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Bioconjugation

Cyclopropenes as Chemical Reporters for Dual Bioorthogonal and Orthogonal Metabolic Labeling of DNA

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Abstract: Dual bioorthogonal labeling enables the investigation and understanding of interactions in the biological environment that are not accessible by a single label. However, applying two bioorthogonal reactions in the same environment remains challenging due to cross-reactivity. We developed a pair of differently modified 2′-deoxynucleosides that solved this issue for dual and orthogonal labeling of DNA. Inverse-electron demand Diels–Alder and photoclick reactions were combined to attach two different fluorogenic labels to genomic DNA in cells. Using a small synthetic library of 1- and 3-methylcyclopropenyl-modified 2′-deoxynucleosides, two 2′-deoxyuridines were identified to be the fastest-reacting ones for each of the two bioorthogonal reactions. Their orthogonal reactivity could be evidenced in vitro. Primer extension experiments were performed with both 2′-deoxyuridines investigating their replication properties as substitutes for thymidine and evaluating subsequent labeling reactions on the DNA level. Finally, dual, orthogonal and metabolic fluorescent labeling of genomic DNA was demonstrated in HeLa cells. An experimental procedure was developed combining intracellular transport and metabolic DNA incorporation of the two 2′-deoxyuridines with the subsequent dual bioorthogonal labeling using a fluorogenic cyanine-styryl tetrazine and a fluorogenic pyrene-tetrazole. These results are fundamental for advanced metabolic labeling strategies for nucleic acids in the future, especially for live cell experiments.

Introduction

Over the last decades, bioorthogonal labeling has become a valuable tool for probing biomolecules in living systems, allowing us to study biological processes by imaging on a molecular level.[1] The method relies on a small functional group, the chemical reporter, which is introduced into the biomolecule of interest, keeping the perturbation of the living system at a minimum until the biomolecule is labeled with a fluorophore through a bioorthogonal reaction. The bioorthogonal reaction is required to proceed at physiological temperature and pH with a high reaction rate, yield and selectivity.[2] A selection of powerful bioorthogonal reactions has been established for proteins, glycans and nucleic acids.[3] Despite these substantial advances, the studies of living biological systems often require simultaneous tracking of multiple targets. Most biological processes are driven by the interaction of different biomolecules, hence labeling methods must be extended to multiple targets by using different bioorthogonal reactions. However, many of the existing bioorthogonal reactions give undesired cross-reactions if they are combined in one pot. For example, both the strain-promoted azide-alkyne cycloaddition (SPAAC) and the inverse electron-demand Diels–Alder (IEDDA) reaction use strained alkenes or alkynes as a starting material. Several approaches to construct mutually bioorthogonal *and* orthogonal pairs have been introduced.[4] One is to exploit different reaction mechanisms, as shown successfully by the group of Jaeschke.[5] They attached two different labels on DNA using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) in combination with the IEDDA reaction.[5a] Wittmann and co-workers achieved a triple orthogonal labeling of cell-surface glycans by applying the CuAAC, SPAAC and the photoclick reaction.[6] A second approach is to create orthogonality by modulating the reactivity of functional groups. Prescher and co-workers discovered that the reactivity of methylcyclopropenes can be strongly influenced by differing substitution pattern.[7] 1-Methylcyclopropenes are powerful candidates for IEDDA reactions with tetrazines.[8] A methyl group at the 3-position of the cyclopropene, however, creates a steric clash in the transition state of the IEDDA reaction. Yet these molecules readily undergo photoclick reactions with nitrilimines generated in situ from tetrazoles.[7,9] This reactivity is further promoted by carboxyl substituents that lower the frontier orbitals. Metabolic labeling of nucleic acids using bioorthogonal chemistry[1a] started with the copper-catalyzed alkyne-azide cycloaddition by the group of Salic,[10] and was further developed for copper-free reactions by the groups of

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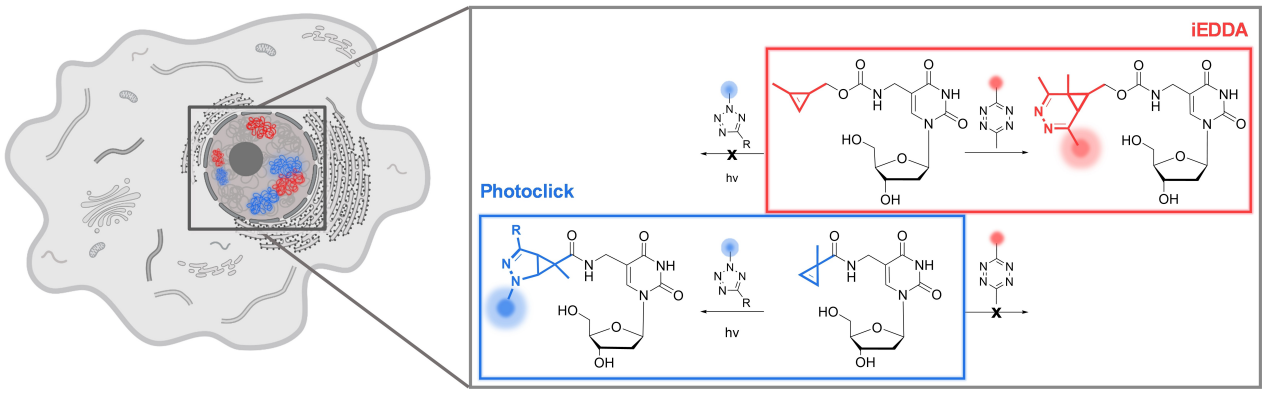

Figure 1. Dual bioorthogonal and orthogonal metabolic labeling of DNA by a combination of IEDDA and photoclick reaction using two isomeric methylcyclopropenes as 2′-deoxyuridine modifications.

