

# Regulation of Transcription through Light-Activation and Light-Deactivation of Triplex-Forming Oligonucleotides in Mammalian Cells

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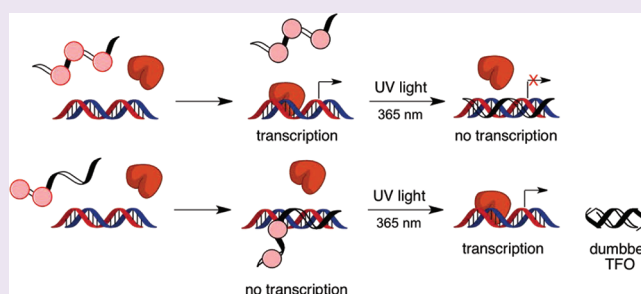
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## S Supporting Information

**ABSTRACT:** Triplex-forming oligonucleotides (TFOs) are efficient tools to regulate gene expression through the inhibition of transcription. Here, nucleobase-caging technology was applied to the temporal regulation of transcription through light-activated TFOs. Through site-specific incorporation of caged thymidine nucleotides, the TFO:DNA triplex formation is blocked, rendering the TFO inactive. However, after a brief UV irradiation, the caging groups are removed, activating the TFO and leading to the inhibition of transcription. Furthermore, the synthesis and site-specific incorporation of caged deoxycytidine nucleotides within TFO inhibitor

sequences was developed, allowing for the light-deactivation of TFO function and thus photochemical activation of gene expression. After UV-induced removal of the caging groups, the TFO forms a DNA dumbbell structure, rendering it inactive, releasing it from the DNA, and activating transcription. These are the first examples of light-regulated TFOs and their application in the photochemical activation and deactivation of gene expression. In addition, hairpin loop structures were found to significantly increase the efficacy of phosphodiester DNA-based TFOs in tissue culture.



Triplex-forming oligonucleotides (TFOs) bind double-stranded DNA in a sequence-specific manner for a variety of different functions and applications, including the inhibition of protein–DNA binding,<sup>1</sup> gene expression,<sup>2</sup> and DNA replication.<sup>3</sup> TFOs have also been applied to induce site-specific DNA damage,<sup>4</sup> to enhance DNA recombination,<sup>5</sup> and to perform DNA mutagenesis.<sup>6,7</sup> Recently, TFOs have even been used as electrochemical sensors for double-stranded DNA.<sup>8</sup> Currently, TFOs and decoy oligonucleotides are one of the few gene-regulating tools used to inhibit transcription, in contrast to traditional antisense agents that regulate translation of a given gene. TFOs block transcription factors from binding to their genomic recognition site by forming a DNA triplex structure within a promoter sequence (Figure 1),<sup>9</sup> whereas decoy oligonucleotides directly sequester the target transcription factors.<sup>10</sup> One advantage of TFOs over RNA-targeting antisense agents in the regulation of gene expression is the lower copy number of genomic DNA compared to the higher copy number of mRNA.<sup>6</sup>

TFOs are single-stranded oligonucleotides that can recognize polypurine- or polypyrimidine-rich regions of double-stranded DNA by binding in the major groove of DNA through Hoogsteen hydrogen bonds.<sup>1,11</sup> For example, adenine can hybridize to an adenine:thymine base pair forming an T:A:T triplex (Figure 1B), while guanine can bind through reverse Hoogsteen hydrogen bonds to a guanine:cytosine base pair forming a G:G:C triplex structure (Figure 1C).<sup>2,6,7,12</sup>

One current limitation of TFOs is that their activity cannot be controlled with spatial or temporal resolution, thus preventing their application in the precise dissection of biological processes. A solution to this problem can be found in “caging” technologies that have been developed to photochemically regulate biological function with high spatiotemporal resolution.<sup>13–20</sup> By placing a photolabile protecting group (“caging” group) onto the base of a nucleotide, its ability to undergo hybridization is disrupted, rendering the oligonucleotide functionally inactive. After brief UV irradiation, the caging groups are cleaved, and oligonucleotide activity is restored.<sup>21–25</sup> Here, we demonstrate that this methodology can be extended to the photochemical regulation of Hoogsteen base-pairing and thus the activation and deactivation of TFO function and gene transcription in live cells.

Cyclin D1 was selected as a model system for the design of light-controlled TFOs because of its critical regulatory role in the cell cycle.<sup>26–28</sup> Cyclin D1 is essential in the transition from G<sub>1</sub> to S phase in the cell cycle, and the misregulation of this process has been associated with a number of neoplastic diseases.<sup>29,30</sup> Overexpression of cyclin D1 causes multiple

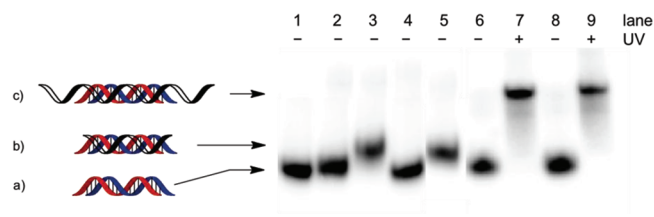
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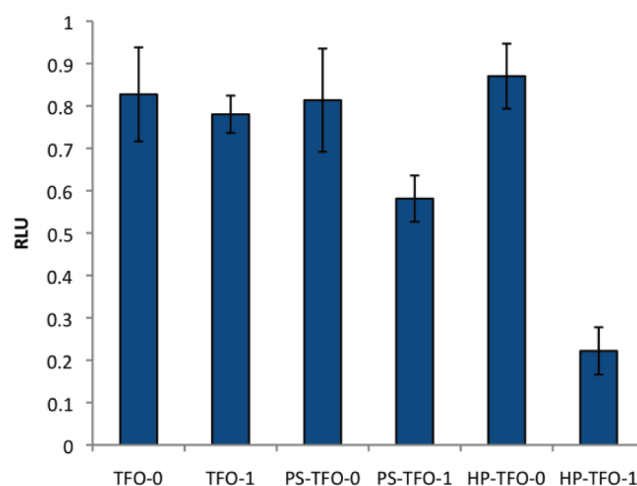
Gel shift assays were performed in order to examine if the backbone modifications or the introduction of the hairpin loop structures has an effect on the formation of oligonucleotide triplexes. Gratifyingly, the phosphodiester TFO-1, phosphorothioate PS-TFO-1, and the hairpin-protected HP-TFO-1 efficiently bind to the cyclin D1 duplex DNA target to form triplex structures, inducing a corresponding gel shift (Figure 2,



