Supporting Information

Fluorescent probes for ecto-5′-nucleotidase (CD73)

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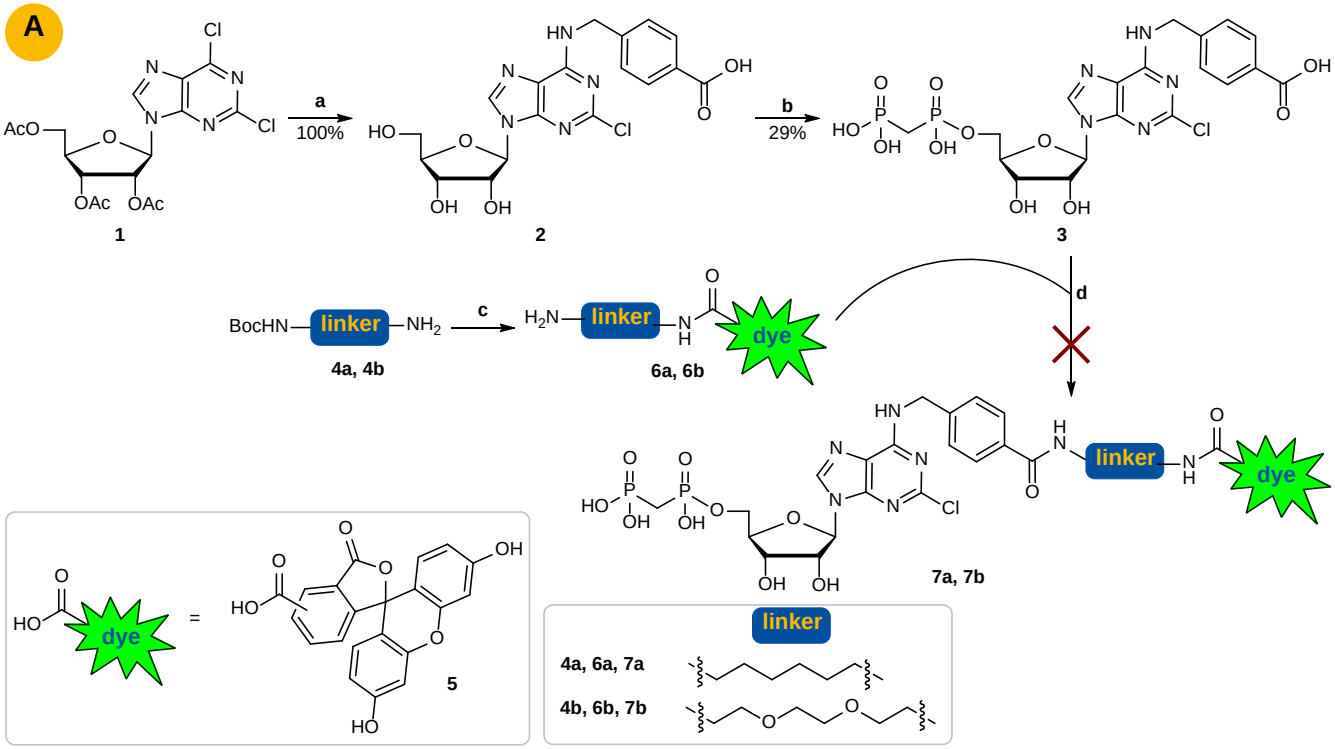
Chemistry