Luedtke[11] and Spitale.[12] These studies show that the size of the chemical reporter is the most critical parameter for efficient metabolic labeling of genomic DNA, the smaller the better. Smaller reporters induce minimal perturbation to the living system and are better accepted by the metabolic enzymes. In comparison to more reactive but larger chemical reporters such as *trans*-cyclooctene, cyclopropenes offer the best compromise of small size and reactivity.[13] Additionally, cyclopropenes are sufficiently stable as well as reactive in the cellular environment due to their moderate ring strain.[8a,14] Our group has previously established the use of 1-methylcyclopropenyl-modified nucleosides for the labeling of DNA in vitro through IEDDA reaction with fluorogenic tetrazine-modified dyes.[15]

Inspired by these promising findings we herein further expand the application range of methylcyclopropenes as chemical reporters for nucleic acid imaging (Figure 1). In this work, we describe for the first time the dual bioorthogonal *and* orthogonal metabolic labeling of DNA by a combination of IEDDA and photoclick reaction using two isomeric methylcyclopropenes as small chemical reporters. In comparison to previously published examples of dual labeling of DNA, this method does not rely on the use of cytotoxic copper or other transition metal catalysts.[5,15b]

Results and Discussion

Cyclopropenyl-Modified 2′-Deoxynucleosides with Orthogonal Reactivity

The 2′-deoxynucleosides **1**–**5** were modified with 1-methylcyclopropyl substituents as functional groups for IEDDA reactions with tetrazines (Figure 2, red). A carbamate or urea moiety was placed adjacent to the cyclopropene, as electron-donating substituents have been shown to increase the rate of the IEDDA reaction.[8a,14a] The 2′-deoxynucleosides **6** and **7** have the 3-methylcyclopropyl substituent as a reactive group attached through a carboxyl group to render it more electron-deficient for the photoclick reaction with nitrilimes (Figure 2, blue). The nucleosides differ in the

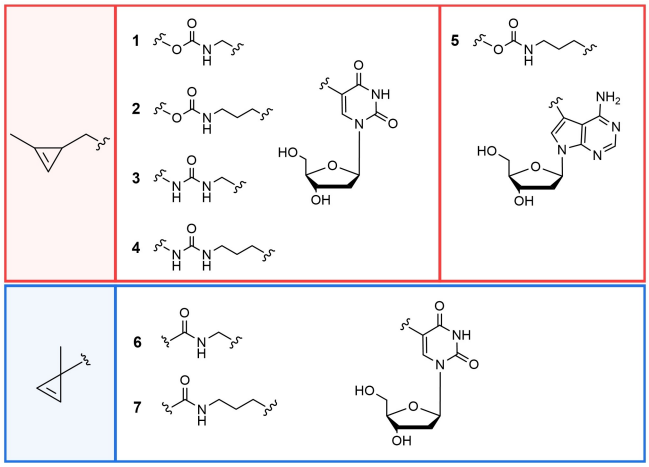

Figure 2. 1-Methylcyclopropenyl-modified 2′-deoxynucleoside **1**–**5** for IEDDA reactions and 3-methylcyclopropenyl-modified 2′-deoxyuridines **6** and **7** for photoclick reactions.

linker length between the DNA base and reactive group, namely the methyl linker in **1**, **2** and **6**, and the propyl linker in **3**–**5** and **7**. The modification was placed at the 5-position of 2′-deoxyuridines (dU), except of **5** which is a 7-deaza-2′-deoxyadenosine (dzA) modified at the 7-position to investigate the influence of the nucleobase. The modular approach allows the straightforward synthesis of these modified 2′-deoxynucleosides from 5-aminomethyl/5-aminopropyl-2′-deoxyuridine or 7-aminopropyl-7-deaza-2′-deoxyadenosine as building blocks (see SI, Scheme S1–S7). The cyclopropenes were accessible by rhodium-catalyzed [2+1]-cycloaddition of a TMS-protected alkyne and a diazo compound.[8b,9] They were coupled with the nucleoside building block in the form of a cyclopropenyl alcohol, amine or carboxylic acid using either carbonyl diimidazole, *p*-nitrophenyl chloroformate or *N*-hydroxy-succinimide as an activating agent, depending on the desired linkage.

The chemoselectivity of the 2′-deoxynucleosides **1**–**5** in the IEDDA reaction was explored using the dipyridyl-substituted tetrazine **8** (Scheme S8 and Figures S1–S5). This tetrazine is a literature-known and standard substrate to

allow the comparison of the rate constants.[8a,16] The second-order rate constants k_2 were determined at pseudo-first-order conditions using HPLC analysis (Table 1 and Figures S6–S10). The rate constants k_2 for the IEDDA reaction are in the range of 0.12–0.27 $M^{-1}s^{-1}$ and show no significant systematic effect of the structural variations, neither of the linker lengths, methyl in **1**–**2** vs. propyl in **3**–**5**, nor of the type of attached nucleobase, dU in **1**–**4** vs. dzA in **5**. We expected that 1-methylcyclopropenes attached via urea are more reactive compared to the corresponding urethane derivates due to the strong electron-donating effect of the nitrogen in the urea. Additionally, Devaraj and co-workers demonstrated that 1-methylcyclopropenes with an adjacent nitrogen atom of an amide function react more rapidly with tetrazines than those conjugated to an oxygen atom via urethane.[17] Such an effect, however, was not observed for the IEDDA reactions with our 2′-deoxynucleosides. The 2′-deoxynucleosides **1** and **3** with the urethane linkage show a similar reactivity as the 2′-deoxynucleosides **2** and **4** with the urea linkage. In conclusion, the 2′-deoxynucleoside **1** shows the fastest IEDDA reactivity. We therefore focused on this candidate in the following experiments (see below). The photoclick reaction of 3-methylcyclopropenyl-modified 2′-deoxynucleosides **6** and **7** was performed using tetrazole **9** as it is a literature-known diaryl tetrazole (Scheme S9 and Figures S11–S12).[9] In the HPLC analysis (Figures S13–S14), the photoclick reactions of **6** and **7** give significantly higher second-order rate constants of 64.4 and 31.1 $M^{-1}s^{-1}$, respectively. As 2′-deoxynucleoside **6** shows the highest photoclick reactivity, it was applied in the subsequent experiments evaluating the orthogonality of both reactions in vitro.