**Figure 2.** Investigation of triplex formation *via* gel shift assays. The radiolabeled cyclin D1 target sense strand was annealed to its complement and was incubated with a 5-fold excess of TFO at 37 °C for 4 h. Lane 1: Cyclin D1 target duplex. Lane 2: TFO-0. Lane 3: TFO-1. Lane 4: PS-TFO-0. Lane 5: PS-TFO-1. Lane 6: HP-TFO-0. Lane 7: HP-TFO-1. Lane 8: CHP-TFO-1 –UV. Lane 9: CHP-TFO-1 + UV. (a) Unbound DNA duplex. (b) DNA duplex bound to a TFO. (c) DNA duplex bound to a hairpin-protected TFO.

lanes 3, 5, and 7). As expected, the negative control TFOs TFO-0, PS-TFO-0, and HP-TFO-0 did not form triplex structures, as evident by the lack of a shifted radiolabeled target DNA. Since no difference in triplex-forming ability was observed between the phosphodiester and phosphorothioate-modified oligonucleotides, both species were subsequently tested in mammalian tissue culture.

Next, the effect of triplex formation on cyclin D1-driven expression of a luciferase reporter gene in live cells was investigated. TFOs were transfected into human embryonic kidney (HEK) 293T cells together with a dual reporter system encoding firefly luciferase and *Renilla* luciferase. The cyclin D1 promoter was present upstream of the firefly luciferase gene (pCyclin D1  $\Delta$ -944),<sup>44</sup> enabling the measurement of promoter activity by quantifying firefly luciferase expression. The *Renilla* luciferase plasmid (pRL-TK) was used as a control to account for differences in cell viability and transfection efficiency. As expected on the basis of the gel shift assays shown in Figure 2, the negative controls (TFO-0 and PS-TFO-0) showed no inhibition of firefly luciferase expression (Figure 3). Also, TFO-1, which consists of DNA with a simple phosphodiester linkage, showed no inhibition of firefly gene expression. This is most likely the result of fast intracellular degradation of the TFO, since unmodified DNA is quickly degraded by exonucleases.<sup>45</sup> Surprisingly, the substantially more stable phosphorothioate PS-TFO-1 led to only a marginal inhibition (25–40%) of firefly luciferase expression, despite optimization of transfection conditions (see Supporting Information, Figures 1 and 2). This low level of transcriptional inhibition could potentially be the result of TFO sequestering through nonspecific binding of the phosphorothioate DNA to proteins<sup>7</sup> or the result of PS-TFO:DNA triplexes having a dissociation constant 10-fold higher than that of phosphodiester TFO:DNA triplexes.<sup>46</sup> Importantly, the hairpin-protected TFO (HP-TFO-1) induced a >70% inhibition of luciferase expression, while the corresponding negative control HP-TFO-0 showed no gene knockdown. This demonstrated, for the first time, that hairpin-stabilized deoxyoligonucleotides containing regular phospho-

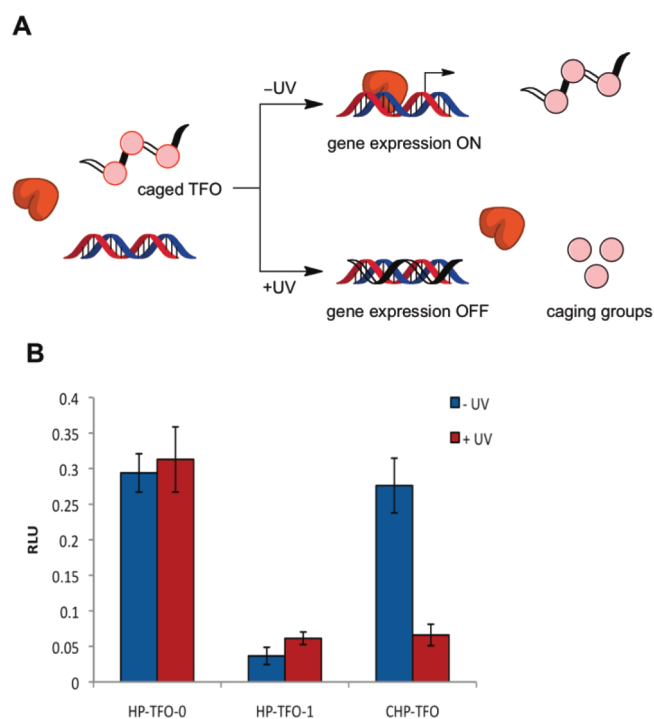


**Figure 3.** Intracellular inhibition of cyclin D1 promoter-driven transcription using modified TFOs. HEK 293T cells were co-transfected with pCyclin-D1  $\Delta$ -944, pRL-TK, and TFOs. Relative luciferase units (RLU) represent the firefly luciferase signal under control of the cyclin D1 promoter normalized to the *Renilla* luciferase signal as a control. Error bars represent standard deviations from three independent experiments.

diester bonds could be effectively applied as triplex-forming gene regulatory agents in mammalian cell culture.

Since HP-TFO-1 demonstrated the greatest inhibition of gene transcription, we synthesized a corresponding caged analogue (CHP-TFO-1). On the basis of previous studies investigating the effect of multiple caged thymidines on the inhibition of Watson–Crick base-pairing in DNA:DNA and DNA:RNA duplexes,<sup>24,25</sup> caged thymidine nucleotides were incorporated every 3–6 nucleotides throughout the TFO binding site in HP-TFO-1, generating CHP-TFO-1 bearing four NPOM caging groups (Table 1). To determine if the caged hairpin TFO forms a triplex structure, a gel shift assay was performed. Gratifyingly, the installation of 4 caging groups on selected thymidine residues completely prevented CHP-TFO-1 from undergoing DNA triplex formation (Figure 2, lane 8), as the gel shows only a band for the unbound cyclin D1 target duplex. CHP-TFO-1 is as inactive as the corresponding negative control TFO HP-TFO-0 (lane 6). However, upon UV irradiation the caging groups are removed and the hairpin TFO binds to the cyclin D1 target site forming a triplex structure and induces a significant gel shift (lane 9), identical to the shift observed for the positive control TFO shown in lane 7 (HP-TFO-1).

