COMMUNICATION

Opto-epigenetic modulation of DNA methylation with a photo-responsive small-molecule approach**

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Controlling the functional dynamics of DNA within living cells is essential in biomedical research. Epigenetic modifications such as DNA methylation play a key role in this process, yet there are no chemical tools available for the spatial and temporal modulation of this modification. Here we present a small-molecule approach to modulate DNA methylation with the precision of light. The strategy uses a light-tuneable version of a clinically used drug (5-aza-2′-deoxycytidine) to alter the catalytic activity of DNA methyltransferases, the enzymes that methylate DNA. The photo-regulated molecule provides unprecedented control over demethylation in targeted cells and may be applied to answer biological questions such as the role of epigenetic reprogramming in tissue development or regenerative medicine.

The methylation of DNA at position 5 of cytosines is chemically a very simple but biologically one of the most important modifications of DNA. It influences many biological processes in humans such as the regulation of cell function, cellular reprogramming, and organismal development[1-7]. Biological effects of higher methylation levels at promoters are mediated by lowering the transcription of genes either via blocking binding of transcription factors, or by recruiting unique methyl-recognizing proteins that lower gene expression. Altered levels of methylation are also associated with several diseases[8-11] including cancer[8,12-16].

Driven by the growing importance of DNA methylation in biomedical research, there is a strong interest to experimentally lower or increase methylation levels[17-21] to study, for example, the role of epigenetic reprogramming in tissue development or regenerative medicine[22-23]. Optical control is of particular relevance given the high spatial and temporal resolution of light. Often, the approach is implemented with photosensitive small molecules of tuneable bioactivity[24-28]. These can be used without the need for genetic engineering of cells leading to powerful applications within cell biology[29]. Yet, despite the importance of DNA methylation in biology, no light-tuneable small-molecule tool has been developed to manipulate methylation levels in cells.

Here we present a photo-mediated small-molecule strategy that modulates methylation in targeted cells. At the approach's centre is an inhibitor that interferes with DNA methyltransferases (DNMTs), the enzymes responsible for DNA methylation[30] including the maintenance DNA methyltransferase 1 (DNMT1)[31]. The inhibitor's bioactivity becomes tuneable with light by chemical derivatization with a photocage. As schematically illustrated in Figure1a, the attached photocage renders the inhibitor biologically inactive. However, light exposure cleaves off the photocage to restore the original inhibitory effect (Figure1a). The photocaged molecule is hence expected to maintain methylation levels in the dark, while light should decrease methylation levels following replication of cells[32] (Figure 1a).