The orthogonality of the IEDDA reaction and the photoclick reaction (Schemes S10 and S11) was elucidated using two identical batches of an equimolar mixture of 2′-deoxynucleosides **1** and **6** in PBS:MeCN (1:1). These two 2′-deoxyuridines were identified to be the fastest reacting ones in our small library. The HPLC and LC–MS analysis of the first batch with tetrazine **8** revealed a clear selectivity for the formation of the desired IEDDA product **DA1–8** with **1** and no cross-reactivity of **6** to any undesired product (Figure 3a). The photoclick experiment in the second batch with tetrazole **9** also showed high selectivity towards the formation of the desired product **PC6–9** (Figure 3b). Overall, the 2′-deoxynucleosides **1** and **6** show an orthogonal reactivity which is the prerequisite for the dual bioorthogonal and metabolic labeling in cells.

Table 1: Second-order rate constants k_2 of the IEDDA reaction of the 2′-deoxynucleosides **1–5** with tetrazine **8** and the photoclick reaction of **6** and **7** with tetrazole **9**. For the IEDDA reaction, 2′-deoxynucleosides **1–5** (0.50 mM in PBS buffer:MeCN 1:1) were treated with tetrazine **8** (0.10 mM), incubated at room temperature and analyzed by HPLC hourly. The photoclick experiments were performed with 2′-deoxynucleosides **6** and **7** (0.50 mM in PBS:MeCN 1:1) and tetrazole **9** (0.10 mM) under irradiation with a 308 nm LED at 20 °C for 600 s with a sample being collected every 60 s.

	Selectivity	k_2 [$M^{-1}s^{-1}$]
1	IEDDA reaction with **8**	0.27 ± 0.02
2	IEDDA reaction with **8**	0.12 ± 0.01
3	IEDDA reaction with **8**	0.13 ± 0.03
4	IEDDA reaction with **8**	0.14 ± 0.01
5	IEDDA reaction with **8**	0.27 ± 0.01
6	Photoclick reaction with **9**	64.4 ± 5.0
7	Photoclick reaction with **9**	31.8 ± 1.1

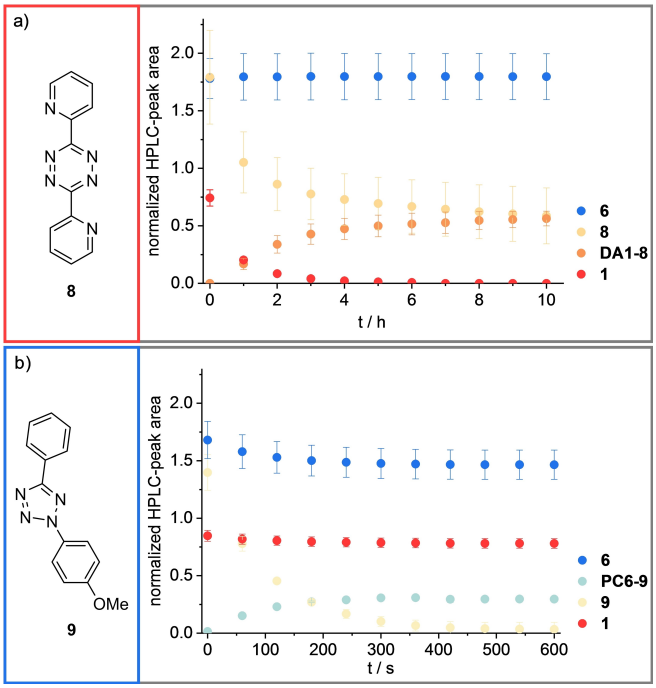

Figure 3. Orthogonal reactivity of **1** and **6** analyzed by HPLC. A mixture of **1** and **6** (0.50 mM in PBS:MeCN 1:1) was treated with a) 0.50 mM tetrazine **8** or b) 0.50 mM tetrazole **9**. The IEDDA reaction batch was incubated at room temperature for 10 h. The photoclick reaction batch was irradiated with a 308 nm LED at 20 °C for 10 min. The normalized HPLC peak areas of **1**, **6** and **8**, **9** as well as IEDDA product **DA1–8** and photoclick product **PC6–9** were plotted against the reaction time.