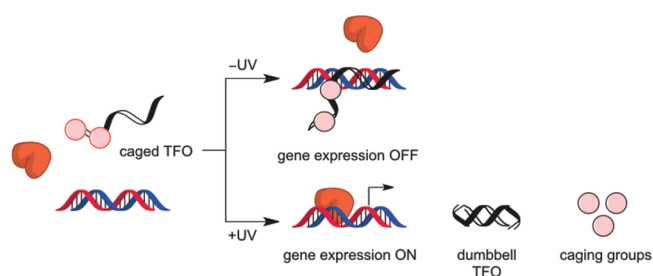
These results set the stage for the investigation of the light-activation of the caged hairpin TFO in cell culture. We hypothesized that the caged CHP-TFO-1 would be inactive when transfected into mammalian cells, leading to gene expression. A brief UV irradiation, however, will remove the caging groups, activate the TFO, and lead to the suppression of gene expression (Figure 4A). To this end, HEK 293T cells were co-transfected with plasmids encoding firefly luciferase (pCyclin D1  $\Delta$ -944), *Renilla* luciferase (pRL-TK), and with the TFO antisense agents. As expected, the negative control hairpin HP-TFO-0 had no effect on firefly luciferase activity regardless of UV irradiation (Figure 4B). HP-TFO-1 reduced luciferase expression by approximately 80%, in agreement with the previous assay (Figure 3). The caged hairpin TFO (CHP-TFO-1) was inactive, showing full luciferase expression



**Figure 4.** Photochemical activation of caged TFOs in mammalian cells. (A) Schematic of light-activated gene silencing using caged TFOs. The sequence of the TFO that binds to the targeted double-stranded DNA is blocked through light-removable protecting groups (caging groups). UV irradiation removes the caging groups, restores TFO activity, and inhibits gene expression. (B) Dual-Luciferase reporter assay in the presence and absence of UV irradiation (365 nm, 2 min). Relative luciferase units (RLU) represent the firefly luciferase signal under control of the cyclin D1 promoter normalized to the *Renilla* luciferase signal as a control. Error bars represent standard deviations from three independent experiments.

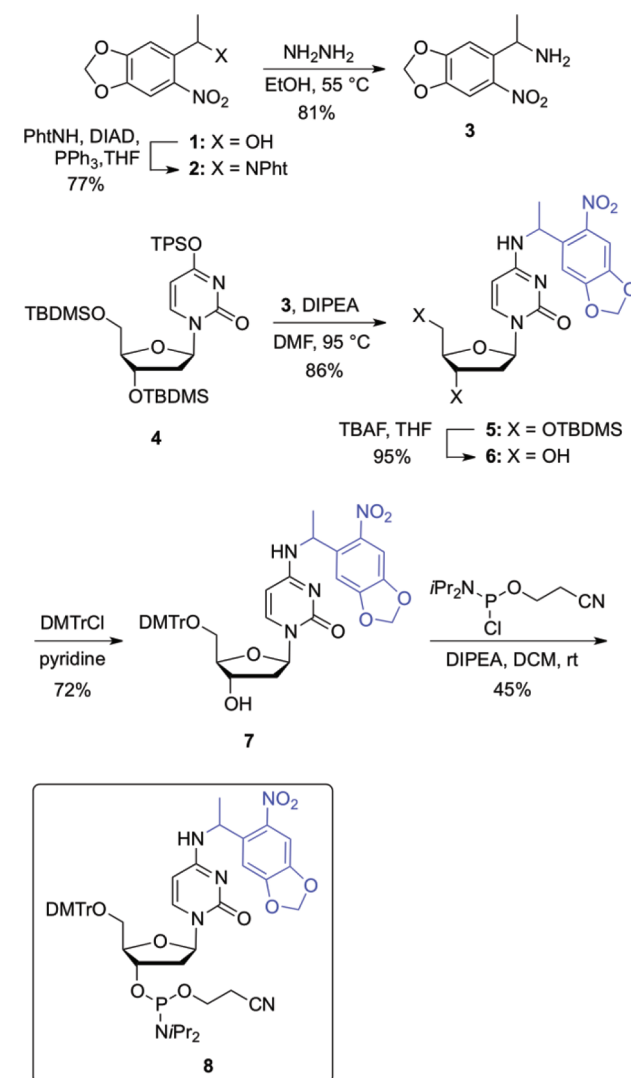
(comparable to the negative control HP-TFO-0) and demonstrating that intracellular triplex formation and thus gene silencing could be completely inhibited through the installation of nucleobase caging groups at defined thymidine residues. After brief UV irradiation (365 nm) of the transfected HEK 293T cells, the caging groups were removed, triplex-forming ability was restored, and a 78% inhibition of gene expression was observed, comparable to that of the noncaged HP-TFO-1. The optimal irradiation condition was found to be 2 min, and no increased inhibition was observed with prolonged exposure to UV light (see Supporting Information, Figure S3). The resulting level of reporter gene activity before and after UV irradiation demonstrates a highly efficient light-switching of an inactive, caged TFO (CHP-TFO-1) to a fully active, transcription-inhibiting TFO (HP-TFO-1).

In addition to the photochemical activation of TFOs and thus photochemical inhibition of transcription, we hypothesized that by designing a caged TFO dumbbell structure we can achieve *photochemical inhibition* of TFO activity and therefore light-activation of transcription. In the caged form, the TFO dumbbell will bind DNA, forming a triplex structure and thereby inhibiting gene expression. After irradiation, the caging groups are cleaved and the TFO will now deactivate itself by forming a DNA dumbbell structure that cannot bind to the promoter region anymore, leading to an activation of gene expression (Figure 5).



**Figure 5.** Schematic of the light-activation of gene expression (gene transcription) using caged dumbbell TFOs. The TFO is active before UV irradiation and becomes inactive through decaging and subsequent blocking of the double-stranded DNA targeting site by dumbbell formation.

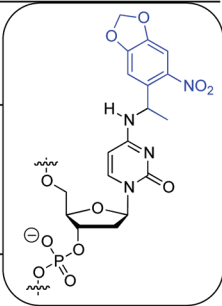
Due to the prevalence of guanines in the TFO targeting region, the caged thymidine phosphoramidite could not be applied, and a caged deoxycytidine phosphoramidite (Figure 6) needed to be developed in order to create a light-deactivatable TFO. Previously, 1-(2-nitrophenyl) ethyl caged deoxycytidine



**Figure 6.** Synthesis of the nitropiperonyl ethyl (NPE)-caged deoxycytidine phosphoramidite 8. The light-removable caging group is shown in blue. PhtNH = phthalimide, TPS = triisopropylbenzenesulfonyl, TBDMS = *tert*-butyldimethylsilyl, DMTr = dimethoxytrityl.

