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Supporting Information

A Bioorthogonal Chemical Reporter of Viral Infection**

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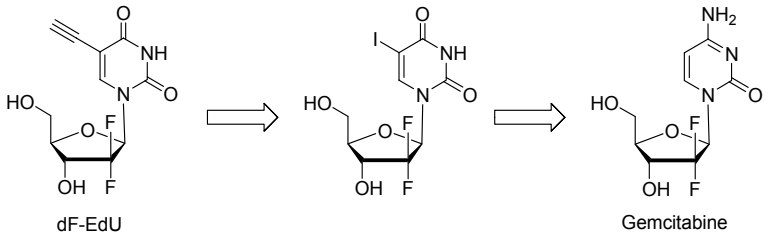
I. Materials and Methods

General synthetic methods and reagents. DAPI and EdU were purchased from Sigma Aldrich. THPTA was synthesized as previously described.[1] AlexaFluor 488 and 594 azides were purchased from Life Technologies. All other reagents were obtained in the highest commercial grades from Sigma Aldrich, TCI Europe and ABCR, and used without further purification. All non-aqueous reactions were conducted under nitrogen atmosphere using anhydrous solvents. NMR spectra were measured with a *Bruker AV2-400* (400 MHz for ^{1}H, 100 MHz for ^{13}C) and *Bruker AV2-500* (500 MHz for ^{1}H, 150 MHz for ^{13}C, 202 MHz for ^{31}P). Chemical shifts (δ) are given in parts per million (ppm) and are reported relative to residual solvent peaks: $CHCl_3$ (δ_H 7.26, δ_C 77.0 ppm), DMSO (δ_H 2.50, δ_C 39.5 ppm), D_2O (δ_H 4.79), MeOH (δ_H 3.31, δ_C 49.0 ppm). Coupling constants (*J*) are given in Hertz (Hz). ^{13}C-spectra were recorded broadband proton decoupled. High-resolution electrospray mass spectra were recorded on a Bruker maXis QTOF-MS instrument. Electrospray Ionization (ESI) mass spectra were obtained on a quadrupole ion trap instrument equipped with an atmospheric pressure ion (API) source. Masses are given as *m/z*.

II. Synthesis of dF-EdU

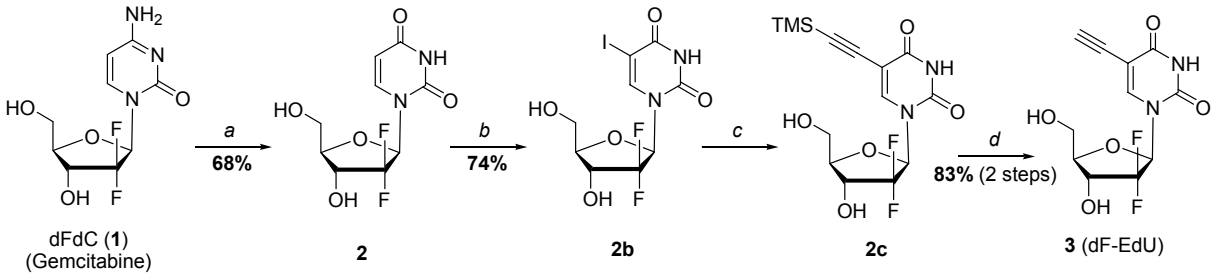
The synthesis of dF-EdU was envisioned starting from Gemcitabine (Scheme S1). Accordingly, the 5-ethynyl group would be introduced via Sonogashira reaction of 2'-deoxy-2',2'-difluoro-5-iodouridine with TMS-protected acetylene and subsequent TMS removal. 2'-Deoxy-2',2'-difluoro-5-iodouridine is a literature known compound that has been prepared by glycosylation of 5-iodouracil with an activated derivative of 2'-deoxy-2',2'-difluororibose.[2] However, it should also be feasible to obtain 2'-deoxy-2',2'-difluoro-5-iodouridine by electrophilic iodination of 2'-deoxy-2',2'-difluorouridine. The latter compound can be conveniently synthesized from commercially available Gemcitabine by hydrolytic deamination.[3,4]



Scheme S1. Retrosynthetic analysis of dF-EdU.

According to a modified procedure,[3] Gemcitabine (**1**) was converted to 2'-deoxy-2',2'-difluorouridine (**2**) using isopentyl nitrite in a mixture of aqueous HCl and 1,4-dioxane with a yield of 68% (Scheme S2). Subsequent electrophilic iodination with iodine and cerium(IV) ammonium nitrate in glacial acetic acid gave 2'-deoxy-2',2'-difluoro-5-iodouridine (**2b**) in a yield of 75%. A Sonogashira reaction of **2b** with ethynyltrimethylsilane proceeded to completion according to thin layer chromatography, yet despite extensive purification efforts by silica gel column chromatography using various eluents, it was not possible to separate product **2c** from

contaminating triethylammonium salts which were carried over into the final step of TMS removal using sodium hydroxide. Following purification, the final product 2'-deoxy-2',2'-difluoro-5-ethynyluridine (**3**, dF-EdU) was obtained in high purity (>95%) with a yield of 83% over two steps. The final product was fully characterized by ^{1}H-NMR, ^{13}C-NMR, and high resolution mass spectrometry.



Scheme S2. Synthesis of 2'-deoxy-2'-difluoro-5-ethynyluridine (dF-EdU, **3**) from Gemcitabine in four steps with an overall yield of 42%. *a*) isopentyl nitrite, 0.1 N HCl / H_2O / 1,4-dioxane, N_2, 70 °C, o/n; *b*) $Ce(NH_4)_2(NO_3)_6$, I_2, HOAc, N_2, 80 °C, 2 h; c) ethynyltrimethylsilane, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, N_2, RT, 60 min; d) NaOH/ H_2O/ MeOH, N_2, RT, 15 min.

