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Unintended Consequences of Expanding the Genetic Alphabet

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Supporting Information

ABSTRACT: The base pair d5SICS·dNaM was recently reported to incorporate and replicate in the DNA of a modified strain of *Escherichia coli*, thus making the world's first stable semisynthetic organism. This newly expanded genetic alphabet may allow organisms to store considerably more information in order to translate proteins with unprecedented enzymatic activities. Importantly, however, there is currently no knowledge of the photochemical properties of d5SICS or dNaM—properties that are central to the chemical integrity of cellular DNA. In this contribution, it is shown that excitation of d5SICS or dNaM with near-visible light leads to efficient trapping of population in the nucleoside's excited triplet state in high yield. Photoactivation of these long-lived, reactive states is shown to photosensitize cells, leading to the generation of reactive oxygen species and to a marked decrease in cell proliferation, thus warning scientists of the potential phototoxic side effects of expanding the genetic alphabet.

A six-letter genetic code was replicated in vivo for the first time in 2014 using the hydrophobic base pair d5SICS·dNaM [i.e., 2-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-methylisoquinolinethione and 2-methoxy-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-naphthalene, respectively].[1] This breakthrough enables semisynthetic organisms to be grown with an expanded genetic alphabet and may soon allow them to code for unnatural amino acids in order to generate proteins with novel activities.[2] The expanded genetic alphabet has a variety of other promising in vitro and in vivo applications, such as increasing the specificity of aptamers and nucleic acid catalysts that are developed through Systematic Evolution of Ligands by EXponential enrichment (SELEX)[3] and allowing DNA to be site-specifically modified in order to deliver cargo or to identify DNA lesions.[4]

It is recognized that the canonical nucleosides of cellular DNA are exceedingly prone to spontaneous mutation and to damage by reactive oxygen species (ROS), free radicals, carcinogenic substances, and many other external antagonists.[5] UV radiation is one such external factor that constantly damages cellular DNA,[6] but cells can frequently repair UV-induced lesions through ubiquitous enzymatic mechanisms.[7] With the expansion of the genetic alphabet, the question arises as to whether the incorporation of unnatural DNA base pairs into cells can adversely affect the integrity of the genetic code and the viability of the cells upon exposure to sunlight or even conventional laboratory lighting.

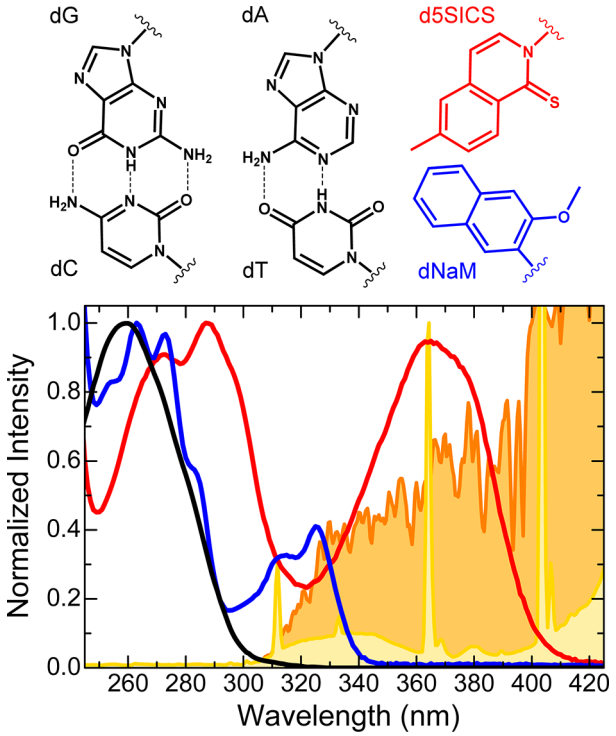

Figure 1. (top) Molecular structures of the canonical DNA base pairs guanosine·cytidine (dG·dC) and adenosine·thymidine (dA·dT) compared with that of the unnatural hydrophobic base pair d5SICS·dNaM. The 2-deoxyribose groups have been excluded for clarity. (bottom) Absorption spectra of calf thymus DNA (black), d5SICS (red), and dNaM (blue) overlaid with the average AM1.5 solar spectrum reaching the earth's surface[9] (orange) and the emission spectrum from standard fluorescent lighting (yellow). All of the spectra have been normalized to their maximum between 250 and 400 nm.