Table 2. Structure of the Caged Cytidine Residue and Sequences of Synthesized Triplex-Forming Oligonucleotides<sup>a</sup>

DNA	Sequence
DB-TFO-1	$\begin{array}{c} 5' \quad 3' \\ \text{A} \quad \text{GCGCGCGATGCACCCACCCACCCACCCCATAGCGCGCG} \quad \text{A} \\ \text{A} \quad \text{CGCGCGCTACGTGGGTGGGGTGGGGGTATCGCGCGC} \quad \text{A} \end{array}$
CDB-TFO-1	$\begin{array}{c} 3' \text{--ATGCACCCACCCACCCACCCCATAGCGCGCG} \quad \text{A} \\ \text{A} \quad \text{GCGCGCG} \text{--}5' \quad \text{A} \\ \text{A} \quad \text{CGCGCGCTACGTGGGTGGGGTGGGGGTATCGCGCGC} \quad \text{A} \end{array}$
CDB-TFO-2	$\begin{array}{c} 3' \text{--ATGCACCCACCCACCCACCCCATAGCGCGCG} \quad \text{A} \\ \text{A} \quad \text{GCGCGCG} \text{--}5' \quad \text{A} \\ \text{A} \quad \text{CGCGCGCTACGTGGGTGGGGTGGGGGTATCGCGCGC} \quad \text{A} \end{array}$



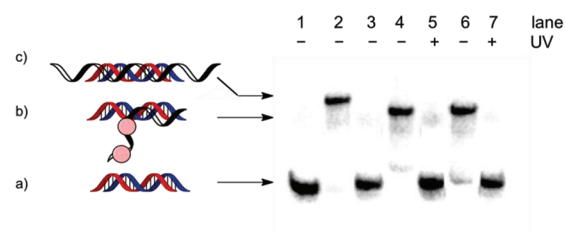
<sup>a</sup>C denotes a caged cytosine nucleotide (see insert).

has been synthesized and used in the activation of aptamers using light.<sup>47</sup> However, it has been shown that the efficiency of photolysis of the 2-nitrobenzyl caging group depends upon the nature of its aromatic substituents.<sup>48</sup> Electron-rich substituents on the benzene ring and a methyl substituent in the benzylic position enhance the photolytic properties of such caging groups, enabling the use of long-wavelength UV light of 365 nm, and thus are advantageous for applications in biological systems.<sup>49,50</sup> In order to take advantage of the electron-donating nature of the methylenedioxy moiety and the  $\alpha$ -methyl group, a nitropiperonyl ethyl (NPE)-caged deoxycytidine phosphoramidite was designed for the synthesis of caged DNA oligonucleotides containing light-activatable deoxycytidine residues. The synthesis of the caged phosphoramidite **8** commenced with the coupling of phthalimide with the known alcohol **1** in the presence of diisopropyl azodicarboxylate and triphenylphosphine in THF, affording the corresponding nitrodione **2** in 77% yield.<sup>52</sup> The dione **2** was reacted with hydrazine in refluxing ethanol for 2 h, delivering the amine **3** in 81% yield. The amine **3** was subsequently coupled with TBDMS-protected triisopropylbenzenesulfonyl activated deoxyuridine **4** (synthesized in 2 steps from 2' deoxyuridine)<sup>53</sup> in the presence of diisopropyl ethyl amine (DIPEA) in DMF at reflux conditions for 12 h to obtain the NPE-caged deoxycytidine nucleoside **5** in 86% yield. The TBDMS groups on **5** were removed through treatment with TBAF in tetrahydrofuran at low temperature to give the corresponding alcohol **6** (95% yield). Selective tritylation of the 5' hydroxyl group of **6** with dimethoxytritylchloride in pyridine was carried out at RT and delivered the compound **7** in 72% yield. Finally, activation of the 3' hydroxyl group of **7** was achieved by reacting it with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in the presence of DIPEA in dichloromethane providing the desired caged deoxycytidine phosphoramidite **8** as a white solid in 45% yield.

In order to test the photochemical activation of gene expression with light-deactivatable TFOs, the corresponding noncaged DNA oligonucleotides and caged DNA oligonucleotides containing NPE-protected cytosine residues were synthesized (Table 2). The caged cytosine nucleotides were site-specifically incorporated every 3–4 bases along the self-complementary inhibitor sequence of the TFO molecule. This design renders the TFO binding site open to hybridization to its double-stranded DNA target in the caged form; however,

UV irradiation will remove the caging groups, inducing formation of a dumbbell structure that blocks the binding site and deactivates TFO function. In turn, this leads to the light-activation of transcription. On the basis of our results with the hairpin TFO (HP-TFO-1) described above, the hairpin loop structures were incorporated into the TFO to provide cellular stability to the DNA.

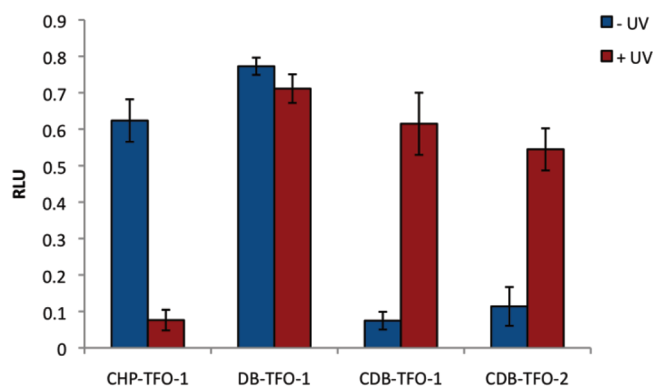
The light-deactivation of TFOs was first investigated using a gel shift assay (Figure 7). The radiolabeled double-stranded



**Figure 7.** Investigation of triplex formation *via* gel shift assays. The <sup>32</sup>P-radiolabeled cyclin D1 target duplex DNA was incubated with the different TFOs at 37 °C for 2 h. Lane 1: Cyclin D1 target duplex. Lane 2: HP-TFO-1. Lane 3: DB-TFO-1. Lane 4: CDB-TFO-1 –UV. Lane 5: CDB-TFO-1 +UV. Lane 6: CDB-TFO-2 –UV, Lane 7: CDB-TFO-2 +UV. (a) Unbound radiolabeled DNA duplex. (b) DNA duplex bound to an active, caged dumbbell TFO (CDB-TFO). (c) DNA duplex bound to a hairpin TFO.

cyclin D1 target sequence was incubated with the noncaged or caged TFOs. The caged dumbbell TFOs were either kept in the dark or were irradiated for 5 min (365 nm). As a positive control, the HP-TFO-1 was used and displayed a significant gel shift indicating the formation of a triplex structure (Figure 7, lane 2) when compared to just the target DNA duplex (lane 1). In the presence of the dumbbell TFO, DB-TFO-1, no gel shift was observed (lane 3), indicating that the double-stranded stem of the TFO does not bind to the target and does not form a triplex structure. The caged TFOs, CDB-TFO-1 and CDB-TFO-2, containing 4 or 5 caged cytosine nucleotides, however, do in fact bind the targeted DNA duplex and form triplex structures (lanes 4 and 6), since dumbbell formation is inhibited by the presence of the caging groups. However, after light irradiation, the caging groups are removed leading to formation of the dumbbell and thus inhibition of TFO binding activity (lanes 5 and 7).