Primer Extension Experiments and Fluorescence Labeling of DNA in Vitro

Primer extension experiments were performed with both 2′-deoxyuridines **1** and **6** to check their replication properties as substitutes for thymidine and evaluate the subsequent IEDDA and photoclick labeling on the DNA level (Scheme S12). The synthesis of triphosphate **10** followed the Ludwig-Eckstein method[18] and is described in the Supporting Information (Scheme S2). The acceptance of **10** for enzymatic incorporation was first tested in running start experiments using a Cy5-marked primer **P1**, template **T1**, and three different commercially available DNA polymerases (Vent (exo−), Deep Vent (exo−) and Hemo KlenTaq)). **10** was recognized as an analog for thymidine and incorporated by all tested DNA polymerases at 37 °C within 5 min of elongation time (Figure 4a and Figure S15). Thus, the cyclopropenyl modification is well tolerated and does not impact the activity of these enzymes. For subsequent postsynthetic labeling experiments, **P1** was elongated using

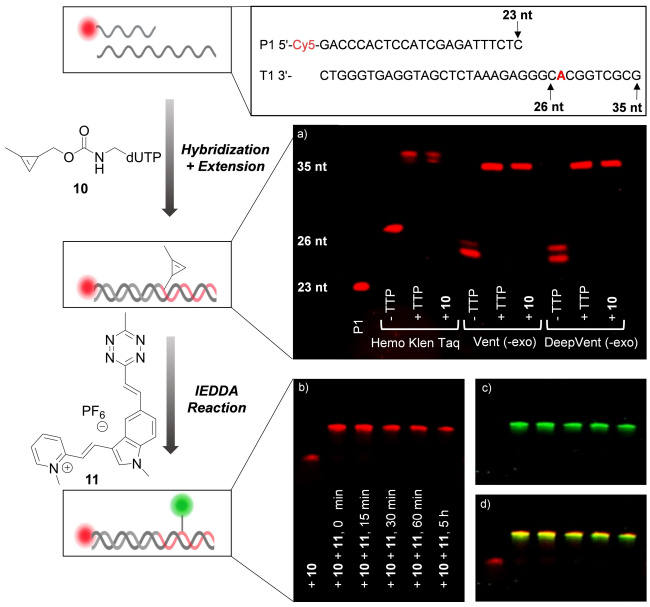

Figure 4. Primer extension experiments with 2′-deoxyuridine triphosphate **10** and subsequent labeling with the tetrazine dye **11** through IEDDA reaction analyzed by PAGE. a) After hybridization with **T1**, **P1** was elongated using Hemo KlenTaq, Vent (exo−), and Deep Vent (exo−) polymerase at 37 °C for 30 min (−TTP: negative controls with only dATP, dCTP and dGTP in the primer extension mixture; +TTP: positive controls with unmodified dNTPs; +**10**: TTP was replaced with **10**). b,c) For IEDDA experiments the extension product was desalinated, lyophilized, redissolved in H_2O and incubated with **11** (1000 eq.) for indicated time periods. a) and b) $\lambda_{exc} = 630 \pm 20$ nm, $\lambda_{em} = 670 \pm 20$ nm; c) $\lambda_{exc} = 470 \pm 20$ nm, $\lambda_{em} = 505 \pm 20$ nm; d) merge of (b) and (c).

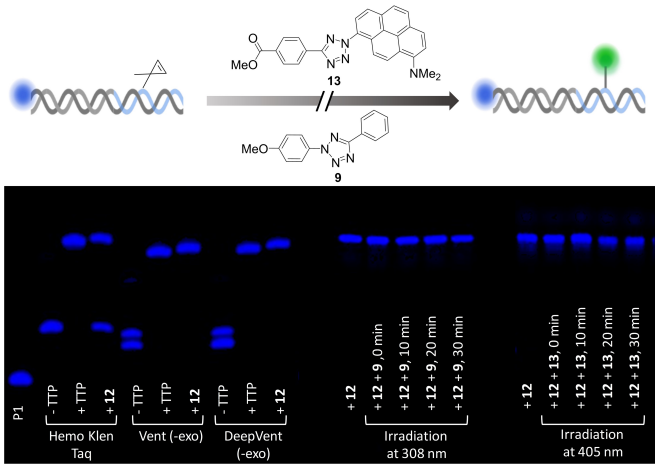

Figure 5. Primer extension experiments with 2′-deoxyuridine triphosphate **12** and photoclick labeling approaches with tetrazoles **9** and **13** analyzed by PAGE. **P1** was annealed with **T1** and elongated with indicated polymerases at 37 °C for 30 min. Positive and negative controls were prepared with and without the addition of TTP, respectively. For labeling experiments, the extension product was prepared by elongating **P1** with Deep Vent (exo−) polymerase at 37 °C for 30 min. Samples were desalinated, lyophilized and redissolved in PBS buffer. Tetrazole **9** or **13** (1000 eq.) was added, and the mixtures were irradiated for the indicated time periods at 308 and 405 nm, respectively.

Deep Vent (exo−) polymerase at 37 °C for 30 min in the presence of **10** to yield the full-length product. The cyclopropenyl-modified oligonucleotide was then desalinated, lyophilized, redissolved and labeled with a 1000-fold excess of tetrazine-dye conjugate **11**. These dyes were specifically developed for the labeling of DNA and showed a very significant acceleration upon transfer from free nucleosides to DNA, reaching rate constants up to 284,000 $M^{-1}s^{-1}$.[19] In the following PAGE analysis, a new gel band with lower gel mobility was detected indicating a successful IEDDA labeling (Figure 4b). The product formation is further evidenced by the band's additional fluorescence in the specific emission range of the cyanine-styryl dye **11**. Remarkably, no starting material was found in the sample drawn immediately after the addition of **11**, which shows a quantitative labeling of the DNA strand within minutes. The large excess of the reaction partner was used to allow direct comparison with our previously published primer extension experiments.[15] Additional experiments with 1, 2, 5, 10 and 100 equiv. of **11** showed that also a fivefold excess of **11** is sufficient for labeling the primer extension product immediately after the addition of the reaction partner (Figure S16).