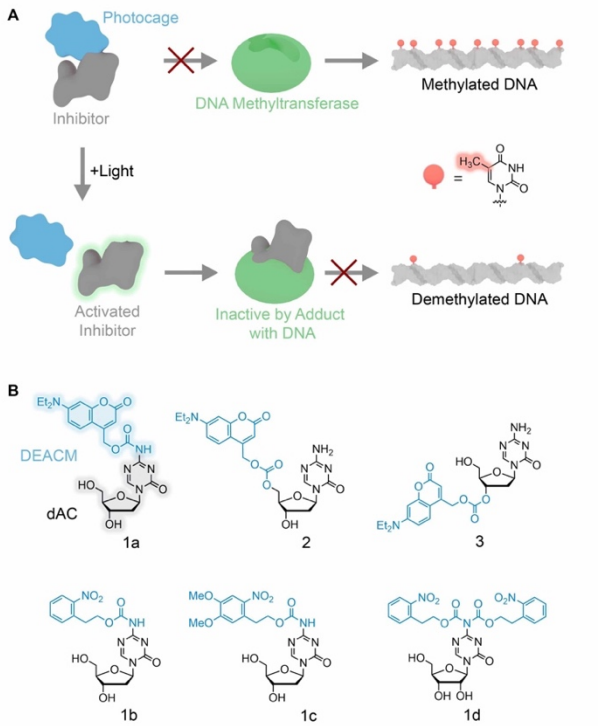

Figure 1. Photocaged derivatives of DNMT inhibitor 5-aza-2′-deoxycytidine (dAC) designed to optically modulate the methylation of DNA. (a) Scheme illustrating the principle of the photo-caging approach. Photocaged inhibitor dAC is biologically inert and allows DNMT to maintain high methylation levels. Exposure to light removes the phototag to restore the inhibitory effect on DNMT to cause lowered DNA methylation with each round of DNA replication. (**b**) Caged DNMT inhibitors N-DEACMOC-dAC (**1a**), N-NPEOC-dAC (**1b**), N-DMNPEOC-dAC (**1c**), bis-NPEOC-AC (**1d**), 5′-DEACMOC-dAC (**2**), 3′-DEACMOC-dAC (**3**).

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Our approach was implemented with DNMT inhibitor 5-aza-2′-deoxycytidine (dAC, decitabine)[32-33] (Figure 1b). The cytidine analogue is a clinically used drug for myelodysplastic syndromes[34] and is being tested against leukemia and solid tumors[18,35] and as sensitizer for immunotherapies[36-37]. dAC is the best choice for the photocaging approach given its high inhibitory effect on DNMTs[38] even though it is also known to undergo slow hydrolysis at the 5-aza-base ring[39]. To exert its inhibitory effect after cellular uptake, dAC is phosphorylated by deoxycytidine kinase in a rate-limiting step[40]. Subsequent phosphorylations to triphosphate lead to the polymerase-mediated incorporation into DNA[40] where the 5-aza-base ring forms an covalent adduct with DNMT. This adduct prevents methylation of DNA in replicating cells but also targets DNMT for proteosomal degradation[41]. Given the tight fit inside the active site of deoxycytidine kinase (Figure S1), we surmised that photocaging dAC would block the rate-limiting step of phosphorylation and hence abolish inhibition of DNMT.

To optically control the activity of dAC, we attached a photocage to each of all possible coupling sites within the nucleoside: the exocyclic NH_2 group of the base, and the 3′ and 5′ OH groups of the deoxyribose (Figure 1b). All three positions were modified as the resulting steric blockade was expected to hinder binding of dAC into the active site of deoxycytidine kinase (Figure S1). It was not possible to predict which position would be synthetically most feasible, yield the best spectroscopic or photolytic properties, or have the biggest biological impact because photocaged dAC has not been reported in the literature. For the chemical derivatization, photocage diethylaminocoumarinyl-4-methyl (DEACM) (Figure 1b) was used given its favourable high extinction coefficient (ε = 16,000 M^{-1} cm^{-1}) and long absorption wavelength (λ = 385 nm) that ensure biocompatibility by avoiding mutagenic irradiation at high intensity in the UV spectral region.

Three DEACM derivatives of dAC **1a**, **2**, and **3** (Figure 1b) were synthesized. In **1a**, the photocage is attached via a carbamate bond to NH_2, while the linkage in **2** and **3** is mediated via a carbonate to 5′ and 3′ OH, respectively (Figure 1b). The synthetic route to **1a** involved modifying NH_2 with the highly reactive chloroformate version of DEACM. The high reactivity helped overcome the amino group's weak nucleophilicity which is a consequence of electron-withdrawal by N5 in the dAC base. Undesired reaction of the 3′- and 5′- OH groups of dAC was suppressed by transient protection with trimethylsilyl (Supporting Information, Supporting Methods)[42]. By comparison, **2** was obtained by modifying the sterically highly accessible 5′ OH without any protecting groups for NH_2 or 3′ OH (Supporting Methods). DEACM was therefore introduced not as chloroformate but as a less reative pentafluorophenyl ester that solely targets 5′ OH. Similarly, **3** was generated by reacting 3′ OH with the same ester of DEACM. The undesired modification of 5′ OH was blocked by protection with triisopropylsilyl (Supporting Methods).