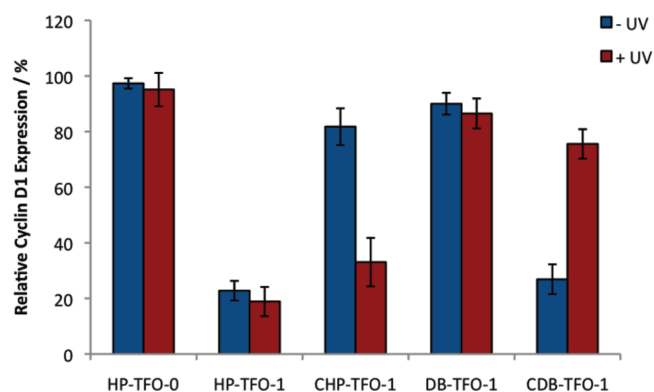
The gel shift results indicate the potential to photochemically deactivate triplex-formation and thus photochemically activate transcription. Thus, the noncaged and caged dumbbell TFOs were tested in cell culture. HEK 293T cells were co-transfected with plasmids encoding firefly luciferase (pCyclin D1  $\Delta$ -944), *Renilla* luciferase (pRL-TK), and with the TFOs. As a positive control for UV exposure, the CHP-TFO-1 was used since we have already established that it has an excellent light-switching behavior from an inactive to an active TFO (see Figure 4). As before, the CHP-TFO-1 shows no inhibition of gene expression until irradiation induces decaging and virtually complete inhibition of luciferase expression (Figure 8). The dumbbell



**Figure 8.** Photochemical activation of gene transcription in mammalian cells. Relative luciferase units (RLU) represent the firefly luciferase signal normalized to a *Renilla* luciferase signal as a transfection and cell viability control. Error bars represent standard deviations from three independent experiments.

TFO DB-TFO-1 does not inhibit gene expression, as expected, based on the gel shift results (see Figure 7). Gratifyingly, the CDB-TFO-1 is an active TFO, inhibiting luciferase transcription until irradiated with UV light of 365 nm (5 min), which removes the caging groups and deactivates the TFO through dumbbell formation, leading to a substantial increase in luciferase activity. Similarly, the CDB-TFO-2 (carrying 5 caging groups) inhibits gene expression before irradiation followed by restoration of gene expression after a brief UV exposure. These results demonstrate that the caged dumbbell TFOs are complementary tools to the caged TFOs. Together they enable the promoter-specific light-activation and light-deactivation of transcription with excellent efficiency, allowing caged TFOs to be used as photochemical on/off switches for gene function.

On the basis of the successful light-activation and light-deactivation of gene activity in a classical luciferase reporter system, the caged TFOs were subsequently tested for their photochemical regulation of the endogenous cyclin D1 gene. Due to the tightly packed nature of chromatin DNA compared to plasmid DNA, the light regulation of an endogenous gene using caged TFOs needed to be validated. Toward this goal, the human breast cancer cell line (MDA-MB-231) was transfected with the previously synthesized caged TFOs, and cells were either irradiated (365 nm) or kept in the dark. After 48 h, cellular RNA was isolated and subjected to quantitative real-time PCR (qRT PCR) analysis. Serving as a negative control, HP-TFO-0 displayed no inhibition of cyclin D1 expression, while HP-TFO-1 suppressed cyclin D1 expression by 80% (Figure 9). Thus, the inhibition of expression of an endogenous gene is in good agreement with inhibition of reporter gene expression. The caged hairpin-stabilized TFO CHP-TFO-1 was



**Figure 9.** Photochemical activation and deactivation of cyclin D1 expression in mammalian cells. MDA-MB-231 cells were transfected with TFOs, and cells were incubated for 48 h at 37 °C. RNA was isolated, and qRT PCR quantification was performed. Cyclin D1 expression was normalized to the expression of the GAPDH housekeeping gene, and expression levels in untreated cells were set to 100%. Error bars represent standard deviations from three independent experiments.

inactive; however, activity was restored through UV irradiation (5 min, 365 nm) leading to a light-induced knock-down of an endogenous gene. In order to demonstrate photochemical activation of cyclin D1 gene expression, the caged active TFO, CDB-TFO-1, was transfected into MDA-MB-231 cells. The 4 caging groups present in the predumbbell TFO CDB-TFO-1 rendered this silencing agent active with 74% suppression of cyclin D1 expression. Upon UV irradiation, the dumbbell is formed, inactivating the TFO and activating gene expression. The application of the two caged oligonucleotides, CHP-TFO-1 and CDB-TFO-1, demonstrates for the first time the light-activation and light-deactivation of transcription of an endogenous gene. Thus, the developed nucleobase-caging technology is highly versatile and can be applied to any TFO target site, as caged phosphoramidites of all four nucleotides are available.<sup>36,53,54</sup> This methodology will enable transcriptional studies of gene expression with high spatial and temporal resolution using caged TFOs.

**Summary.** Caged triplex-forming oligonucleotides (TFOs) were synthesized and successfully applied in the photochemical regulation – activation and deactivation – of transcription in mammalian cells. In the course of these studies, several important discoveries and methodology advancements were made. Specifically, the first physiologically active TFOs composed of standard DNA and stabilized with intramolecular hairpins at the 5' and 3' termini were synthesized. These reagents enabled stronger inhibition of gene expression than standard phosphorothioate-stabilized TFOs. Through the direct incorporation of caging groups on nucleobases within the TFO molecules, light-induced inhibition of a specific promoter (cyclin D1) and thus inhibition of gene transcription was achieved. Gel shift studies verified that the installed caging groups disrupt reverse Hoogsteen hydrogen bonding of the TFO to the target DNA duplex and that binding can be restored through decaging and activation of the TFO *via* a brief UV irradiation. In addition, a caged TFO design was developed that enables the light-activation of gene expression. This was achieved through the synthesis of a new caged deoxycytidine phosphoramidite and its incorporation into dumbbell-forming TFOs. Here, the TFO molecule is active and inhibits transcription until UV irradiation renders it inactive through

nucleobase decaging and DNA dumbbell formation, leading to light-activation of transcription. These methodologies were validated in human cells by photochemically controlling the transcription of a transiently transfected reporter gene (luciferase) and an endogenous gene (cyclin D1).

