC\(_{50}\) was in both cases >100 \( \mu \text{M}\).
(Supporting Information). These data show that delivery of the prodrug to the organelle followed by its chemical activation leads to mitochondrial membrane depolarization and subsequent cell death at much lower efficient concentration.

**CONCLUSION**

In this work, we show that structural fine-tuning of 1,2,4,5-tetrazines enables their redesign into mitochondriotropic reagents. The structural features leading to their spontaneous enrichment in the organelle constitute the combination of a positive, delocalized charge and sufficient lipophilicity. These attributes are manifested in the new isoquinolinium mitochondrial localization signal. The optimized mitochondriotropic tetrazines are not toxic at working concentrations as determined on various cell lines and one-cell mouse embryos. We demonstrate the utility of this approach by tetrazine-triggered fluorophore release and by reactivation of mitochondrial uncoupler niclosamide from the respective precursors. By delivery and subsequent mitochondrial release, the potency of the latter compound was improved about 50-fold when compared to the TCO-caged prodrug and over 100-fold with respect to the parent unmodified compound. We believe that the ability to perform abiotic chemical reactions within particular organelles will open new avenues to manipulate and study biological processes at the subcellular level and that this strategy could be useful for the development of advanced organelle-targeted drug delivery systems. Finally, the general concept presented here could be applied to the redesign of other popular bioorthogonal reagents and possibly be extended to other important cellular organelles.

**ASSOCIATED CONTENT**

* Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.0c00053.

Synthetic procedures, characterization data for new compounds, kinetic measurements, compound stability studies, additional cellular experiments, and cytotoxicity measurements (PDF)

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**REFERENCES**

(1) Row, R. D.; Prescher, J. A. Constructing New Bioorthogonal Reagents and Reactions. Acc. Chem. Res. 2018, 51 (5), 1073–1081.
(2) Sletten, E. M.; Bertozzi, C. R. Bioorthogonal Chemistry: Fishing for Selectivity in a Sea of Functionality. Angew. Chem., Int. Ed. 2009, 48 (38), 6974–6998.
(3) Patterson, D. M.; Nazarova, L. A.; Prescher, J. A. Finding the Right (Bioorthogonal) Chemistry. ACS Chem. Biol. 2014, 9 (3), 592–605.
(4) Spicer, C. D.; Pashuck, E. T.; Stevens, M. M. Achieving Controlled Biomolecule-Biomaterial Conjugation. Chem. Rev. 2018, 118 (16), 7702–7743.
(5) Boutureira, O.; Bernardes, G. J. L. Advances in Chemical Protein Modification. Chem. Rev. 2015, 115 (5), 2174–2195.
(6) McKay, C. S.; Finn, M. G. Click Chemistry in Complex Mixtures: Bioorthogonal Bioconjugation. Chem. Biol. 2014, 21 (9), 1075–1101.
(7) Debets, M. F.; van Hest, J. C. M.; Rutjes, F. P. J. T. Bioorthogonal labelling of biomolecules: new functional handles and ligation methods. Org. Biomol. Chem. 2013, 11 (38), 6439–6455.
(8) Takaoka, Y.; Ojida, A.; Hamachi, I. Protein Organic Chemistry and Applications for Labeling and Engineering in Live-Cell Systems. Angew. Chem., Int. Ed. 2013, 52 (15), 4088–4106.
(9) Borrmann, A.; van Hest, J. C. M. Bioorthogonal chemistry in living organisms. Chem. Sci. 2014, 5 (6), 2123–2134.

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Author Contributions
R.D. performed all cell experiments; determined the cytotoxicity of niclosamide, TCO-TPP-niclosamide, TCO-niclosamide and its combination with tetrazines; and was involved in the manuscript preparation. J.G. together with A.V. synthesized, isolated, and characterized all synthetic compounds. J.G. was involved in preparation of the manuscript. J.K. and M.M. performed cytotoxicity experiments on various cell lines. H.F. performed all experiments with one-cell embryos. M.D. conducted the computational structural optimization and calculated the logP values. M.V. supervised the project and prepared the manuscript for publication.

Notes
The authors declare no competing financial interest.

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Development of background-free tamo fluorescent probes for intracellular live cell imaging. Nat. Commun. 2016, 7 (1), 11964.

(30) Knall, A. C.; Slugovc, C. Inverse electron demand Diels-Alder (iEDDA)-initiated conjugation: a (high) potential click chemistry scheme. Chem. Soc. Rev. 2013, 42 (12), 5131–5142.