Equivalent experiments were performed with triphosphate **12**, prepared from 2′-deoxynucleoside **6**, for photoclick labeling (Figure 5). In running start experiments, the full-length product was obtained when elongating **P1** in the presence of **12** with the three previously used polymerases, showing the acceptance of **12** as a thymidine substitute. The subsequent photoclick labeling was attempted with the pyrene-tetrazole conjugate **13**. This dye can be photoactivated by visible light (405/450 nm LEDs), and the subsequent reaction of the nitrilimine is fluorogenic.[3c] The postsynthetic DNA labeling attempts, however, were not successful, neither with tetrazole **13** nor with **9**, likely due to the insolubility of the oligonucleotide samples in MeCN. We know from our photoclick experiments in vitro and in cells that this chemistry requires substantial amounts of organic solvent, the most suitable is MeCN.[3c] Although this is an experiment with a negative outcome, it is an important result because it helped us to adjust the protocol for the dual metabolic labeling in a cellular environment, as described below.

Metabolic Labeling of DNA

To prepare the metabolic incorporation of nucleosides **1** and **6** into cellular DNA for subsequent dual bioorthogonal labeling we first examined their cellular toxicity through MTT assay (Figure S17). A cell viability above 75 % was observed after incubation with both 2′-deoxynucleosides at 0.50 mM for 48 h. At 0.10 mM no toxicity was observed, which is the concentration pursued in the following cell experiments. To increase metabolic labeling efficiency by pressuring cells to incorporate the modified 2′-deoxyuridines **1** and **6** we applied our recently published and optimized cell culture conditions.[13] HeLa cells were cultured in minimum

essential medium (MEM-α) without nucleosides and L-glutamine. The latter is known to be an essential nitrogen source for the cellular nucleoside synthesis.[20] Metabolic labeling of genomic DNA with IEDDA and photoclick reaction using the 2′-deoxynucleosides **1** and **6** was first investigated for each reaction separately. In initial IEDDA metabolic labeling experiments, cells were incubated with 0.10 mM **1** for 48 h to ensure at least one cell division. After fixation, the cells were labeled with tetrazine-cyanine-styryl dye **11** (Figure S18), which has the advantage of being a turn-on fluorophore eliminating the need for additional washing steps.[19] However, no nuclear staining was observed which could be explained by the inefficient metabolization of the modified nucleoside or the inaccessibility of cyclopropenyl moiety in the chromatin structure. To address the latter, we included a denaturation step before the labeling reaction (Figure S19), but again no staining was observed. In this regard, the instability of the cyclopropenyl moiety under the strongly acidic conditions used for DNA denaturation could also be problematic. We recently showed that the size of the reactive group for IEDDA reactions significantly influences the efficiency of the metabolic labeling of DNA.[13] It is also known that the most critical enzymes in the cellular nucleoside metabolism are the kinases converting the 2′-deoxynucleosides into the corresponding triphosphates.[5b] To circumvent the phosphorylation by cellular kinases and thereby potentially increase incorporation efficiency we decided to synthetically convert **1** into triphosphate **10** and deliver it into HeLa cells using a synthetic nucleoside triphosphate transporter (SNTT) developed by Kraus and co-workers.[21] Recently, this transporter has been successfully applied in introducing 2′-deoxynucleotides modified with large functional groups into cellular DNA by Hocek and co-workers to image nascent DNA during cell division.[22] HeLa cells were therefore incubated with the modified 2′-deoxynucleoside triphosphate **10** (10 μM) and SNTT (10 μM) in tricine buffer for 10 min. After a 2 h incubation period in MEM-α medium without glutamine and nucleosides, cells were fixed and treated with tetrazine-cyanine-styryl dye **11** overnight. The cells revealed clear fluorescent staining in the nucleus (Figure S20). Control experiments with only **10** or **11**, and **10** without SNTT showed no fluorescence indicating a successful labeling reaction of cellular DNA modified with 1-methylcyclopropenes. The localization of the observed fluorescence in the cellular DNA was confirmed by co-staining with Hoechst as a DNA-specific dye (Figure S21) which further evidences the metabolic incorporation of the modified substrate into the genomic DNA. Additional washing steps were not required due to the strong fluorogenicity of **11**. Compared to our recently published metabolic labeling using a 1-methylcyclopropenyl-modified 2′-deoxyuridine with a longer alkyl linker,[13] the incorporation and labeling of **10** using the SNTT leads to a more intense fluorescent signal (Figure S22). To assess metabolic labeling by photoclick reaction using modified 2′-deoxynucleotide **6**, HeLa cells were incubated with 0.10 mM **6** for 48 h. The cells were then fixed, subjected to HCl denaturation, incubated with pyrene-tetrazole **13** (30 μM) for 1 h and irradiated with a 405 nm LED for 20 min. Since the photolysis of the tetrazole requires the presence of organic solvents, **13** was supplied as a solution in MeCN. In contrast to **1**, **6** was accepted by the cellular metabolism and cell images showed a strong fluorescence in the nucleus in the range of 420 nm to 470 nm ($\lambda_{exc} = 405$ nm) (Figure S23). The metabolic incorporation of **6** was further corroborated by staining the cellular DNA with DRAQ5 where a strong co-localization of both fluorescent probes was observed (Figure S24). With both labeling procedures in hand, we further moved to dual metabolic labeling experiments, incorporating the 3-methylcyclopropenyl moiety via nucleoside **6** and the 1-methylcyclopropene using triphosphate **10** using SNTT (Figure 6a). Control experiments investigating the chemoselectivity of the isomeric cyclopropenes in a cellular environment were performed initially in two separate experiments. To ensure distinct detection of the two differently labeled products in separate fluorescence readout channels we used the tetrazine-modified cyanine-styryl dye **14** (Scheme S13), a modified version of **11** bearing a quinoline substituent instead of pyridine with a characteristic fluorescence from 600 to 650 nm.[23] When introducing triphosphate **10** in the cell using the SNTT in the first control experiment, the subsequent labeling with tetrazole **13** samples showed no significant fluorescence in the cell nuclei (Figure 6b). With **6**, however, fluorescent staining was observed (Figure 6c) evidencing that the photoclick reaction works specifically with the DNA metabolically modified with 3-methylcyclopropenes from **6**. In the second control experiment, HeLa cells were supplied with 2′-deoxynucleoside **6** and incubated with tetrazine-dye **14**. Again, no significant fluorescence in the cells could be detected by microscopy (Figure 6d) while the labeling with **10** and **14** led to significant nuclear staining (Figure 6e). Taking both types of control experiments together, they support the orthogonality of photoclick and IEDDA labeling *in* the cell. To finally evidence the dual *and* orthogonal labeling of genomic DNA, the following workflow was established combining all necessary intracellular transport and labeling steps. HeLa cells were incubated with the 2′-deoxynucleoside **6** (100 μM) for 48 h and subsequently treated with a solution of **10** (10 μM) and SNTT (10 μM) in tricine buffer. After incubation in the medium for only 2 h, cells were fixed and treated with the tetrazine-dye **14** (1 μM in MEM) overnight to allow the IEDDA labeling. Genomic DNA was then subjected to HCl denaturation and finally, cells were irradiated at 405 nm in the presence of the tetrazole-dye **13** (30 μM in MeCN) to induce the photoclick labeling. The images of these cells revealed strong fluorescence located in the nucleus in both emission channels (Figure 6f; 600–650 nm for IEDDA labeled DNA and 420–470 nm for photoclick products). Most remarkably, the fluorescence signals do not completely overlay which supports the result of a successful dual labeling of the genomic DNA by two different fluorescent labels. Although both substrates are modified thymidines and can follow the same cellular pathway, we assume that the chemical reporters are not incorporated during the same cell division due to the different metabolic labeling approaches that were used. The sequential incubation of the