TFOs are versatile inhibitors of transcription and have been applied to the knock-down of a wide range of genes,<sup>12,55</sup> despite several factors that may limit their applicability, including electrostatic repulsion between the three negatively charged DNA strands, limited sequence variation, and the potential formation of secondary DNA structures.<sup>55,56</sup> However, several nucleotide modifications have been developed for enhanced triplex stabilization, inhibition of secondary structures, and improved binding affinity<sup>57,58</sup> and could be combined with this caging technology to further improve its applicability in tissue culture and in model organisms.

The discoveries reported here, intracellular function of DNA-based TFOs through stabilization by terminal hairpins and their photochemical activation and deactivation using caged nucleobases, have implications in the precise regulation of gene promoter activity in tissue culture and multicellular organisms.<sup>59,60</sup> Applications of caged TFOs range from basic biological studies of gene expression to new gene therapeutic approaches in high spatial and temporal resolution.

## METHODS

**DNA Synthesis Protocol.** DNA synthesis was performed using an Applied Biosystems Model 394 automated DNA/RNA synthesizer and standard  $\beta$ -cyanoethyl phosphoramidite chemistry. The caged hairpin triplex-forming oligonucleotide was synthesized on a 40 nmol scale, with a low volume solid-phase support obtained from Glen Research. Standard synthesis cycles provided by Applied Biosystems were used for all normal bases using 2 min coupling times. The coupling time was increased to 10 min for the positions at which the caged deoxythymidine modified phosphoramidites were incorporated. The caged dumbbell triplex-forming oligonucleotides were synthesized using the 0.2  $\mu$ M scale with 1000 Å solid phase supports obtained from Glen Research. Reagents for automated DNA synthesis were also obtained from Glen Research. The NPE-caged deoxycytidine phosphoramidite was resuspended in anhydrous acetonitrile to a concentration of 0.1 M. Standard synthesis cycles provided by Applied Biosystems with 25 s coupling times were used for all bases.

**Gel Shift Assays.** Gel shift assays were performed as previously described.<sup>32</sup> In short, cyclin D1 target sense strand was 5' radiolabeled with <sup>32</sup>P- $\gamma$  ATP (MP Biomedicals) and annealed to its complementary strand. A 5-fold excess of TFO (500 nM) was incubated with the radiolabeled duplex (100 nM) in TBM (89 mM Tris, 89 mM boric acid, 10 mM MgCl<sub>2</sub>, pH 7.4) buffer at 37 °C for 4 h. Aliquots were kept in the dark or irradiated for 2 min (CHP-TFO-1) or 5 min (CBD-TFOs) using a transilluminator (365 nm, 25 W). After irradiation, the TFO/dsDNA target mixtures were incubated for 30 min at 30 °C and analyzed on a 16% TBM gel and imaged with a Typhoon FLA 7000.

**TFO Inhibition of Gene Expression in Mammalian Cells.** Human embryonic kidney (HEK) 293T cells were grown at 37 °C, 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Hyclone), supplemented with 10% fetal bovine serum (Hyclone) and 10% streptomycin/penicillin (MP Biomedicals). Cells were passaged into a 96-well plate (200  $\mu$ L per well,  $\sim 1 \times 10^4$  cells per well) and grown to  $\sim 70\%$  confluence within 24 h. The medium was changed to Optimem (Invitrogen), and the cells were co-transfected with pCyclin D1  $\Delta$ -944 (0.15  $\mu$ g, gift from Dr. Linda Schuler, University of Wisconsin, Madison), pRL-TK (0.15  $\mu$ g, Promega), and TFO DNA (0.5  $\mu$ M) using X-tremeGene (3:2 reagent/DNA ratio, Roche). All transfections were performed in triplicate. Cells were incubated at 37 °C for 4 h, the transfection medium was removed and replaced with standard growth medium, and the cells were incubated for an additional 24 h. After the

24 h incubation, the medium was removed, and the cells were assayed with a Dual-Luciferase Reporter Assay system (Promega) using a Biotek Synergy 4 microplate reader. The firefly luciferase signal was normalized to the *Renilla* luciferase signal for each of the triplicates, the data were averaged, and standard deviations were calculated.

**Light Regulation of TFOs.** HEK 293T cells were passaged into a 96-well plate (200  $\mu$ L per well,  $\sim 1 \times 10^4$  cells per well) and grown to  $\sim 70\%$  confluence within 24 h. The medium was changed to Optimem, and the cells were co-transfected with pCyclin D1  $\Delta$ -944 (0.15  $\mu$ g), pRL-TK (0.15  $\mu$ g, Promega), and TFO DNA (0.5  $\mu$ M) using X-tremeGene. All transfections were performed in triplicate. Cells were incubated at 37 °C for 4 h, and the transfection medium was removed. Selected wells were briefly irradiated with a transilluminator (365 nm, 25 W) for 2 min (CHP-TFO-1) or 5 min (CBD-TFOs). The medium was then replaced with standard growth medium, and the cells were incubated for an additional 24 h. After the 24 h incubation, the medium was removed, and the cells were assayed with a Dual-Luciferase Reporter Assay system using a Biotek Synergy 4 microplate reader. Firefly luciferase signal was normalized to the *Renilla* luciferase signal for each of the triplicates, the data was averaged, and standard deviations were calculated.