2'-Deoxy-2',2'-difluorouridine (2). 2'-Deoxy-2',2'-difluorocytidine (**1**, Gemcitabine; 1.0 g, 3.8 mmol) was mixed with 1,4-dioxane (1.33 ml), H_2O (2.53 ml), and 1 N aq. HCl (133 µl). Isopentyl nitrite (5.9 eq, 22.3 mmol, 2.62 g, 3.00 ml) was added, and the mixture was stirred at 70 °C overnight. After addition of H_2O (10 ml), the aqueous layer was extracted with Et_2O (2 x 20 ml). The combined organic layers were washed with H_2O (3 x 10 ml) and concentrated under reduced pressure. Silica gel column chromatography (CH_2Cl_2 / MeOH, 20:1 to 9:1) gave **2** (682 mg, 2.58 mmol, 68%) as a slightly yellow solid. The data of the ^{1}H- and ^{13}C-NMR spectra were in good agreement with those published in the literature.[5] ^{1}H-NMR (500 MHz, CD_3OD) δ: 7.86 (d, *J* = 8.16, 1 H, *H*-6), 6.14 (dd, *J* = 5.39, *J* = 10.26, 1 H, H-1'), 5.73 (d, *J* = 8.11, 1 H, *H*-5), 4.28 (td, *J* = 8.14, *J* = 12.06, 1 H, *H*-3'), 3.89-3.95 (m, 2 H, *H*-4', *H*-5'), 3.78 (dd, *J* = 3.19, *J* = 12.52, 1 H, *H*-5''). ^{13}C-NMR (150 MHz, CD_3OD) δ: 165.8 (*C*-4), 152.1 (*C*-2), 142.1 (*C*-6), 124.1 (t, *J* = 258.5, *C*-2'), 103.1 (*C*-5), 85.5 (t, *J* = 21.5, *C*-1'), 82.9 (*C*-4'), 70.4 (t, *J* = 22.0, *C*-3'), 60.6 (*C*-5'). HR-ESI-MS (MeOH, neg. mode): $[M-H]^-$ calculated for $C_9H_{10}F_2N_2O_5$ 263.04850; found 263.04870.

2'-Deoxy-2',2'-difluoro-5-iodouridine (2b). Compound **2** (425 mg, 1.61 mmol) was dissolved in glacial HOAc (25 ml). $Ce(NH_4)_2(NO_3)_6$ (0.5 eq, 0.804 mmol, 441 mg) and I_2 (0.6 eq, 0.965 mmol, 245 mg) were added, and the mixture was heated to 80 °C for 2 h. The solution was then concentrated under reduced pressure and the residue was co-evaporated with toluene/EtOH (2:1; 3 x 30 ml). After silica gel column chromatography (CH_2Cl_2 / MeOH, 30:1 to 20:1), **2b** (468 mg, 1.20 mmol, 75%) was obtained as a yellow solid. The data of the ^{1}H- and ^{13}C-NMR spectra were in good agreement with those published in the literature.[2] ^{1}H-NMR (400 MHz, CD_3OD) δ: 8.42 (s, 1 H, *H*-6), 6.11 (dd, *J* = 4.52, *J* = 9.04, 1 H, H-1'), 4.32 (td, *J* = 8.35, *J* = 12.36, 1 H, *H*-3'), 3.90-3.98 (m, 2 H, *H*-4', *H*-5'), 3.78 (dd, *J* = 2.65, *J* = 12.63, 1 H, *H*-5''). ^{13}C-NMR (100 MHz, CD_3OD) δ: 162.5 (*C*-4), 151.8 (*C*-2), 146.2 (*C*-6), 120.8 (t, *J* = 258.9, *C*-2'), 85.5 (t, *J* = 32.6, *C*-1'), 82.9 (*C*-4'), 70.0 (t, *J* = 25.9, *C*-3'), 69.2 (*C*-5), 60.1 (*C*-5'). HR-ESI-MS (MeOH, neg. mode): $[M-H]^-$ calculated for $C_9H_9F_2IN_2O_5$ 388.94514; found 388.94562.

2'-Deoxy-2',2'-difluoro-5-(2-(trimethylsilyl)ethynyl)-uridine (2c). Compound **2b** (450 mg, 1.15 mmol), $Pd(PPh_3)_2Cl_2$ (0.1 eq, 0.12 mmol, 81 mg) and CuI (0.1 eq, 0.12 mmol, 22 mg) were dissolved in DMF (3 ml). Et_3N (6 ml) and ethynyltrimethylsilane (5 eq, 5.77 mmol, 567 mg, 815 µl) were added, and the mixture was stirred at room temperature for 60 min. After evaporation of the solvents under reduced pressure, the residue was purified using silica gel column chromatography (CH_2Cl_2 / MeOH, 40:1 to 30:1). A second purification using acetonitrile as the eluent failed to remove all traces of triethylammonium salts, and therefore no yield for product **2c** (381 mg) can be given. ^{1}H-NMR (500 MHz, CD_3OD) δ: 8.23 (s, 1 H, *H*-6), 6.13 (dd, *J* = 5.41, *J* = 9.48, 1 H, H-1'), 4.32 (td, *J* = 8.40, *J* = 12.24, 1 H, *H*-3'), 3.90-3.97 (m, 2 H, *H*-4', *H*-5'), 3.79 (dd, *J* = 2.84, *J* = 12.67, 1 H, *H*-5''), -0.21 (s, 9 H, $Si(CH_3)_3$). ^{13}C-NMR (100 MHz, CD_3OD) δ: 163.7 (*C*-4), 151.0 (*C*-2), 145.1 (*C*-6), 123.9 (t, *J* = 258.5, *C*-2'), 101.3 ($Me_3SiC^{ethynyl}$), 99.7 (*C*-5), 96.8 ($C^{ethynyl}$), 83.0 (*C*-1'), 82.9 (*C*-4'), 70.1 (t, *J* = 23.0, *C*-3'), 60.2 (*C*-5'), -0.1 ($Si(CH_3)_3$). HR-ESI-MS (MeOH, neg. mode): $[M-H]^-$ calculated for $C_{14}H_{18}F_2N_2O_5Si$ 359.08803; found 359.08786.