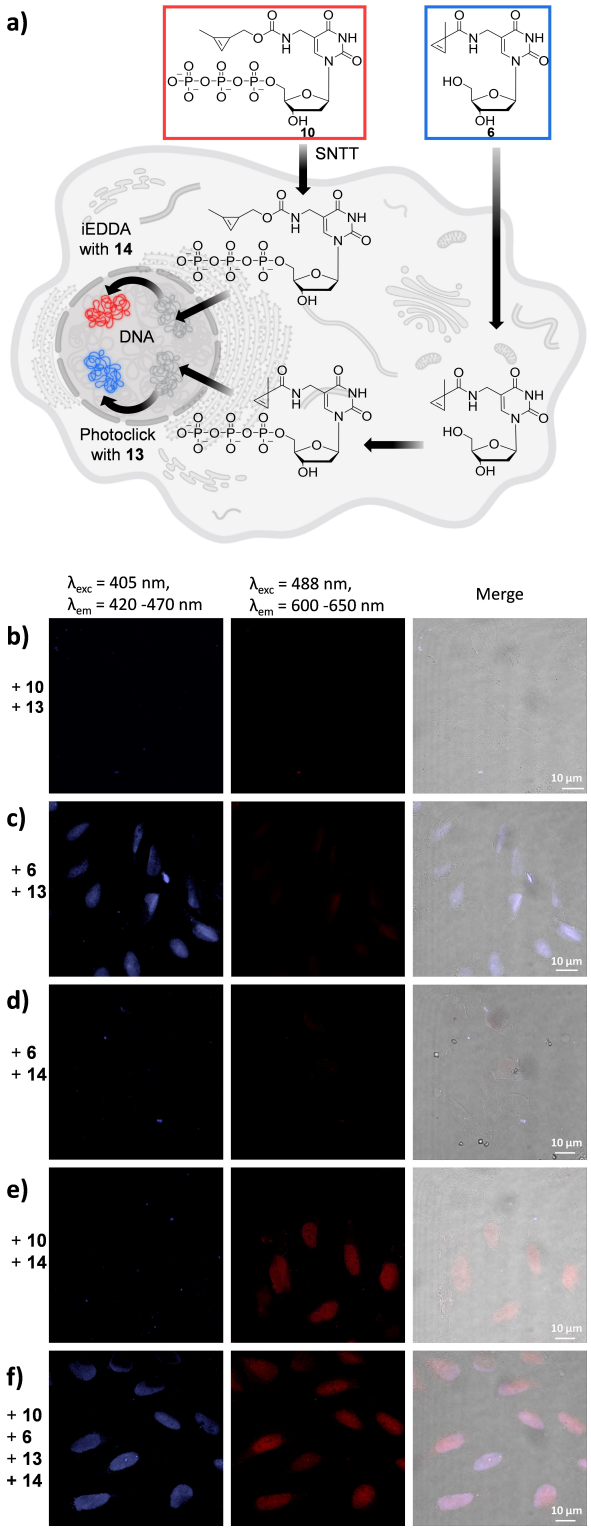

Figure 6. a) Dual and orthogonal metabolic labeling of DNA using 2′-deoxynucleoside triphosphate **10** for the IEDDA reaction and 2′-deoxynucleoside **6** for the photoclick reaction. b–e) Chemoselective reactivity of isomeric cyclopropenes in HeLa cells. f) Dual labeling with orthogonal IEDDA and photoclick reaction in HeLa cells. Cells were incubated with **6** (100 µM) for 48 h and then treated with a **10** (10 µM) and SNTT in tricine buffer for 10 min. After incubation in MEM for 2 h, cells were fixed and treated with **14** (1 µM in MEM) overnight. Cells were subjected to permeabilization and DNA denaturation and after incubation for 1 h irradiated in the presence of **13** for 20 min.

cells, first for 48 h with the 2′-deoxynucleoside **6** that has to undergo the kinase pathway for phosphorylation, and then for 2 h with the 2′-deoxynucleoside triphosphate **10** leads to the incorporation of both substrates at different time periods for each cell. Any of the control experimentes without **10**, **6**, **13** and/or **14** do not give any significant fluorescence inside the cells (Figure S25).