**Quantitative RT-PCR.** MDA-MB-231 cells were passaged into 6-well plates (2 mL per well,  $\sim 2 \times 10^5$  cells per well) and grown to  $\sim 70\%$  confluence within 24 h. The medium was changed to Optimem (Invitrogen), and the cells were transfected with 0.5  $\mu$ M TFOs using X-tremeGENE transfection reagent (3:2 reagent/RNA ratio, Roche). Cells were incubated at 37 °C for 4 h, the transfection medium was removed, and 1 mL of PBS (pH 7.4) was added. The cells were irradiated for 5 min on a UV transilluminator (365 nm, 25W). DMEM media was added, the cells were incubated at 37 °C, 5% CO<sub>2</sub> for 48 h. RNA was isolated with TRIZOL reagent (Invitrogen). cDNAs were synthesized with Superscript Reverse Transcriptase II (Invitrogen), and quantitative RT PCRs were performed with cyclin D1 forward primer 5' CCTGTCTACTACCGCTCA, cyclin D1 reverse primer 5' CAGTCCGGGTCACACTTGA,<sup>61</sup> GAPDH forward primer 5' TGCACCACCACTGCTTAGC, and GAPDH reverse primer 5' GGCATGGACTGTGGTCATGAG. The threshold cycles (Ct) of each sample were normalized to the GAPDH housekeeping gene and relative to untreated cells. For each of the triplicates, the data was averaged, and standard deviations were calculated.

**2-(1-(6-Nitrobenzo[d][1,3]dioxol-5-yl)ethyl)isoindoline-1,3-dione (2).** To a stirring solution of the alcohol 1 (2.0 g, 9.4 mmol) in THF (25 mL) at 0 °C under an argon atmosphere were added phthalimide (1.4 g, 9.4 mmol) and triphenylphosphine (2.9 g, 11.2 mmol). Diisopropyl azodicarboxylate (2.20 mL, 11.2 mmol) was slowly added into the reaction mixture at 0 °C and was stirred for another 4 h at RT. The solvent was removed under reduced pressure, and the residue was diluted with CH<sub>2</sub>Cl<sub>2</sub> (20 mL). The organic layer was washed with a saturated aqueous solution of NaHCO<sub>3</sub> (2  $\times$  20 mL) and brine (20 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and evaporated under reduced pressure. The thus obtained residue was purified by column chromatography on silica gel using CH<sub>2</sub>Cl<sub>2</sub>/hexanes (1:4) to afford the dione 2 (2.4 g, 77% yield) as a white solid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  7.79–7.76 (m, 2H), 7.69–7.66 (m, 2H), 7.34–7.31 (m, 2H), 6.08–6.04 (m, 3H), 1.91 (d, *J* = 6.8 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  168.3, 151.8, 147.4, 143.1, 134.3, 132.5, 131.8, 123.5, 108.7, 105.4, 103.1, 46.4, 18.8. HRMS-ESI (*m/z*) [M + Na]<sup>+</sup> calcd for C<sub>17</sub>H<sub>12</sub>N<sub>2</sub>O<sub>6</sub> 363.0588, found 363.0592.

**1-(6-Nitrobenzo[d][1,3]dioxol-5-yl)ethanamine (3).** To a stirring solution of the dione 2 (2.4 g, 7.0 mmol) in dry EtOH (30 mL) at RT under an argon atmosphere was slowly added NH<sub>2</sub>NH<sub>2</sub> (0.55 mL, 17.6 mmol), and the mixture was refluxed for 1 h. The reaction mixture was then cooled to 0 °C for 15 min, and diethyl ether (200 mL) was added while stirring vigorously. The formed precipitate was filtered off and washed with diethyl ether (10  $\times$  5 mL). The residue was purified by column chromatography on silica gel using CH<sub>2</sub>Cl<sub>2</sub>/MeOH (9:1) with 1% TEA to obtain the amine 3 (1.2 g, 81% yield) as a yellow solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.32 (s, 1H), 7.22 (s, 1H), 6.06 (dd, 2H), 4.63 (q, *J* = 6.6 Hz, 1H), 1.59 (s, 2H), 1.37 (d, *J* = 6.6 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$  152.1, 146.5,

139.8, 106.5, 105.1, 102.9, 46.2, 24.8. HRMS-ESI ( $m/z$ ) [ $M + H$ ]<sup>+</sup> calcd for C<sub>9</sub>H<sub>10</sub>N<sub>2</sub>O<sub>4</sub> 211.0713, found 211.0717.

**1-((2R,4S,5R)-4-((tert-Butyldimethylsilyloxy)-5-(((tert-butyl)dimethylsilyloxy)methyl)tetrahydrofuran-2-yl)-4-((1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl)amino)pyrimidin-2(1H)-one (5).** To a stirring solution of the sulfonate **4** (1.0 g, 1.3 mmol) in dry DMF (5 mL) at RT under an argon atmosphere was slowly added DIPEA (0.72 mL, 4.4 mmol) followed by the amine **3** (0.58 g, 2.7 mmol). The reaction mixture was then heated to 90 °C overnight. The reaction mixture was cooled to RT, poured into a saturated aqueous solution of NaHCO<sub>3</sub> (30 mL), and extracted using EtOAc (3 × 20 mL). The combined organic layer was washed with saturated aqueous solution of NaHCO<sub>3</sub> (3 × 10 mL) and brine (10 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel using EtOAc with 1% TEA to obtain compound **5** (0.80 g, 89% yield) as a pale yellow solid. <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>COCD<sub>3</sub>): δ 7.82 (dd,  $J = 24$  Hz, 7.6 Hz, 1H), 7.58 (br, 1H), 7.43 (s, 1H), 7.14 (d,  $J = 12$  Hz, 1H), 6.20–6.16 (m, overlap, 3H), 5.82 (dd,  $J = 24$  Hz, 7.6 Hz, 1H), 5.73 (br, 1H), 4.47 (br, 1H), 3.90–3.83 (m, overlap, 3H), 2.31–1.99 (m, 2H), 1.53 (d,  $J = 6.8$  Hz, 3H), 0.93–0.88 (m, 18 H), 0.12–0.08 (m, 12 H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>COCD<sub>3</sub>): δ 163.7, 155.7, 155.6, 153.2, 153.1, 141.0, 139.2, 139.0, 106.9, 106.7, 105.6, 105.5, 104.2, 94.9, 94.8, 88.1, 86.3, 72.5, 72.3, 63.4, 47.2, 47.0, 42.3, 42.2, 26.3, 26.1, 22.3, 18.9, 18.5, -4.4, -4.6, -5.2, -5.3. HRMS-ESI ( $m/z$ ) [ $M + H$ ]<sup>+</sup> calcd for C<sub>30</sub>H<sub>48</sub>N<sub>4</sub>O<sub>8</sub>Si<sub>2</sub> 649.3083, found 649.3081.