2'-Deoxy-2',2'-difluoro-5-ethynyluridine (3, dF-EdU). Compound **2c** (256 mg including Et_3NH^+ salts) was dissolved in MeOH (5 ml) and cooled to 0 °C. 5 N aq. NaOH (250 µl) was added, and the mixture was stirred at room temperature for 15 min. After neutralization with 1 N aq. HCl and concentration under reduced pressure, silica gel column chromatography (CH_2Cl_2 / MeOH, 20:1) gave **3** (171 mg, 0.593 mmol, 83% over two steps) as a white solid. ^{1}H-NMR (400 MHz, d_6-DMSO) δ: 11.91 (bs, 1 H, N*H*), 8.26 (s, 1 H, *H*-6), 6.32 (d, *J* = 7.16, 1 H, 3'-O*H*), 6.04 (t, *J* = 7.35, 1 H, H-1'), 5.42 (t, *J* = 5.19, 1 H, 5'-O*H*), 4.19-4.26 (m, 1 H, *H*-3'), 4.17 (s, 1 H, $H^{ethynyl}$), 3.85-3.88 (m, 1 H, *H*-4'), 3.77-3.81 (m, 1 H, *H*-5'), 3.61-3.66 (m, 1 H, *H*-5''). ^{13}C-NMR (100 MHz, CD_3OD) δ: 163.8 (*C*-4), 151.0 (*C*-2), 145.3 (*C*-6), 122.3 (t, *J* = 258.6, *C*-2'), 100.3 (*C*-5), 85.4 (dd, *J* = 24.4, *J* = 40.5, *C*-1'), 83.3 ($CH^{ethynyl}$), 82.9 (*C*-4'), 75.5 ($C^{ethynyl}$), 70.0 (dd, *J* = 19.5, *J* = 26.5, *C*-3'), 60.1 (*C*-5'). HR-ESI-MS (MeOH, neg. mode): $[M-H]^-$ calculated for $C_{11}H_{10}F_2N_2O_5$ 287.04850; found 287.04890.

III. Protein expression, nucleoside phosphorylation assays, crystallization and X-ray analyses

Protein expression and purification. The full length human cytosolic thymidine kinase (hTK1) "hTK1W" was produced and purified according published procedures.[6] The thymidine kinase of herpes simplex virus type 1 (HSV1-TK) was expressed as a PreScission protease-cleavable glutathione-S-transferase fusion protein by the plasmid pGEX-6P-2-TK. A glutathione affinity chromatography was employed to purify the GST-HSV1-TK. HSV1-TK was then isolated following established protocols.[7,8]

Analytical phosphorylation. Analytical phosphorylation reactions were conducted in aqueous solutions containing 50 mM HEPES, pH 7.5, 5 mM ATP, 5 mM $MgCl_2$ and 5% DMSO (v/v) in a final volume of 70 µl. All reactions contained 1.0 – 1.75 mM of nucleoside, and 8 – 10 µg of HSV1-TK or hTK. Reaction mixtures were pre-incubated for 2 min at 37 °C, and initiated by addition of ATP. Reactions were stopped after various time intervals (30 min, 60 min and 90 min) by addition a 10X solution of 50 mM EDTA (final concentration 5 mM). The quenched reactions were stored at -22°C prior to their analysis using ion-exchange chromatography on a Merck HITACHI LaChrom (Gynkotek HPLC, Münich, Germany) system equipped with a pump (L-7100), autosampler (L-7200) and a UV detector (L-7400). The analyses were performed using a LiChroCART® 250-4 cartridge column packed with a stationary phase made of spherical partical of silica (LiCrospher® 100 RP-18 endcapped, (5 µm)). The mobile phase for analysis contained aqueous 200 mM NaH_2PO_4, 25 mM tetrabutylammonium-hydrogen sulfate (TBAHS), and 1 – 2 %. methanol (HPLC grade). The separation was performed isocratically with a flow-rate of 1 ml/min. Control experiments were run in parallel to evaluate background ATP hydrolysis in presence of nucleoside but no enzyme (Control 1), or in presence of the enzyme but no nucleoside (Control 2). Control reactions were stopped at 60 min when performing the phosphorylation reactions with HSV1-TK. The mean of at least triplicate measurements are presented.

Preparative phosphorylation: 2'-Deoxy-2',2'-difluoro-5-ethynyluridine-5'monophosphate (4, dF-EdUMP). Compound **3** (3 mg, 10 µmol) was enzymatically phosphorylated in an aqueous buffer containing 50 mM Tris-HCl, $MgCl_2$, pH 7.5, 9 mM ATP and 5% DMSO (v/v) in a final volume of 4 ml. dF-EdU and HSV1-TK (128 µg) were pre-incubated for 2 min at 37°C. The reaction was initiated by addition of ATP, incubated for 90 min at 37 °C, and stopped by filtration through a Millipore Ultrafree-4 centrifugal filter (10 kDa cutoff). The filtrate was separated by strong anion exchange chromatography on Q-Sepharose™ Fast Flow (GE Healthcare) and an ammonium bicarbonate gradient. The product eluted along with some ATP at ~0.38 M NH_4HCO_3. Fractions containing the desired product were purified to homogeneity by C8 reversed phase HPLC and a mobile phase of aqueous 0.1 M triethyl ammonium acetate buffer (pH = 7.3) with a non-linear gradient of 1% to 8% of CH_3CN to furnish the triethylammonium salt of **4** as a colorless solid. ^{1}H-NMR (500 MHz, D_2O) δ: 8.18 (s, 1 H, *H-6*), 6.27 (m, 1 H, *H-1'*), 4.50 (td, *J* = 8.20, J = 11.90, 1 H, *H-3'*), 4.23-4.31 (m, 2 H, *H-4'*, *H-5'*), 4.15-4.20 (m, *J* = 2.65, 1 H, *H-5''*), 3.65 (s, $H^{ethynyl}$). ^{31}P-NMR (202 MHz, D_2O) δ: 0.81 (s, 1P). HR-ESI-MS (MeCN, neg. mode): $[M-H]^-$ calculated for $C_{11}H_{10}F_2N_2O_8P$ 367.01483; found 367.01493.