Additional photocaged compounds were made to demonstrate that the synthetic route is generic. For example, synthesis of **1b** and **1c** carrying a nitrophenyl group on the amino group (Figure 1b) showed that a chromophore other than DEACM can be attached to dAC. **1b** and **1c** also served as reference compounds for the spectroscopy analysis (see below). Similarly, preparation of nitrophenyl-modified azacytidine **1d** (Figure 1b) proved that the clinically used ribonucleotide version of dAC can be equipped with a photocage (see Supporting Methods for synthetic routes of **1b-d**).

DEACM-dAC derivatives **1a**, **2**, and **3** were examined to probe whether the spectroscopic properties are influenced by the chromophore's attachment site. As shown in Figure 2a, all compounds exhibited strong absorption (ε = 7000, 7300, and 8100 M^{-1} cm^{-1}, respectively) at a biocompatible wavelength of λ = 365 nm (Table 1). The values are similar to unconjugated DEACM (ε = 7000 M^{-1} cm^{-1}, Figure S2)[43] and imply that coupling to the three attachment points of dAC did not affect absorption. The data for compounds **1b-d** showed similar findings (Table 1, Figure S2).

Uncaging efficiency, by contrast, was influenced at which site of dAC the chromophore was attached. The rates for photo-induced uncaging were determined by exposing the DEACM-dAC conjugates to light at λ = 365 nm of moderate intensity at 145 μW cm^{-2} and at ambient temperature of 25 °C. The time-dependent decrease of caged dAC and the increase of cleaved dAC and DEACM was quantified with HPLC (Figure 2b). The analysis revealed for compound **1a** a fast uncaging rate of $k = 1.15 \times 10^{-3}$ s^{-1} equivalent to a 50% recovery of dAC within a half-life of $t_{1/2}$ = 11 min (Figure 2c). By contrast, 5′ OH derivative **2** exhibited a more than 4-fold slower rate of $k = 4.83 \times 10^{-4}$ s^{-1} (Figure 2c, Figure S3), possibly due to a quenching interaction between the photocage and proximal triazine nucleobase. In support of this interpretation, **3** with DEACM at more distant 3′ OH to triazine had a fast photolysis rate at $k = 1.10 \times 10^{-3}$ s^{-1} equivalent to $t_{1/2}$ = 10 min (Figure 2b and 2c, Figure S3). The likely mechanism for uncaging is shown in Figure S4.

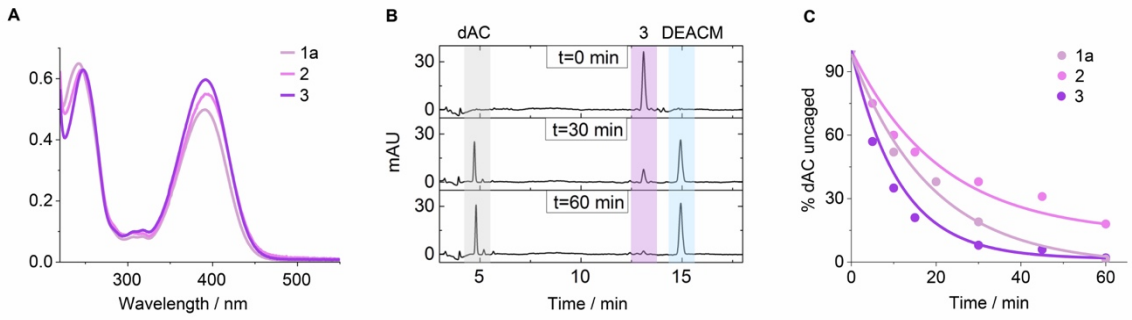

Figure 2. Spectroscopic and photochemical analysis of photocaged dAC versions 1a, 2 and 3. (a) UV-vis absorption spectra of photocaged dAC compounds **1a**, **2**, and **3** at 50 μM in DMSO/Water (5/95). (b) HPLC traces for the photodeprotection of **1a**. The initial peak corresponding to caged **1a** disappears upon irradiation at 365 nm to yield uncaged dAC and free DEACM-OH. (c) Time course for photo-induced uncaging of **1a**, **2** and **3** at λ = 365 nm.