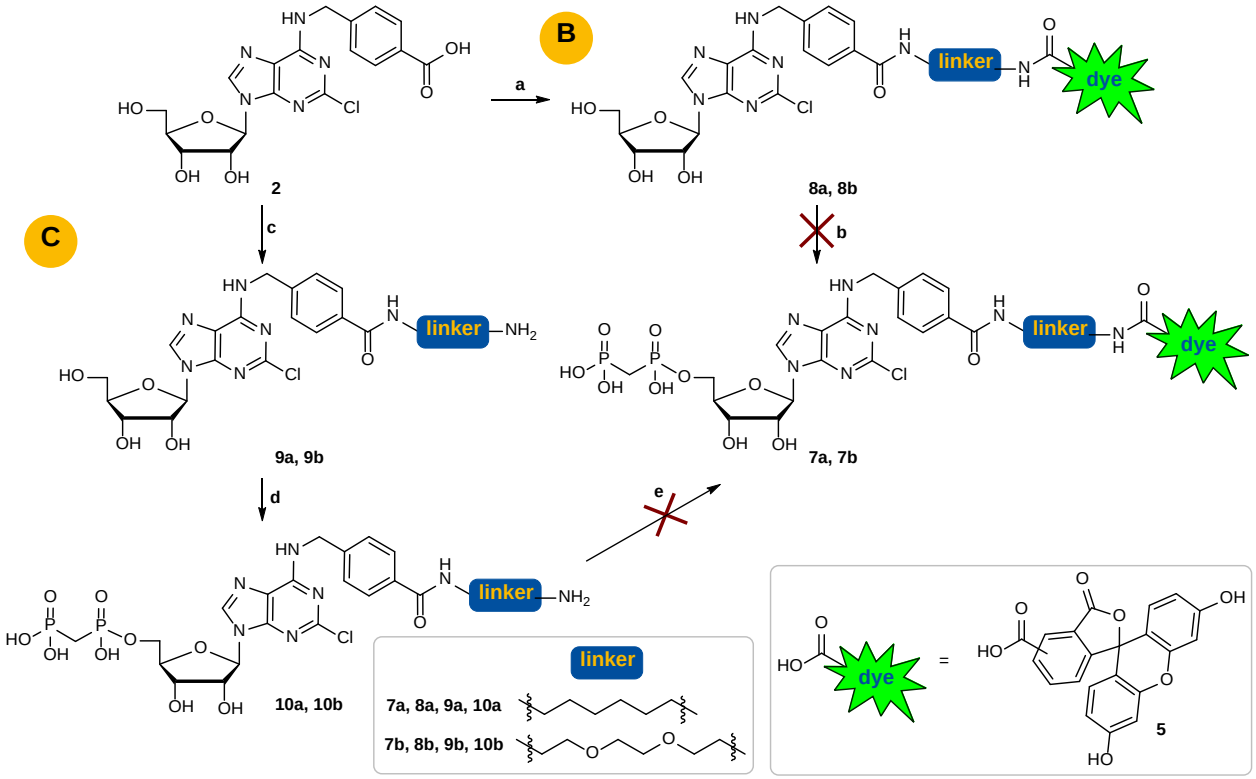
The synthesis of compounds **2**, **3**, **6a**, **6b**, **7a**, **7b**, **8a**, **8b**, **9a**, **9b**, **10a**, **10b**, **11a**, **11b**, **13**, **14a**, **14b** was carried out as described in Schemes **S1-S3**.

Scheme S1. Pathway A[a]