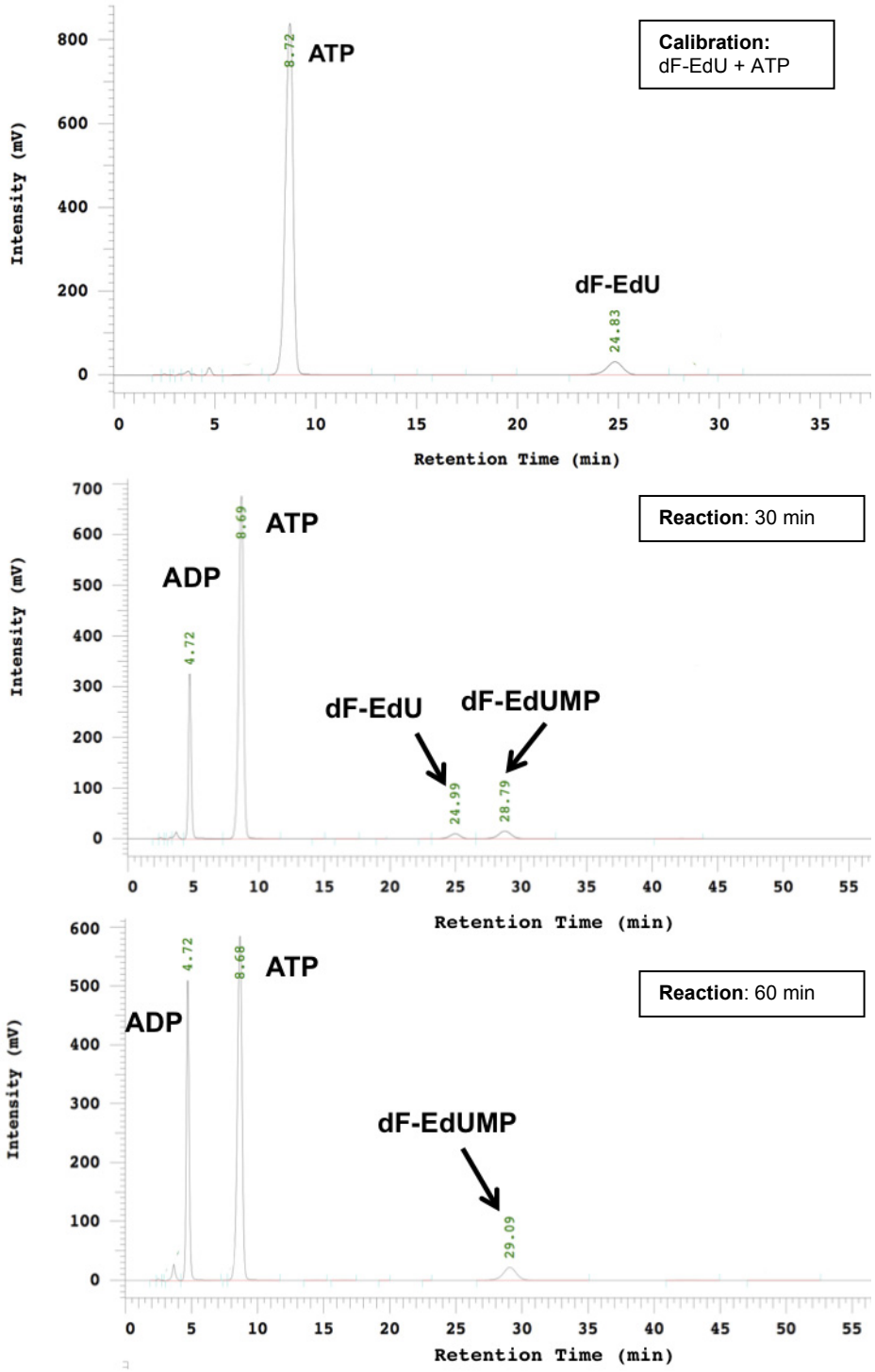


Figure S1. HPLC chromatograms (absorbance at 257 nm) illustrating phosphorylation of dF-EdU by HSV-1 TK. Preparative-scale phosphorylation reactions (described above) were used to confirm the identity of dF-EdUMP.

Protein crystallization. HSV1-TK crystals were grown in sitting drops at 23 °C using vapor diffusion. HSV1-TK crystals appeared in crystallization solutions containing 0.9 – 1.2 M Li_2SO_4, 1 mM DTT and 0.1 M HEPES at pH 7.5–8.0.[8] After one week, the crystals reached their maximal size. The crystals were then removed from their mother liquor and stored in a stabilizing solution containing 1.1 M Li_2SO_4 and 0.1 M HEPES at pH 7.5. The complex of HSV1-TK with dF-EdU was obtained by soaking the crystals in 1.75 mM of dF-EdU (prepared in stabilizing solution) for 45 min. The crystals were immerged for 10 s in a cryoprotection solution (stabilizing solution containing 27% ethylene glycol) and were plunged into liquid nitrogen and kept frozen in liquid nitrogen until data collection. The crystals belonged to the orthorhombic space group $C222_1$ with typical unit cell parameters of a = 113.4 Å, b = 116.4 Å, c = 108.1 Å, $\alpha = \beta = \gamma = 90$ °.