The molecular structures of the base pairs making up the expanded genetic alphabet are presented in Figure 1 along with a comparison of the absorption spectra of d5SICS, dNaM, and random double-stranded DNA at physiological pH. Contrary to the canonical nucleosides, which efficiently absorb wavelengths of light higher in energy than 300 nm,[8] d5SICS and dNaM absorb strongly in the near-visible range (Table 1). As shown in Figure 1, wavelengths in this spectral range are highly abundant in the solar spectrum reaching the Earth's surface and in the

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Table 1. Ground-State Absorptions, Triplet-State Properties, And Singlet Oxygen Quantum Yields of d5SICS and dNaM in PBS and in Acetonitrile Solutions Compared to Those of the Canonical DNA Base Thymidine

DNA nucleoside	λ_{max} (nm) (ε, $M^{-1}cm^{-1}$)[a]	τ_T[b] (μs)	Φ_T[c]	Φ_Δ[d]
	Aqueous Phosphate-Buffered Saline (pH 7.4)			
d5SICS	365 (6629)	1.4 ± 0.1	0.85 ± 0.17	0.36 ± 0.02
dNaM	325 (1893)	70 ± 5	0.28 ± 0.07	n.d.[e]
thymidine	267 (9860)[13]	25[14]	0.014[f]	<0.01[15]
		Acetonitrile		
d5SICS	371 (6731)	0.21 ± 0.02	0.94 ± 0.15	0.42 ± 0.02
dNaM	325 (2520)	5.3 ± 0.4	0.65 ± 0.12	0.23[g]
thymidine	261 (n.d.[e])[16]	2.0[17]	0.069[14]	0.07 ± 0.01[15]

[a]Lowest-energy absorption maximum and corresponding extinction coefficient. [b]Triplet decay lifetime under N_2-saturated conditions. [c]Triplet quantum yield. [d]Singlet oxygen quantum yield in O_2-saturated solution. [e]n.d. = not determined. [f]From ref 14 upon 267 nm excitation, the triplet yield of thymidine 5′-monophosphate decreases with decreasing excitation energy.[18] [g]From ref 19 for the NaM chromophore.

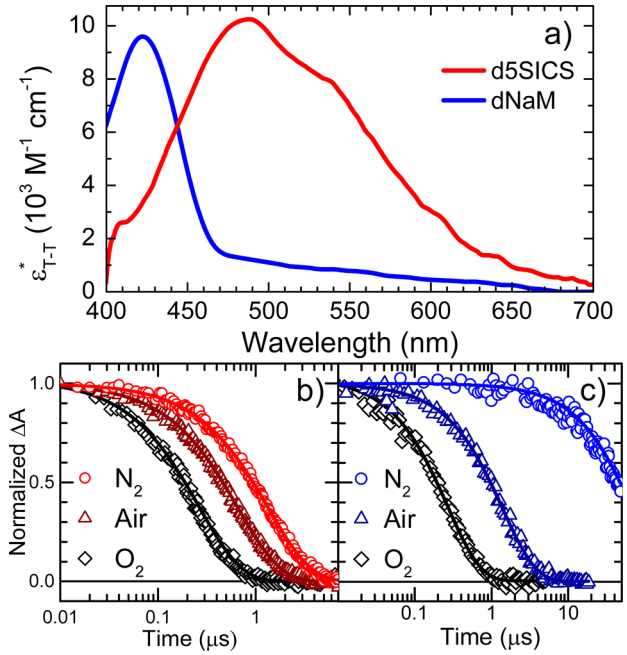

Figure 2. (a) Transient absorption spectra taken at a time delay of 65 ns for d5SICS and dNaM in PBS following 390 and 325 nm excitation, respectively. These represent the triplet−triplet absorption spectra of the lowest-energy excited triplet states of the unnatural nucleosides. (b, c) Representative triplet-state decay traces were taken from the triplet−triplet absorption maxima for (b) d5SICS and (c) dNaM under N_2-, air-, and O_2-saturated conditions. The solid lines represent the best global fits of the data to a single-exponential decay function used to obtain the triplet decay lifetimes given in Tables 1 and S1. All of the experiments were performed at concentrations of ca. 0.3 mM.

emission spectrum of standard fluorescent laboratory lighting. Hence, the photochemical stability of these unnatural nucleosides is crucial if they are to safely expand the genetic alphabet.