Table 1. Spectroscopic and photolytic properties of photocaged DNMT inhibitors

	λ_{max} [a]	λ_{max} [a]	ε_{254} [b]	ε_{365} [b]	k / s^{-1} [c]	$t_{1/2}$ / min	Φ_{365} [d]	$\varepsilon \times \Phi_{365}$ [e]
1a	391	10000	11000	7000	1.10×10^{-3}	11	2.32×10^{-2}	162
1b	233	16400	9000	200	8.33×10^{-5}	139	1.23×10^{-1}	24.7
					3.33×10^{-4}*			
1c	348	5000	10400	4000	6.67×10^{-5}	173	1.89×10^{-3}	7.50
					1.00×10^{-4}*			
1d	260	14800	12100	460	n/a	n/a	n/a	n/a
2	395	11000	11400	7300	4.83×10^{-4}	24	9.98×10^{-3}	72.9
3	392	12000	11700	8100	1.15×10^{-3}	10	2.29×10^{-2}	185

Successful uncoupling of the photocage from the nucleobase was also found for control nucleotides **1b**–**d** whose spectroscopic and photolytic properties were in line with literature value for nitrophenyl (Table 1 and Figure S3). Nevertheless, the absorption wavelengths of **1b**–**d** are too low for subsequent cell work. By comparison, compound **3** has a long absorption wavelength and the fastest photolysis.

Analysis of **3** determined its stability in the absence of light. Unmodified dAC is known to have a slightly reduced stability due to hydrolysis at the 5-aza-base ring leading to a half-life of 2200 min at 25 °C[39]. By comparison, **3** had a related half-life at 690 min which reflects partial hydrolysis of the ring and the carbonate linkage to the photocage, as determined by MS (Figure S5). This half-life is almost 70-times longer than the half-life for photo-induced uncaging of **3** and 7-times longer than the subsequent incubation duration to cells. Reflecting its good stability and fast deprotection rate under cell-compatible illumination, compound **3** was used for subsequent biological investigations.

To test whether methylation levels in cells can be controlled with light, **3** was added to hypermethylated human cancer cell lines SaOS2 and T24[44]. Concomitantly exposing cells to light was expected to induce demethylation due to photo-uncaging of **3** (Figure 3b) while no illumination was anticipated to maintain methylation (Figure 3a). Consequently, cells were incubated with 0.1 µM **3** and either illuminated for 1 h at 365 nm and 25 °C, or kept in the dark. Treatment of cells with unmodified dAC served as positive control for demethylation (Figure 3c). After incubation with the small molecules, the medium was changed, cells were grown for 24 h, genomic DNA was isolated and enzymatically digested, and the nucleotide content analysed with Liquid Chromatography coupled with tandem Mass Spectrometry (LC-MS).

Figure 3d and 3e summarize the cellular levels of methylated C as percentage of the total cytosine pool for $SaSO_2$ and T24 cells, respectively. Exposure to **3** without illumination maintained a high level of methylated DNA (Figure 3d and 3e, **3**), thereby confirming that photocaged dAC was biologically inactive at the tested conditions. However, incubation with **3** and simultaneous exposure to light caused a drastic reduction in methylated DNA (Figure 3d and 3e, **3**-light) to a level almost identical to uncaged dAC (Figure 3d and 3d, dAC), while light exposure in the absence of **3** did not affect methylation (Figure 3d and 3e, 0). The data demonstrate that our strategy of light-induced demethylation is successful; by photolysis of **3**, dAC's biological inhibition was reactivated to block DNA methyl transferases within cells. Our approach was also confirmed by demethylation at a concentration of 0.5 µM **3** (Figure S6). At 1.5 µM or higher, the compound leads do demethylation without light exposure, possibly because **3** is hydrolytically inactivated by enzymes. Control experiments where cells were solely exposed to light did not lead to altered methylation levels (data not shown).