Table S1. Data processing and refinement statistics. Numbers in brackets are for the highest shell resolution of 2.21 Å – 2.1 Å.

Data Collection	
Data set	HSV1-TK : ***dF-EdU***
Resolution limits (Å)	27.4 – 2.1
No. of measured reflections	259263
No. of unique reflections	41988
Redundancy(1)	6.2 (5.3)
Completeness (%)	99.9 (99.6)
I/σ(I)	13.5 (3.9)
R_{sym} (%)	8.5 (33)
R_{merge} (%)	9.3 (36.7)
Refinement and final model	
No. of reflections	41946
No. of omitted reflections	1988
No. of proteins residues (A/B)	306 / 311
No. of water molecules	248
No. of sulfate ions	5
No. of bound ligand	2
R (%) / R_{free} (%)	17.8 / 22.2
Mean on B-factors (Å^2)	
Protein / solvent	22.2 / 27.4
Compound	16.3
RMSD. from ideal geometry	
Bond length (Å)	0.008
Bond angles (°)	1.07

X-ray data collection, structure solution and refinement. X-ray data were collected on beam-line x06sa of the synchrotron Swiss Light Source located at the Paul Scherrer Institute in Villigen, Switzerland. The beam-line was tuned at the wavelength of 1 Å and a Mar CCD detector was used. Each dataset was obtained from a single crystal. Raw diffraction images were indexed and integrated with iMosflm version 1.0.7 using Mosflm version 7.0.9.[9,10] Data scaling, merging and reduction was carried out with programs of the CCP4 suite.[11]

Relevant statistics are given in Table S1. The structure of HSV1-TK complexed with dF-EdU was determined by the molecular replacement method carried out with the program PHASER[12] using the model of HSV1-TK containing the substrate analogue *N*-Methyl-DHBT as a search probe (PDB code entry 3F0T).[13] The model of HSV1-TK complexed with the ethynyl nucleoside dF-EdU was refined by alternating all along the procedure, rounds of refinement performed with the program PHENIX version 1.8.1[14] with inspection of electron density maps and manual rebuilding sessions with the graphical program COOT version 0.7.[15] During the refinement of the structure, a residual density was identified in the (F_{obs} - F_{calc}) electron density map contoured at 2.5σ. For the both subunits A and B of the HSV1-TK homodimer present in the asymmetric unit, this residual density map was located within the nucleoside-binding pocket of HSV1-TK. In each case, the LigandFit program localized the ethynyl nucleoside analog within this residual electron density map.[16,17] The sulfate ions were introduced also directly in the ATP-binding pocket. The eLBOW program was used to generate the geometry restraint information of dF-EdU for performing the final refinement step with the newly introduced ligand.[18] Water molecules were introduced at the end of the refinement procedure. The stereochemical quality of the final refined model was assessed with MolProbity.[19] The relevant statistics of model refinement are reported in Table S1. Protein structure figures were produced using the program PyMOL (http://www.pymol.org). The coordinates and the structure factors of the structure of HSV1-TK:dF-EdU have been deposited in the Protein Data Bank under the accession code 4OQL.

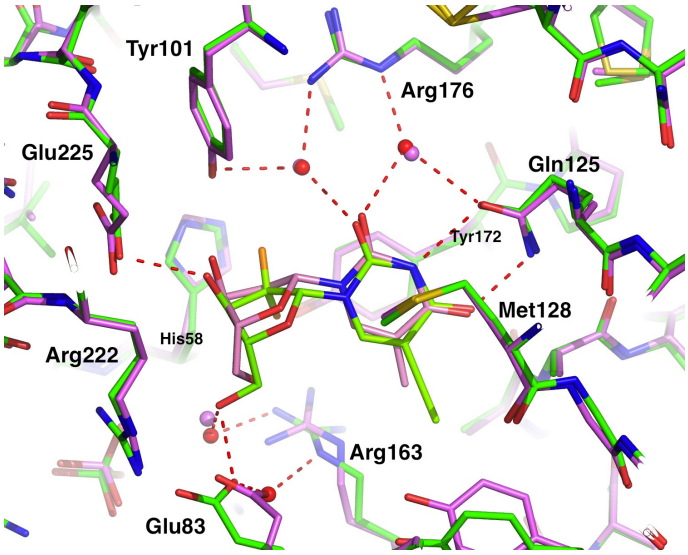


Figure S2. Comparison of dF-EdU and dT bound in the active site of HSV1-TK (subunit A) by superimposition of HSV1-TK:dF-EdU (green; PDB entry code 4OQL) and HSV1-TK:dT crystal structures (violet; PDB entry code 1KIM).[20] Hydrogen bounds, depicted with red dashed lines, are shown only for HSV1-TK bound to dF-EdU.