Broadband transient absorption spectroscopy[10] revealed that both unnatural nucleosides populate long-lived (i.e., microseconds) transient species following photoexcitation. The transient absorption spectra and representative decay traces following excitation of d5SICS and dNaM at 390 and 325 nm, respectively, are shown in Figure 2 in aqueous phosphate-buffered saline (PBS) at pH 7. . On the microsecond time scale, d5SICS and dNaM both exhibit a single transient absorption species (Figures 2a and S2), which decays monotonically with a lifetime of 1.4 ± 0.1 and 70 ± 5 μs, respectively, in N_2-saturated PBS solutions. These long-lived transient species are readily quenched by molecular oxygen (O_2) (Figure 2b,c), and the magnitudes of their lifetimes decrease linearly with increasing O_2 concentration in both PBS and acetonitrile solutions (Table S1 and Figures S3 and S4). The long-lived nature of these transient species and the efficient quenching by O_2 are hallmarks of reactive transient species with triplet spin multiplicity.[11] Hence, the transient spectra shown in Figures 2a and S3a are assigned to the triplet−triplet absorption spectra of the lowest-energy triplet states of both d5SICS and dNaM. The spectrum of dNaM is nearly identical to the triplet−triplet absorption spectrum reported previously for its chromophore 2-methoxynaphthalene (NaM),[12] providing further support for this assignment.

The triplet quantum yields of both unnatural nucleosides were determined, as described in detail in the Supporting Information. A nearly unity triplet quantum yield was calculated for d5SICS in both PBS and acetonitrile following 390 nm excitation (0.85 ± 0.17 and 0.94 ± 0.15, respectively). The triplet yield of dNaM is solvent-dependent, with values of 0.28 ± 0.07 in PBS and 0.65 ± 0.12 in acetonitrile, following excitation at 325 nm. The more than 2-fold increase in the triplet yield of dNaM upon going from PBS to acetonitrile could play a role in vivo as the dielectric environment experienced in acetonitrile solution may more closely mimic that experienced by dNaM within double-stranded DNA.[20]

The significant triplet quenching by O_2 observed for d5SICS and dNaM (Figure 2) suggests that both unnatural nucleosides generate substantial amounts of ROS upon near-visible excitation. In order to scrutinize this hypothesis, time-resolved emission spectroscopy was used to monitor the generation of singlet oxygen by its characteristic phosphorescence emission at 1270 nm.[10,21] Figure S5 demonstrates that d5SICS is able to generate singlet oxygen efficiently in PBS solution, with a quantum yield of 0.36 ± 0.02 under O_2-saturated conditions, whereas an even greater yield of 0.42 ± 0.02 was measured in O_2-saturated acetonitrile (Table 1). The fraction of O_2 quenching events that effectively generate singlet oxygen (S_Δ) was determined to be 0.45 for d5SICS in PBS on the basis of a kinetic analysis of the triplet lifetimes under different concentrations of d5SICS and O_2.[10] These experimental results suggest that excitation of d5SICS by near-visible light within a cellular environment may lead to significant generation of ROS. The in vitro cell studies presented below provide strong evidence for this hypothesis.

The O_2 quenching experiments presented in Figure 2c suggest that the triplet state of dNaM can also lead to a significant generation of singlet oxygen.[22] In fact, under equal experimental conditions (i.e., equal reactant concentrations), the triplet lifetime of dNaM in PBS decreases 260-fold upon going from N_2- to O_2-saturated solution, whereas that of d5SICS decreases only 2.5-fold (see Table S1). These experimental results are consistent with the singlet oxygen quantum yield of 0.23 and the S_Δ value of 0.96 previously reported for the NaM chromophore in acetonitrile solution.[19]