**1-((2R,4S,5R)-4-Hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-4-((1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl)amino)pyrimidin-2(1H)-one (6).** To a stirring solution of compound **5** (0.80 g, 1.2 mmol) in dry THF (15 mL) at 0 °C was slowly added a 1.0 M solution of TBAF (3.6 mL, 3.6 mmol). After 30 min, the ice bath was removed, and the reaction mixture was stirred for another 3 h at RT. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel using CH<sub>2</sub>Cl<sub>2</sub>/MeOH (9:1) with 1% TEA to yield compound **6** (0.48 g, 95% yield) as a white solid. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 8.26–8.24 (m, 1H), 7.77 (d,  $J = 7.2$  Hz, 1H), 7.54 (s, 1H), 7.09 (d,  $J = 3$ , 1H), 6.19–6.17 (m, 2H), 6.09–6.03 (m, 1H), 5.79 (d,  $J = 7.5$  Hz, 1H), 5.55–5.49 (m, 1H), 5.19 (t,  $J = 3.6$  Hz, 1H), 4.93 (t,  $J = 5.4$  Hz, 1H), 4.16 (br, 1H), 3.74 (br, 1H), 3.51 (br, 2H), 2.12–2.01 (m, 1H), 1.94–1.82 (m, 1H), 1.45 (d,  $J = 6.8$  Hz, 3H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ 162.2, 154.6, 151.9, 146.4, 141.6, 140.5, 137.7, 105.7, 105.6, 104.6, 104.3, 94.3, 87.2, 84.9, 70.4, 70.3, 61.3, 45.3, 45.2, 21.9, 21.8. HRMS-ESI ( $m/z$ ) [ $M + H$ ]<sup>+</sup> calcd for C<sub>18</sub>H<sub>20</sub>N<sub>4</sub>O<sub>8</sub> 421.1354, found 421.1358.

**1-((2R,4S,5R)-5-((Bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxytetrahydrofuran-2-yl)-4-((1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl)amino)pyrimidin-2(1H)-one (7).** To a stirring solution of compound **6** (0.20 g, 0.47 mmol) in dry pyridine (1.0 mL) at RT was added DMTCl (0.19 g, 0.57 mmol). After 12 h, MeOH (0.2 mL) was added into the reaction mixture in order to quench unreacted DMTCl. The solvent was removed under reduced pressure, and the residue was dissolved in dichloromethane (15 mL). The organic layer was washed with 5% citric acid (2 × 5 mL), saturated aqueous solution of NaHCO<sub>3</sub> (5 mL), and brine (5 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated under reduced pressure, and the obtained residue was purified by column chromatography on silica gel using CH<sub>2</sub>Cl<sub>2</sub>/MeOH (20:1) with 1% TEA to give compound **7** (0.26 g, 72% yield) as a white solid. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>COCD<sub>3</sub>): δ 8.02 (s, 1H), 7.81–7.76 (m, 1H), 7.52–7.43 (m, 3H), 7.34–28 (m, 6H), 7.24–7.11 (m, 2H), 6.89–6.86 (m, 4H), 6.24–6.15 (m, 3H), 5.74–5.65 (m, 2H), 4.50–4.47 (m, 2H), 4.01–3.99 (m, 1H), 3.78 (s, 6H), 3.35 (d,  $J = 2.7$  Hz, 2H), 2.34–2.28 (m, 1H), 2.15–2.09 (m, 1H), 1.52 (d,  $J = 6.8$  Hz, 3H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>COCD<sub>3</sub>): δ 163.8, 159.6, 155.7, 153.2, 153.1, 147.6, 146.0, 143.2, 141.1, 139.1, 139.0, 136.7, 136.5, 131.0, 129.0, 128.6, 127.6, 113.9, 106.8, 106.7, 105.6, 104.2, 94.8, 87.2, 86.8, 86.4, 86.3, 79.2, 71.6, 71.5, 64.2, 55.5, 47.2, 47.0, 42.1, 42.0, 22.3. HRMS-ESI ( $m/z$ ) [ $M + H$ ]<sup>+</sup> calcd for C<sub>39</sub>H<sub>38</sub>N<sub>4</sub>O<sub>10</sub> 723.2661, found 723.2658.

**(2R,3S,5R)-2-((Bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-4-((1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl)amino)-2-oxypyrimidin-1(2H)-yl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite (8).** To a stirring solution of compound **7** (0.20 g, 0.27 mmol) in dry dichloromethane (2 mL) at 0 °C under an argon atmosphere were added DIPEA (0.24 mL, 1.38 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.12 mL, 0.55 mmol). After 20 min, the ice bath was removed, and the reaction mixture was stirred for another 2 h at RT. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel using CHCl<sub>3</sub>/CH<sub>3</sub>COCH<sub>3</sub> (20:1) with 1% TEA to afford a white solid. The white solid was dissolved in dichloromethane (0.5 mL) and precipitated in diethyl ether (20 mL) in order to remove the excess of chlorophosphoramidite reagent that was coeluted with the desired product. The precipitate was filtered to obtain the phosphoramidite **8** (0.11 g, 45% yield) as an off white solid. <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>COCD<sub>3</sub>): δ 7.84–7.78 (m, 1H), 7.48–7.44 (m, 4H), 7.36–7.32 (m, 6H), 7.24–7.22 (m, 1H), 7.12 (d,  $J = 12$  Hz, 1H), 6.90–6.87 (m, 4H), 6.27–6.16 (m, 3H), 5.74–5.71 (m, 1H), 5.68–5.66 (m, 1H), 4.65 (br, 1H), 4.14–4.09 (m, 1H), 3.70 (br, 6H), 3.72–3.54 (m, 2H), 3.42–3.35 (m, 2H), 2.76–2.72 (m, 1H), 2.63–2.60 (m, 1H), 2.52–2.39 (m, 1H), 2.28–2.16 (m, 1H), 1.53 (d,  $J = 6.8$  Hz, 3H), 1.27–0.83 (m, 12 H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>COCD<sub>3</sub>): δ 163.7, 159.7, 155.4, 151.7, 145.9, 141.1, 136.5, 131.0, 129.0, 128.7, 127.7, 114.0, 106.8, 106.7, 105.6, 104.3, 94.9, 87.3, 86.4, 63.9, 59.6, 59.4, 55.5, 47.0, 46.9, 43.9, 43.8, 41.0, 24.9, 24.8, 22.2, 20.7. <sup>31</sup>P NMR (121 MHz, CD<sub>3</sub>COCD<sub>3</sub>, reference H<sub>3</sub>PO<sub>4</sub> = 0.00 ppm): δ 146.55, 146.50, 146.39, 146.34.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Optimization of transfection conditions, luciferase assay conditions, and UV irradiation; NMR spectra of synthesized compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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