Conclusion

Our results show for the first time the dual bioorthogonal *and* orthogonal metabolic labeling of genomic DNA in cells as fundamental platform technology. The IEDDA and the photoclick reactions were combined by means of two isomeric methylcyclopropenes as bioorthogonally reactive functional groups attached to two 2′-deoxynucleosides. Cyclopropenes are small and show enhanced reactivity due to their ring strain as compared to simple vinyl groups, and thus provide the best compromise of size and reactivity in the cellular environment. A small library of these compounds was synthesized and revealed **1** and **6** as the candidates with the highest IEDDA and photoclick reactivity. The orthogonality was demonstrated using these two 2′-deoxynucleosides in vitro. Primer extension with various DNA polymerases showed that the 2-deoxyuridine triphosphates of **1** and **6** are recognized as substitutes of thymidine and incorporated into DNA in vitro. To demonstrate the dual bioorthogonal and orthogonal labeling of genomic DNA in HeLa cells, a precise experimental sequence was worked out combining all necessary requirements of intracellular transport of the two 2′-deoxyuridines and labeling steps with a fluorogenic cyanine-styryl tetrazine and a fluorogenic pyrene-tetrazole in the right order. These labeling reactions were successful; the images of these cells revealed strong but not completely overlaying fluorescence in both emission ranges (600–650 nm for the IEDDA product and 420–470 nm for the photoclick products) located in the nucleus. Although both chemical reporters were introduced as modified thymidine residues, the difference was gained by the pathways of metabolic labeling: **10** was already phosphorylated by organic synthesis, whereas **6** had to undergo the complete pathway of metabolic labeling, including phosphorylation by the cellular kinases. These results set the basis for advanced metabolic labeling strategies for nucleic acids in the future, also regarding live cell experiments. Many applications now become feasible. For example, the method can be envisioned for the metabolic labeling of cellular RNA or a therapeutic oligonucleotide delivered into the cell in addition to labeling the genomic DNA by two different bioorthogonal and orthogonal reactions.

Supporting Information

The authors have cited additional references within the Supporting Information.[24]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: 2′-deoxyuridine · bioconjugation · DNA polymerase · dyes · oligonucleotides

[1] a) D. Ganz, D. Harijan, H. A. Wagenknecht, *RSC Chem. Biol.* **2020**, *1*, 86–97; b) S. L. Scinto, D. A. Bilodeau, R. Hincapie, W. Lee, S. S. Nguyen, M. Xu, C. W. Am Ende, M. G. Finn, K. Lang, Q. Lin, J. P. Pezacki, J. A. Prescher, M. S. Robillard, J. M. Fox, *Nat. Rev. Methods Primers* **2021**, *1*; c) R. E. Bird, S. A. Lemmel, X. Yu, Q. A. Zhou, *Bioconjugate Chem.* **2021**, *32*, 2457–2479.

[2] J. A. Prescher, C. R. Bertozzi, *Nat. Chem. Biol.* **2005**, *1*, 13–21.

[3] a) D. S. Liu, A. Tangpeerachaikul, R. Selvaraj, M. T. Taylor, J. M. Fox, A. Y. Ting, *J. Am. Chem. Soc.* **2012**, *134*, 792–795; b) C. M. Cole, J. Yang, J. Seckute, N. K. Devaraj, *ChemBioChem* **2013**, *14*, 205–208; c) L. Rieger, B. Pfeuffer, H. A. Wagenknecht, *RSC Chem. Biol.* **2023**, *4*, 1037–1042.

[4] a) Y. Hu, J. M. Schomaker, *ChemBioChem* **2021**, *22*, 3254–3262; b) M. Smeenk, J. Agramunt, K. M. Bonger, *Curr. Opin. Chem. Biol.* **2021**, *60*, 79–88; c) D. M. Patterson, J. A. Prescher, *Curr. Opin. Chem. Biol.* **2015**, *28*, 141–149.

[5] a) J. Schoch, M. Staudt, A. Samanta, M. Wiessler, A. Jaeschke, *Bioconjugate Chem.* **2012**, *23*, 1382–1386; b) U. Rieder, N. W. Luedtke, *Angew. Chem. Int. Ed.* **2014**, *53*, 9168–9172.

[6] V. F. Schart, J. Hassenrück, A.-K. Späte, J. E. G. A. Dold, R. Fahrner, V. Wittmann, *ChemBioChem* **2019**, *20*, 166–171.

[7] D. N. Kamber, L. A. Nazarova, Y. Liang, S. A. Lopez, D. M. Patterson, H. W. Shih, K. N. Houk, J. A. Prescher, *J. Am. Chem. Soc.* **2013**, *135*, 13680–13683.