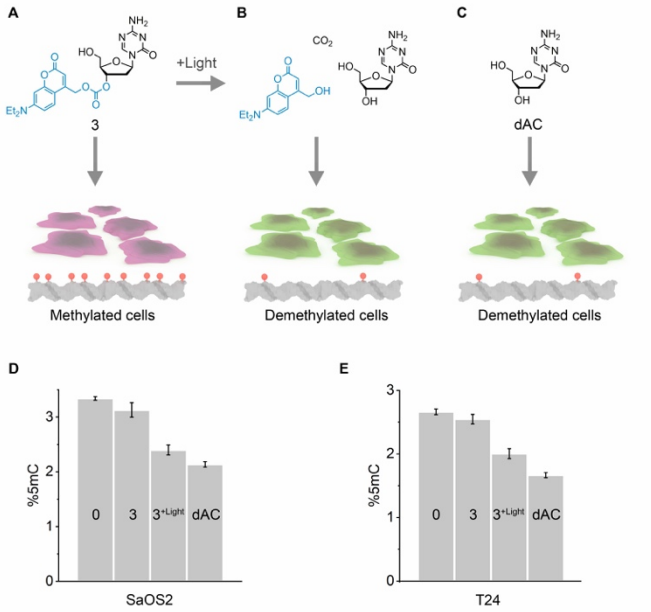

Figure 3. DEACMOC-dAC 3 can be photo-deprotected to re-activate its inhibitory effect on DNMT and lower DNA methylation levels in cells. (a-c) Schematic representation of cell treatment conditions and expected qualitative changes in DNA methylation levels. Treatment with 3 in the absence of light maintains high methylation levels (a), while illumination restores dAC activity to lower DNA methylation (b) to levels close to unmodified dAC (c). The concentration of 3 and dAC was 0.1 µM. (d, e) Treatment-dependent changes in methylation levels in SaOS2 (d) and T24 cell lines (e) for condition in (a-c) and 0 µM dAC, as quantified via LC-MS. DNA methylation levels (%5mC) are expressed as a percentage of total cytosines and analysed in triplicates.

This report describes a pioneering light-gated small-molecule approach to regulate DNA methylation levels within cells. Thereby, our study breaks new ground in two areas. First, the photocaging of the DNA methyl transferase inhibitor achieves optically triggered DNA demethylation. Previously, there has not been any chemical or biochemical tool available for light-induced lowering of cellular methylation levels. Using genetically encoded epigenetic editing, however, targeted DNA demethylation[17] and methylation[45] and light-induced reduction of epigenetically active 5-hydroxymethylcytosine[46] have been reported before. In a wider context, the non-DNA epigenetic mark of histone methylation was

modulated via optically controlled histone methyltransferases and histone deacetylases[47], and via a photoswitchable inhibitor of a deacteylase[48].

Second, our study is the first to prepare photocaged dAC thereby providing rich chemical insight on an epigentically important drug molecule. By preparing a total of six dAC and ribonucleotide versions, we have uncovered information on efficient synthesis but also on how the photocage's attachment site influences photolysis yield. In practical terms, this could improve the future synthesis of photocaged versions of the clinically tested dAC-related drugs such as SGI-110[49], enhanced photocages with self-immolating linkages[50], but also any other photoresponsive nucleotides including those with photoswitches that regulate bioactivity via photoisomerable conformation changes rather than photolysis[24-27].

We expect that the optically addressable DNMT inhibitor will serve as valuable research tool to gain a deeper understanding of epigenetic mechanisms in health and disease. This may include regenerative medicine[51], developmental biology[4], development and progression of cancer[52], and potentially the development of therapeutic routes[18,35,53-55] to treat surface-accessible tissues[56]. In conclusion, our photocaged DNMT inhibitor opens up exciting new avenues in basic and clinical research for epigenetics but also the synthesis of photo-controlled molecules.

Experimental section

Compound synthesis: Detailed experimental procedures on the synthesis of compounds **1a-d**, **2**, **3** and their chemical and optical characterization are described in the Supporting Information.