Table 1 summarizes the primary photochemical properties of d5SICS and dNaM and provides a comparison with the canonical nucleoside thymidine. Although the excited-state dynamics of the canonical DNA bases have been shown to decay primarily on

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ultrafast time scales,[8a−c] thymidine exhibits the highest triplet and singlet oxygen yields of the four canonical DNA nucleosides.[8d,14,15] It is well-documented that photoexcitation of thymidine with ca. 260 nm radiation leads to the formation of cyclobutane pyrimidine dimers and the (6−4) pyrimidone photoproduct—the primary DNA lesions induced by UV radiation.[23] Significantly, our measurements show that the triplet and singlet oxygen yields of d5SICS and dNaM are up to 2 orders of magnitude greater than those of thymidine in aqueous solution. This remarkably higher photoreactivity suggests that exposure of semisynthetic organisms incorporating these unnatural nucleosides to near-visible light should lead to a significantly higher probability of photochemical damage to the cell.

The presumptive phototoxicity of these unnatural nucleosides to living cells was scrutinized in this work using a line of epidermoid carcinoma cells (i.e., A431 cells).[10] The cells were cultured in growth medium containing 0, 50, or 100 μM d5SICS,[24] similar to the culture concentrations used for expanding the genetic alphabet in *Escherichia coli*.[1] After 48 h, the medium was replaced with regular culture medium in order to remove any excess d5SICS not incorporated by the cells. Half of the samples were then exposed to a low dose of near-visible light (5 J/cm^2) in the 350 to 410 nm spectral range, and cell proliferation was determined 3 days after irradiation.[10] Figure 3 shows that the separate treatment of cells with d5SICS or near-visible light had no significant impact on cell survival. However, cells cultured with d5SICS demonstrated a substantial decrease in proliferation upon the same brief exposure to near-visible light.[25] Importantly, the photodynamic effect increases continuously with increasing d5SICS concentration (Figure 3). These in vitro experiments show that micromolar concentrations of d5SICS can photosensitize cells to near-visible light and are therefore detrimental to cell survival.

Initial mechanistic insights regarding the participation of ROS in the oxidatively generated damage to cells can be obtained from an assessment of the intracellular concentrations of ROS.[26] The total intracellular ROS can be correlated with the measured fluorescence intensity of cells pretreated with the ROS reporter dye dichlorodihydrofluorescein diacetate (DCF-DA).[27] Figure 4 presents images of cells cultured with 100 μM d5SCIS, as described above, and incubated with DCF-DA.[10] None of the cells displayed any significant fluorescence prior to irradiation (Figure 4). Some ROS was detected in the control cells following irradiation with a 5 J/cm^2 dose of near-visible light, in agreement with previous in vitro studies.[28] However, the cells cultured with d5SICS exhibited a statistically significant increase in their average fluorescence intensity using the same dose of light, showing that d5SICS causes an increased intracellular concentration of ROS upon exposure to near-visible light.

The increased intracellular concentration of ROS may be correlated with the generation of singlet oxygen by d5SICS upon light activation (Figure S5 and Table 1). However, there is currently some uncertainty due to the nonspecific nature of DCF-DA.[27c,d] Calculations presented in the Supporting Information predict a favorable driving force for electron transfer from the triplet state of d5SICS to molecular oxygen (Table S2), suggesting that the formation of superoxide and hydrogen peroxide may also play a role in these in vitro studies.[29] Furthermore, direct reaction between photoexcited d5SICS and the canonical nucleosides, proteins, and/or other biomolecules in the cell are all possibilities,[30] as has been shown for other thionated nucleosides.[28,31]

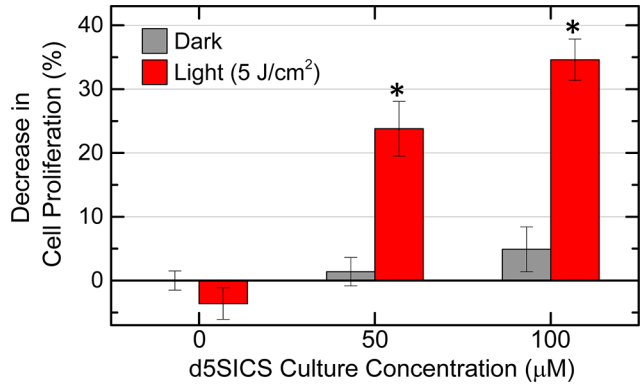

Figure 3. Impact of exposure to near-visible light on the proliferation of epidermoid carcinoma (A431) cells that were treated with increasing concentrations of d5SICS. The decrease in cell proliferation was determined by MTT assay performed in triplicate and in three independent experiments. The error bars represent standard deviations, and * denotes $p < 0.001$.