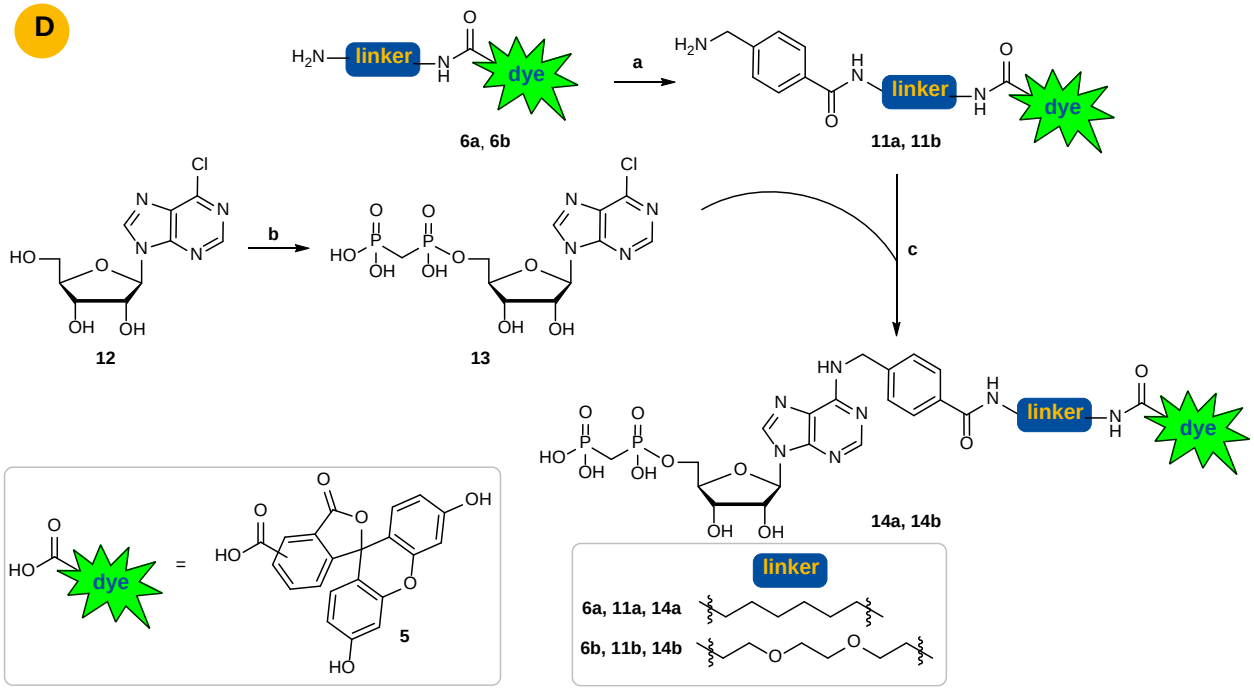
[a]Reagents and conditions: (a) two steps: (i) 4-(aminomethyl)benzoic acid, Et_3N, absolute EtOH, reflux, overnight; (ii) sodium methoxide, methanol, rt, 18 h; (b) two steps: (i) methylenebis(phosphonic dichloride), trimethyl phosphate, Ar, 0 °C, 30 min; (ii) triethylammonium carbonate (TEAC) buffer pH 7.4-7.6, rt, 1 h; (c) **5**, HOBt, DCC, THF, rt, overnight; (ii) 6-8% TFA, DCM, rt, 6 h; (d) HOBt, DCC, THF, rt, overnight.

Scheme S2. Pathways B and C[a]



[a]Reagents and conditions: (a) **6a** or **6b**, HOBt, DCC, THF, rt, overnight; (b) two steps: (i) methylenebis(phosphonic dichloride), trimethyl phosphate, Ar, 0 °C, 30 min; (ii) TEAC buffer pH 7.4-7.6, rt, 1 h; (c) two steps: (i) **6a** or **6b**, HOBt, DCC, THF, rt, overnight. (ii) 6-8% TFA, DCM, rt, 6 h; (d) two steps: (i) methylenebis(phosphonic dichloride), trimethyl phosphate, Ar, 0 °C, 30 min; (ii) TEAC buffer pH 7.4-7.6, rt, 1 h; (e) **5**, HOBt, DCC, THF, rt, overnight.

Scheme S3. Successful pathway D to obtain 14a[a]



[a]Reagents and conditions: (a) 4-(aminomethyl)benzoic acid, HOBt, DCC, THF, rt, overnight; (ii) 6-8% TFA, DCM, rt, 6 h; (b) two steps: (i) methylenebis(phosphonic dichloride), trimethyl phosphate, Ar, 0 °C, 30 min; (ii) TEAC buffer pH 7.4-7.6, rt, 1 h; (c) Et_3N, absolute EtOH, reflux, overnight.