[8] a) D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, *J. Am. Chem. Soc.* **2012**, *134*, 18638–18643; b) J. Yang, J. Seckute, C. M. Cole, N. K. Devaraj, *Angew. Chem. Int. Ed.* **2012**, *51*, 7476–7479; c) A. K. Spate, H. Busskamp, A. Niederwieser, V. F. Schart, A. Marx, V. Wittmann, *Bioconjugate Chem.* **2014**, *25*, 147–154.

[9] Z. Yu, Y. Pan, Z. Wang, J. Wang, Q. Lin, *Angew. Chem. Int. Ed.* **2012**, *51*, 10600–10604.

[10] C. Y. Jao, A. Salic, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 15779–15784.

[11] a) A. Messikommer, K. Seipel, S. Byrne, P. J. M. Valk, T. Pabst, N. W. Luedtke, *ACS Pharmacol. Transl. Sci.* **2020**, *3*, 1225–1232; b) M. Tera, N. W. Luedtke, *Bioconjugate Chem.* **2019**, *30*, 2991–2997.

[12] a) M. Gupta, J. Wang, C. M. Garfio, A. Vandewalle, R. C. Spitale, *Chem. Commun.* **2023**, *59*, 3257–3260; b) M. Gupta, S. R. Levine, R. C. Spitale, *Acc. Chem. Res.* **2022**, *55*, 2647–2659; c) M. Gupta, M. Singha, D. B. Rasale, Z. Zhou, S. Bhandari, S. Beasley, J. Sakr, S. M. Parker, R. C. Spitale, *Org. Lett.* **2021**, *23*, 7183–7187; d) M. Kubota, S. Nainar, S. M. Parker, W. England, F. Furche, R. C. Spitale, *ACS Chem. Biol.* **2019**, *14*, 1698–1707.

[13] D. Ganz, P. Geng, H.-A. Wagenknecht, *ACS Chem. Biol.* **2023**, *18*, 1054–1059.

[14] a) J. Yang, Y. Liang, J. Seckute, K. N. Houk, N. K. Devaraj, *Chem. Eur. J.* **2014**, *20*, 3365–3375; b) J. M. J. M. Ravasco, C. M. Monteiro, A. F. Trindade, *Org. Chem. Front.* **2017**, *4*, 1167–1198.

[15] a) D. Ploschik, F. Ronicke, H. Beike, R. Strasser, H. A. Wagenknecht, *ChemBioChem* **2018**, *19*, 1949–1953; b) U. Reisacher, D. Ploschik, F. Ronicke, G. B. Cserep, P. Kele, H. A. Wagenknecht, *Chem. Sci.* **2019**, *10*, 4032–4037.

[16] R. D. Row, J. A. Prescher, *Acc. Chem. Res.* **2018**, *51*, 1073–1081.

[17] H. Wu, N. K. Devaraj, *Acc. Chem. Res.* **2018**, *51*, 1249–1259.

[18] J. Ludwig, F. Eckstein, *J. Org. Chem.* **1989**, *54*, 631–635.

[19] P. Geng, E. List, F. Rönicke, H.-A. Wagenknecht, *Chem. Eur. J.* **2023**, *29*, e202203156.

[20] J. W. Pelley, in *Elsevier's Integrated Review Biochemistry (Second Edition)* (Ed.: J. W. Pelley), W. B. Saunders, Philadelphia, **2012**, pp. 119–124.

[21] Z. Zawada, A. Tatar, P. Mocilac, M. Budesinsky, T. Kraus, *Angew. Chem. Int. Ed.* **2018**, *57*, 9891–9895.

[22] a) A. Spampinato, E. Kuzmová, R. Pohl, V. Sýkorová, M. Vrábel, T. Kraus, M. Hocek, *Bioconjugate Chem.* **2023**, *34*, 772–780; b) M. Kuba, P. Khoroshyy, M. Lepsík, E. Kuzmová, D. Kodr, T. Kraus, M. Hocek, *Angew. Chem. Int. Ed.* **2023**, *62*, e202307548.

[23] B. Pfeuffer, P. Geng, H.-A. Wagenknecht, *ChemBioChem* **2024**, *25*, e202300739.

[24] a) M. Merkel, S. Arndt, D. Ploschik, G. B. Cserep, U. Wenge, P. Kele, H. A. Wagenknecht, *J. Org. Chem.* **2016**, *81*, 7527–7538; b) A. Gubu, L. Li, Y. Ning, X. Zhang, S. Lee, M. Feng, Q. Li, X. Lei, K. Jo, X. Tang, *Chem. Eur. J.* **2018**, *24*, 5895–5900; c) J. Steinmeyer, H.-A. Wagenknecht, *Bioconjugate Chem.* **2018**, *29*, 431–436.

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Research Articles

Bioconjugation

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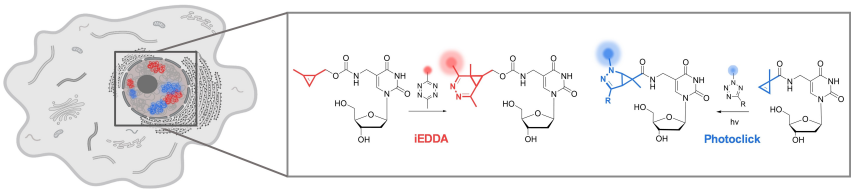
Cyclopropenes as Chemical Reporters for Dual Bioorthogonal and Orthogonal Metabolic Labeling of DNA



Dual biorthogonal and orthogonal metabolic labeling of genomic DNA in HeLa cells was demonstrated by combining the inverse-electron-demand Diels–Alder reaction with a fluorogenic cyanine-styryl-tetrazine dye conjugate and the photoclick reaction with a pyrene-tetrazole using isomeric cyclopropenes as chemical reporters.