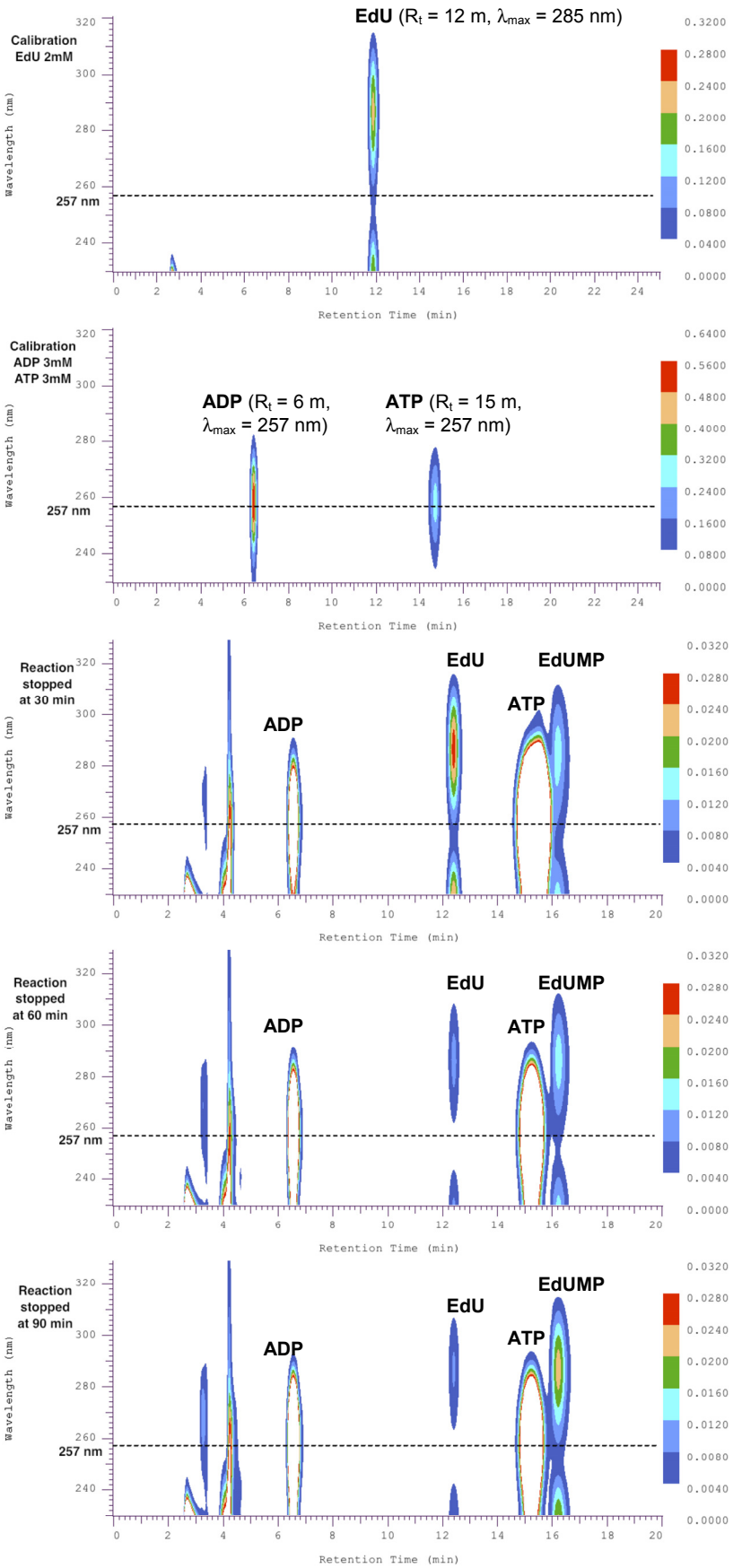


Figure S3. HPLC chromatograms from a diode array detector illustrating phosphorylation of EdU by hTK. Analytical-scale reactions and analyses were conducted as described above.

IV. Cellular and viral labeling

General cell culture conditions. Vero cells were obtained from Dr. Anna Paula de Oliveira, Institute for Experimental Virology, University of Zürich. HeLa, HeLa-HSVtk (+) and HeLa-HSVtk (++) cells were obtained from Pernilla Hoffmann, Section des Sciences Pharmaceutiques, University of Geneva. HeLa, HeLa-HSVtk (+), HeLa-HSVtk (++), and Vero cells were cultivated at 37 °C / 5% CO_2 in DMEM (Life Technologies) containing 4.5 g/l glucose, 10% FCS (Life Technologies), 50'000 units/l Penicillin and 50 mg/l Streptomycin (Sigma Aldrich). A549 cells were cultivated at 37 °C / 5% CO_2 in DMEM (Sigma Aldrich) containing 4.5 g/l glucose, 10% FCS (Life Technologies), 1% non-essential amino acids (Sigma Aldrich), 50'000 units/l Penicillin and 50 mg/l Streptomycin (Sigma Aldrich). Cells were grown to confluency and passaged every 2 – 4 days using Trypsin-EDTA solution (Sigma Aldrich). Cells were counted using trypan blue staining prior to the determination of seeding densities.

Metabolic labeling of cellular DNA. Cells were seeded in 24-well plates containing glass coverslips (VWR; thickness 1.5, diameter 13 mm) at 50'000 – 100'000 cells per well and incubated overnight. The supernatant was removed, and fresh media solutions containing variable nucleoside concentrations (diluted from 2000X stock solutions in DMSO) were added. After incubating for 10 min – 72 h, the cells were fixed in paraformaldehyde (3.7 % in PBS) for 15 min at room temperature, quenched with PBS containing 50 mM glycine and 50 mM NH_4Cl for 5 min, and washed with PBS once. Alternatively, the cells were incubated in media solutions containing additional nucleoside analogs (diluted from 2000X stock solutions in DMSO) for 24 h prior to fixation.

Metabolic labeling of virus-infected cells. Vero cells were seeded in 24-well plates containing glass coverslips (VWR; thickness 1.5, diameter 13 mm) at 100'000 cells per well and incubated overnight. The supernatant was removed, and serum-free media solutions were added. Purified Herpes simplex virus-1 from frozen stocks was added to a final MOI of 10 – 20 infectious particles per cell. Variable nucleoside concentrations (diluted from 2000X stock solutions in DMSO) were added, and infected cells were incubated for 1 – 10 h at 37 °C. Cells were then fixed in paraformaldehyde (3.7 % in PBS) for 15 min at room temperature, quenched with PBS containing 50 mM glycine and 50 mM NH_4Cl for 5 min, and washed with PBS once. Samples were then stained using ICP4 immunostaining and CuAAC as described below.

Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) mediated staining of dF-EdU-modified DNA. Cells were grown and fixed on glass coverslips as described above and incubated upside-down on 50 µl-drops of freshly prepared staining mix (10 µM AlexaFluor azide, 1 mM $CuSO_4$, and 10 mM sodium ascorbate in PBS) for 2 h at room temperature in the dark. In case of GFP-expressing cell lines, THPTA (2 mM) and aminoguanidium HCl (10 mM) were also included in the staining mixtures. The cells were washed with PBS (1 x 2 min), 0.1 % Triton X-100 in PBS (1 x 2 min), and PBS (2 x 2 min). Cells were further stained with DAPI (600 nM in PBS, 15 min, room temperature, dark) and washed with PBS (3 x) and H_2O (1 x). Coverslips were then glued upside-down on microscopy slides using Glycergel (Dako).

Fluorescence microscopy. Images were acquired on a Leica CLSM SP2 microscope (Leica Microsystems) equipped with a HCX PL APO CS 63 x oil immersion objective (NA 1.4) at room temperature. DAPI was excited at 405 nm, and emission was sampled between 415 and 500 nm; GFP was excited at 488 nm, and emission was sampled between 500 and 550 nm; AlexaFluor 488 was excited at 488 nm, and emission was sampled between 500 and 550 nm; AlexaFluor 594 was excited at 594 nm, and emission was sampled between 610 and 660 nm; AlexaFluor 647 was excited at 633 nm, and emission was sampled between 650 and 750 nm. Frames were taken in triplicate and averaged. Image analysis was performed using ImageJ 1.43m (National Institutes of Health, USA) and Imaris x64 7.1.1 (Bitplane).

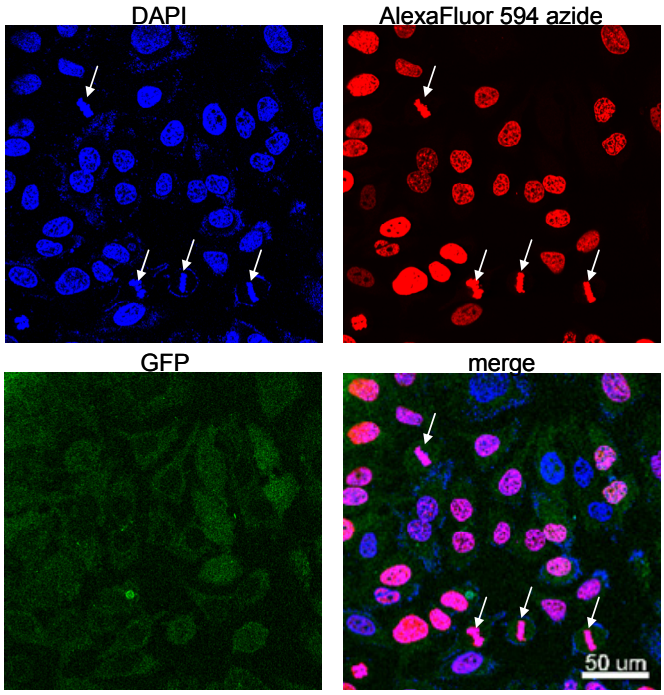


Figure S4. Expanded view of Figure 4b (main paper) illustrating staining of metaphase chromosomes (arrows). HeLa-HSVtk (+) cells were incubated with 1 μM of dF-EdU for 24 h, the cells were fixed, and stained by a solution containing 10 μM of Alexa Fluor 594 azide, 1 mM $CuSO_4$, 10 mM sodium ascorbate, 2 mM THPTA,[1] 10 mM aminoguanidium HCl in PBS for 2 h at room temperature in the dark as described above.

Toxicity assays. Cells were seeded in 96-well plates at a density of 5'000 cells per well and incubated overnight. The supernatant was removed, and fresh media containing variable concentrations of each nucleoside in DMSO was added (final DMSO = 0.05 %). Control samples contained 0.05 % DMSO only. Cells were grown for 24-72 h. The media were removed and fresh media containing 87 μM of resazurin (freshly prepared from an 870 μM stock solution in PBS) was added. After 4 h, the fluorescence at 590 nm (excitation at 560 nm) was measured using a SpectraMax M5 plate reader (Molecular Devices). All compound solutions and activity measures were prepared and measured in three independent trials or more.

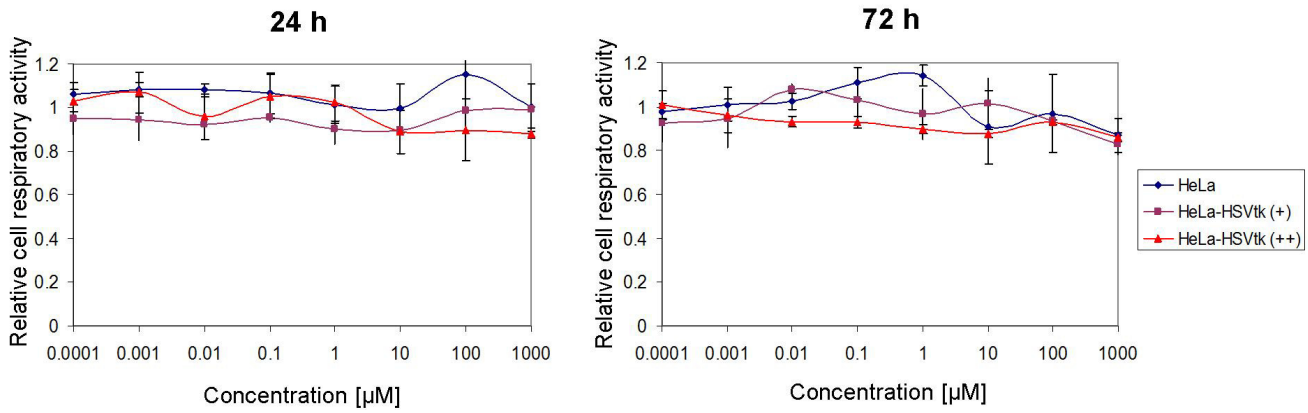


Figure S5. Cytotoxicity of dF-EdU in HeLa cells and in HeLa cells transduced with HSV-1 thymidine kinase. Cells were treated with varying concentrations of dF-EdU for 24 h or 72 h, followed by incubation with resazurin for 4 h. The reduction product resarufin was then quantified using fluorescence to determine cellular respiration. Values are given relative to those of untreated (DMSO only) control cells. Under these same conditions, EdU exhibited an IC_{50} for toxicity = 6 µM in HeLa cells after 72 hours.[21]