Inhibition of human NTPDases

The activity of the most potent fluorescent-labeled CD73 inhibitor **14a** was tested on human NTPDases 1, 2, 3 and 8 in order to assess its selectivity. ATP (100 µM) was used as a substrate, and the effect of 50 µM of **14a** on its hydrolysis by the NTPDases was studied. Compound **14a** did not inhibit any of the NTPDases at this high concentration by more than 50% (see Table S1).

Effects on ADP-activated human $P2Y_{12}$ receptor

Since compound **14a** represents an ADP analog, it was investigated for activation and inhibition of the human $P2Y_{12}$ receptor, which is activated by ADP. $P2Y_{12}$-induced recruitment of β-arrestin was studied according to published procedures using the enzyme (galactosidase) complementation technology.[1–3] The receptor was expressed in CHO-PK1 cells (Eurofins DiscoverX, Fremont, CA, USA).

Table S1. Inhibitory activity of **14a** on human NTPDases and activation/inhibition of the $P2Y_{12}$ receptor

Target	**IC_{50} or EC_{50}** (nM) (% inhibition ± SEM at the indicated concentration[a] or % activation ± SEM)
NTPDase1	>50,000 (36%)
NTPDase2	>50,000 (-39%)
NTPDase3	>50,000 (-9%)
NTPDase8	>10,000 (-14%)
Inhibition $P2Y_{12}$ receptor	>10,000 (-12 ± 2)
Activation $P2Y_{12}$ receptor	>10,000 (16 ± 5)

[a]For antagonist testing, 2-methylthio-ADP was employed as agonist at its EC_{80} concentration (3 µM).

Metabolic stability

The metabolic stability of **14a** was investigated in vitro in human liver microsomes. Compound **14a** was incubated with human liver microsomes (0.5 mg/ml, mixed gender, pooled) in phosphate buffer pH of 7.4 containing reduced nicotinamide adenine dinucleotide phosphate (NADPH). The degradation of **14a** was measured after 0 min, 15 min, 30 min, 45 min and 60 min. Positive controls were carried out with verapamil, negative controls in an incubation medium without NADPH. The experiments were carried out by Pharmacelsus (Saarbrücken, Germany).

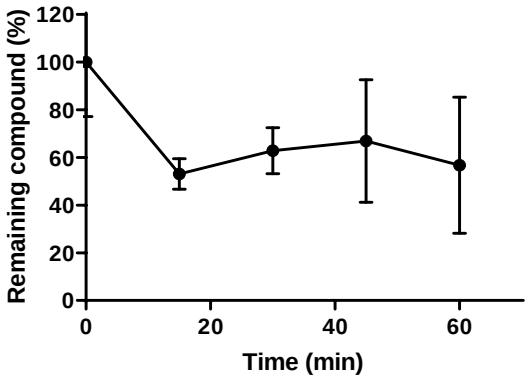


Figure S2. Metabolic stability of **14a** in human liver microsomes.

Compound **14a** was found to be metabolically stable ($t_{½}$ = 116 min) showing an internal clearance of 12 µl/min/mg of protein.

Fluorescence absorption and emission spectra

Fluorescence absorption and emission spectra of probes **14a** and **14b** were recorded in the range from 300 to 800 nm in different media including water, phosphate-buffered saline (PBS) pH 7.4, corresponding to physiological pH value, and in sodium acetate buffer pH 4, respectively. The excitation wavelength was set corresponding to the absorbance maxima of the compounds.