Analysis of photolysis: Into a 2 cm^2 area well of a 24-well tissue culture plate (BD Falcon) were placed 0.5 mL of 100 µM photocaged analogue in DMSO/water (5/95). The solution was irradiated at λ = 365 nm using a Benchtop UV lamp (Model UVGL58, Mineralight lamp, 145 µW cm^{-2}). Aliquots of 20 µL were periodically removed and analysed by HPLC using an Agilent Eclipse C18 column (250 x 4.6 mm, 5 µm) with an elution system of 5-95% acetonitrile in water over 20 min at a flow rate of 1 mL min^{-1}. The percentage of compound converted was determined via integration of HPLC chromatograms.

Testing of the chemical stability: Solutions of **3** or 5′-DEACMOC-dC in DMSO/water (5/95) (1 µM, 1 mL, 1.5 mL Eppendorf® tube) were incubated in the dark at 25 °C using a Thermomixer (Eppendorf® ThermoMixer). Aliquots of 10 µL were periodically removed and analyzed on a Triple Quadrupole 6460 Mass Spectrometer (Agilent Technologies) fitted with an Infinity 1260 LC system (Agilent) and a Hypersil Gold C18 Column (150 x 2.1 mm, 1.9 µm), with an elution system of 5-95% acetonitrile in water with 0.1% formic acid over 20 min at a flow rate of 0.2 mL min^{-1}. Multiple Reaction Monitoring (MRM) was set up and optimised to ensure selective quantitation of the caged-analogue, corresponding photocage and dAC. 2′-Deoxyuridine (dU) and 2′-deoxycytidine were used as internal standards.

Cell culture and treatment with compound 3: Osteosarcoma cell line SaOS-2[57] and urinary bladder carcinoma line T24[58] were grown as an adherent monolayer culture in RPMI 1640 medium (Lonza, #12-702F) supplemented with 10% fetal bovine serum (Gibco, #10500064). Cells were seeded at a density of 2 x 10^5 cells into 10 cm cell culture dishes (Cornig, #430176). Accurate cell counts were determined using a Vi-CELL™ XR cell viability analyzer (Beckman Coulter). 72 h after plating, cells were treated with the photocaged analogue **3** or dAC in fresh medium at final concentrations of 0.1, 0.5 and 1.5 µM. Each treatment was administered in triplicates. Following the addition of fresh medium and treatment, one set of plates were subjected to 1 h of UV illumination at λ = 365 nm. The remaining set of plates served as control and were kept in the dark. After 24 h of further incubation cells were collected and genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, #69504). DNA concentrations were measured using Qubit® dsDNA Assay Kit (Thermo, #Q32854).

Digestion and LC-MS analysis of genomic DNA: 5 µg of genomic DNA was incubated with 5 U of DNA Degradase Plus (Zymo Research) for 4 h at 37 °C. The resulting mixture was spiked with 100 nM of isotope-labelled 2′-deoxycytidine-(^{15}N, D_2) and 5-methyl-2′-deoxycytidine-(D_3) (Toronto Research Chemicals) as internal standards. Synthetic standards 2′-deoxycytidine (C, Sigma), 5-methyl-2′-deoxycytidine (5mC, Berry & Associates) were used to obtain calibration curves in the ranges of 10 – 100 µM for C and 0.5 – 5 µM for 5mdC. 10 µl of nucleic acid digest was injected into an Agilent Infinity 1290 LC system fitted with an Acquity UHPLC HSS T3 column (50 × 2.1 mm, 1.8 µm particle size), maintained at 50 °C, at a flow rate of 300 µL min^{-1}, and a 5 min gradient of 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B) (0-0.5 min 100% A; 0.5-1.4 min 100->70% A; 1.4-2.0 min 70% A; 2.0-3.0 min 70->10% A; 3.0-5.0 min 100% A). The eluent was directed to an Thermo Q Exactive mass spectrometer fitted with a heated electrospray source with temperature set to 350°C. The quantitation was based on the peak area ratio of the analytes to their corresponding isotope-labeled internal standards, and the constructed calibration curves. 5mC levels are expressed as a percentage of total cytosines (C plus 5mC).

Keywords: DNA, cytosine, methylation, photo-caging, epigenetics

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