(31) Oliveira, B. L.; Guo, Z.; Bernardes, G. J. L. Inverse electron demand Diels-Alder reactions in chemical biology. Chem. Soc. Rev. 2017, 46 (16), 4895–4900.

(32) Kozma, E.; Demeter, O.; Kele, P. Bio-orthogonal Fluorescent Labelling of Biopolymers through Inverse- Electron-Demand Diels–Alder Reactions. Bioconjugate Chem. 2017, 18 (6), 486–501.

(33) Kang, K.; Park, J.; Kim, E. Tetrazine ligation for chemical proteomics. Proteome Sci. 2016, 15, 15.

(34) Seckute, J.; Devaraj, N. K. Expanding room for tetrazine ligations in the in vivo chemistry toolbox. Curr. Opin. Chem. Biol. 2013, 17 (5), 761–767.

(35) 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.

(36) Devaraj, N. K.; Weissleder, R. Biomedical applications of tetrazine cycloadditions. Acc. Chem. Res. 2011, 44 (9), 816–827.

(37) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. Tetrazine-based cycloadditions: application to pretargeted live cell imaging. Bioconjugate Chem. 2008, 19 (12), 2297–2299.

(38) Devaraj, N. K.; Upadhayay, R.; Haun, J. B.; Hilderbrand, S. A.; Weissleder, R. Fast and sensitive pretargeted labeling of cancer cells through a tetrazine/trans-cyclooctene cycloaddition. Angew. Chem., Int. Ed. 2009, 48 (38), 7013–7016.

(39) Blackman, M. L.; Rozen, 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.

(40) 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.

(41) Versteegen, R. M.; Ten Hoeve, W.; Rossin, R.; de Geus, M. A. R.; Janssen, H. M.; Robillard, M. S. Click-to-Release from trans-Cyclooctynes: Mechanistic Insights and Expansion of Scope from Established Carbamate to Remarkable Ether Cleavage. Angew. Chem., Int. Ed. 2018, 57 (33), 10494–10499.

(42) Neumann, K.; Gambardella, A.; Bradley, M. The Emerging Role of Tetrazines in Drug-Activation Chemistries. ChemBioChem 2019, 20 (7), 872–876.

(43) Jimenez-Moreno, E.; Guo, Z.; Oliveira, B. L.; Albuquerque, I. S.; Kitowski, A.; Guerreiro, A.; Bouteireau, O.; Rodrigues, T.; Jimenez-Oses, G.; Bernardes, G. J. Vinyl Ether/Tetrazine Pair for the Traceless Release of Alcohols in Cells. Angew. Chem., Int. Ed. 2013, 52 (4), 14112–14116.

(44) Czuban, M.; Srinivasan, S.; Yee, N. A.; Agustin, E.; Koliszk, A.; Miller, E.; Khan, I.; Quinones, I.; Noory, H.; Motola, C.; Volkmer, R.; Di Luca, M.; Trampuz, A.; Royzen, M.; Mejia Oneto, J. M. Bio-Orthogonal Chemistry and Reloadable Biomaterial Enable Local Activation of Antibiotic Prodrugs and Enhance Treatments against Staphylococcus aureus Infections. ACS Cent. Sci. 2018, 4 (12), 1624–1632.

(45) Mejia Oneto, J. M.; Khan, I.; Seebald, L.; Royzen, M. In Vivo Bioorthogonal Chemistry Enables Local Hydrogel and Systemic Pro Drug To Treat Soft Tissue Sarcoma. ACS Cent. Sci. 2016, 2 (7), 476–482.

(46) Wu, H.; Cisneros, B. T.; Cole, C. M.; Devaraj, N. K. Bioorthogonal tetravalent-mediated reactions facilitate reaction turnover in nucleic acid-templated detection of microRNA. J. Am. Chem. Soc. 2014, 136 (52), 17942–17945.

(47) Rossin, R.; Versteegen, R. M.; Wu, J.; Khasanov, A.; Wessels, H. J.; Steenbergen, E. J.; Ten Hoeve, W.; Janssen, H. M.; van Onzen, A.; Hudson, P. J.; Robillard, M. S. Chemically triggered drug release from an antibody-drug conjugate leads to potent antitumour activity in mice. Nat. Commun. 2018, 9 (1), 1484.