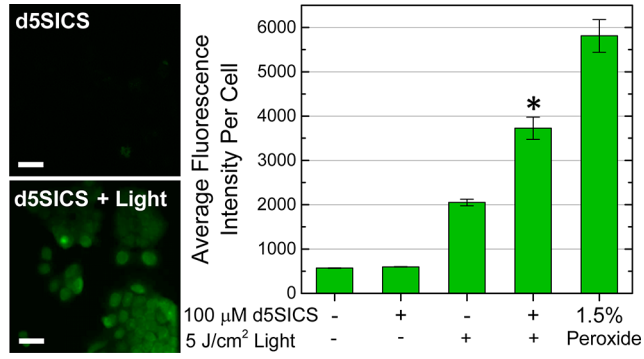

Figure 4. Assessment of total intracellular reactive oxygen species on the basis of the fluorescence of the ROS reporter dye DCF-DA. (left) Representative images of intracellular fluorescence due to ROS generation in cells cultured with d5SICS before and after exposure to 5 J/cm^2 near-visible light (scale bar = 50 μM). (right) Bar graph quantifying the average fluorescence intensity per cell in samples with (+) or without (−) the specified d5SICS or light treatment. Data were taken from three representative images of each sample in two separate experiments. The error bars represent standard errors of the mean, and * denotes $p < 0.001$.

To date, d5SICS and dNaM have been shown to incorporate and replicate efficiently in the DNA of bacterial cells, but it is currently unknown whether these molecules can be efficiently incorporated by other living organisms. The photophysical properties of d5SICS and dNaM reported in this work demonstrate the increased ability of these unnatural bases to act as potent photosensitizers within cells.[32] Photogeneration of these long-lived reactive species can accelerate oxidatively generated damage to DNA and other biomolecules, potentially leading to a variety of genetic mutations such as DNA−DNA and DNA−protein cross-links. Importantly, the phototoxicity results presented herein using a human skin cancer cell line are intended to create awareness in the scientific community of the potential unintended consequences of expanding the genetic alphabet with unnatural nucleosides like those used in this work. Evidently, further research in this regard is warranted.[33]

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06822.

Materials and methods, triplet ε and quantum yield calculations, cell culture and assay details, experimental results in acetonitrile, time-resolved luminescence results, and quantum-chemical calculations and thermodynamic arguments (PDF)

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Notes

The authors declare no competing financial interest.

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(24) Attempts to measure the phototoxic activity of dNaM were not made because it does not significantly absorb at the wavelengths emitted by the near-visible lamp used in this work.

(25) The 5 J/cm^2 of near-visible light used in these experiments is equivalent to less than 15 min of exposure to the average solar spectrum reaching the Earth's surface (Figure 1).

(26) A complete elucidation of the phototoxic mechanism of d5SICS within cells is beyond the scope of the current work but is clearly necessary.

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(29) It should be remarked that the increase in the average fluorescence intensity of these cells was not as dramatic as that observed in control cells treated directly with hydrogen peroxide (Figure 4). This suggests that other photosensitized reaction pathways potentially contribute to the overall decrease in cell proliferation (Figure 3).

(30) Calculations based on thermodynamic arguments predict that both d5SICS and dNaM should act as sinks for oxidative damage because their oxidation potentials are significantly lower than those of the canonical DNA nucleosides (Table S2).[10]

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(32) The potential participation of other relaxation pathways when this unnatural base pair is incorporated into double-stranded DNA is discussed in section 2.5 in the Supporting Information.

(33) The significant phototoxic activity of d5SICS in epidermoid carcinoma cells has the potential to open new opportunities for its use in topical photodynamic therapy applications, as recently proposed for several other thiobase analogues.[28,31b,c]

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