Fluorescence-assisted cell sorting (FACS). Cells were seeded in 6-well plates at 100'000 cells per well and incubated overnight. The supernatant was removed, and fresh media solutions containing varying concentrations of nucleoside analogs (diluted from 2000X stock solutions in DMSO) were added. After incubating for 24 – 72 h, the supernatant was removed. Cells were detached from their surface using Trypsin-EDTA solution (Sigma Aldrich) and pelleted. Alternatively, fresh media solutions containing 10 µM BrdU (diluted from a 2000X stock solution in DMSO) were added, and cells were incubated for additional 24 h prior to Trypsin treatment. After fixation in methanol (-20 °C, 10 min), cell suspensions were either directly treated with CuAAC staining mix, or prepared for BrdU staining as follows. Cells were incubated with 2 N aq. HCl containing 1 % Triton X-100 (30 min, RT), followed by 0.1 N aq. borax (30 min, RT). After blocking with 3 % BSA in PBS (15 min, RT), cells were incubated with mouse monoclonal BrdU antibody-AlexaFluor 647 conjugate (2 µg/ml in PBS; Invitrogen) for 60 min at room temperature in the dark. Cells were then incubated with freshly prepared staining mix (10 µM AlexaFluor 488 azide, 1 mM $CuSO_4$, and 10 mM sodium ascorbate in PBS for alkyne-modified nucleosides; 10 µM AlexaFluor 647 azide, 1 mM $CuSO_4$, 2 mM THPTA and 10 mM sodium ascorbate in PBS for dF-EdU-modified nucleosides; 10 µM AlexaFluor 488 alkyne, 1 mM $CuSO_4$, 2 mM THPTA and 10 mM sodium ascorbate in PBS for azide-modified nucleosides; 10 µM AlexaFluor 488 cyclooctyne in PBS for SPAAC staining of azide-modified nucleosides) for 1-2 h at room temperature in the dark. Optionally, DNA was denatured with 2 M aq. HCl prior to SPAAC. After DAPI staining (10 µM in PBS, 30 min, room temperature, dark), cell suspensions were analyzed using a CyAn ADP 9 flow cytometer (Beckman Coulter) (FL1 for Alexa Fluor 488 and GFP; FL8 for Alexa Fluor 647; FL6 for DAPI). Data was analyzed using Summit 4.3 (Beckman Coulter).

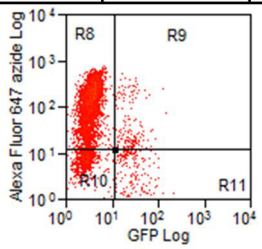
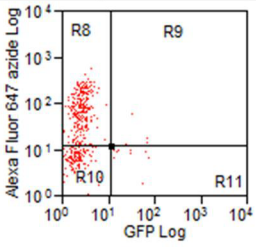
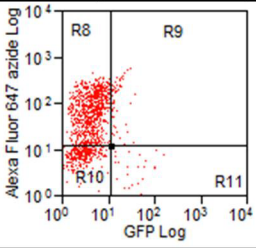
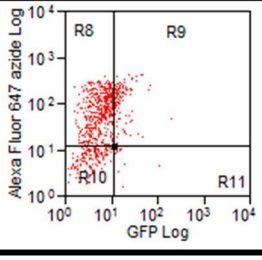
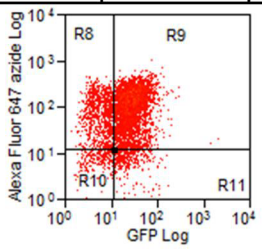
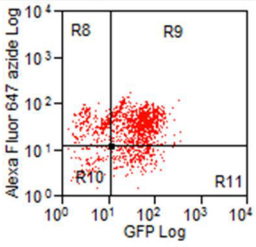
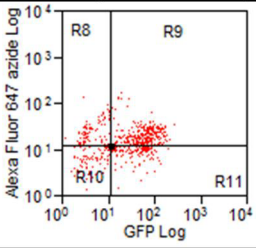
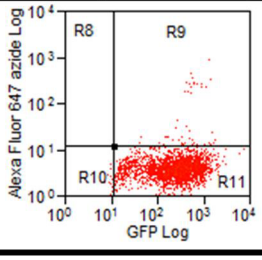
Entry	$CuSO_4$ [mM]	THPTA [mM]	sodium ascorbate [mM]	amino-guanidine [mM]	
1	1	0	10	0	
2	1	0	10	10	
3	1	0.5	10	10	
4	1	1	10	10	
5	1	2	10	10	
6	1	3	10	10	
7	1	5	10	10	
8	0	0	0	0	

Figure S6. Optimization of CuAAC reaction conditions for both GFP signal preservation as well as high CuAAC staining by FACS. HeLa-HSVtk (++) cells were incubated with dF-EdU (10 µM) for 24 h, fixed, and subjected to CuAAC staining by 10 µM of Alexa Fluor 647 azide in the presence of $CuSO_4$ and sodium ascorbate in PBS for 2 h in the presence of variable concentrations of THPTA[1] and aminoguanidinium HCl. Signal intensities of GFP and AlexaFluor 647 in individual cells were measured by FACS and depicted by 2D histograms. Region R8 contains cells with detectable AlexaFluor 647, but no GFP signal; R9, contains both GFP and AlexaFluor 647 signals; R10, contains neither GFP nor AlexaFluor 647 signals; and R11, contains GFP but no AlexaFluor 647 signal. All samples were measured at identical laser voltage and detector gain settings. Events were gated according to untreated samples using forward and side scatter.

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