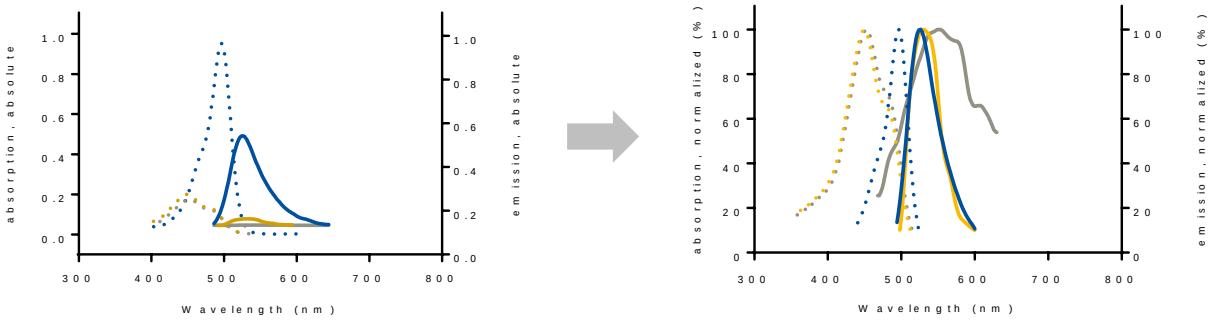


Figure S3. Absorption and emission spectra of **14a**. Depicted are the absorption (dotted lines ···) and emission (solid lines −) curves measured in different media: water (yellow), PBS (blue), and sodium acetate buffer pH 4 (gray). Fluorescence spectra were recorded from 300 - 800 nm.

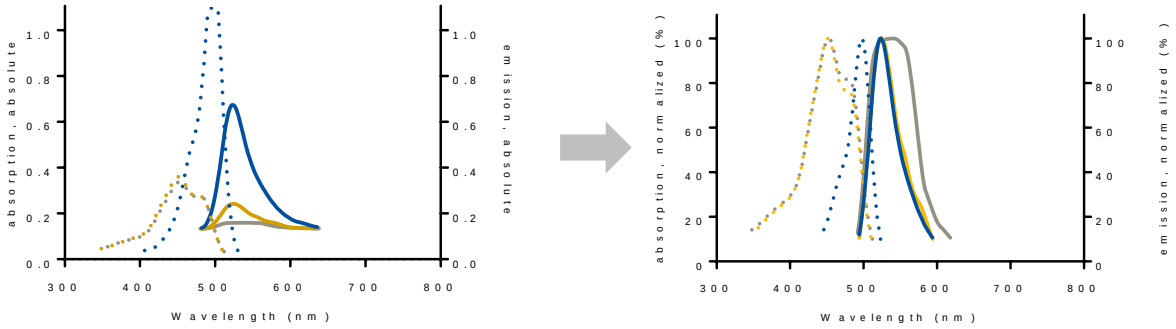


Figure S4. Absorption and emission spectra of **14b**. Depicted are the absorption (dotted lines ···) and emission (solid lines −) curves measured in different media: water (yellow), PBS (blue), and sodium acetate buffer pH 4 (gray). Fluorescence spectra were recorded from 300 -800 nm.

Table S2. Determined absorption and emission maxima and calculated Stokes shifts of **14a** and **14b**. The absorbance maximum of the each compound was used for excitation.[a]

Solvent	14a			14b		
	λ_{ex} (nm)	λ_{em} (nm)	λ_S (nm)	λ_{ex} (nm)	λ_{em} (nm)	λ_S (nm)
H_2O	449.0	530.0	81.0	451.0	524.0	73.0
NaOAc buffer pH 4	452.0	552.0	100.0	453.0	540.0	87.0
PBS	496.0	526.0	30.0	498.0	524.0	26.0

[a]λ_{ex} = absorption maximum; λ_{em} = emission maximum; λ_S = Stokes shift.

Cytotoxicity

Potential cytotoxicity of **14a** and **14b** was determined at a high concentration of 1000 nM, which corresponds to >300-fold of the K_i value of **14a**, and >80-fold of the K_i value of **14b**, by determination of cell proliferation using fluorescence-activated cell sorting (FACS) of the human lung carcinoma cell line A549 (DSMZ, Braunschweig, Germany), which natively expresses CD73. Both fluorescent-labelled compounds showed no difference to control cells without addition of the compounds after incubation for 24h and 48h (Figure S5 and S6).

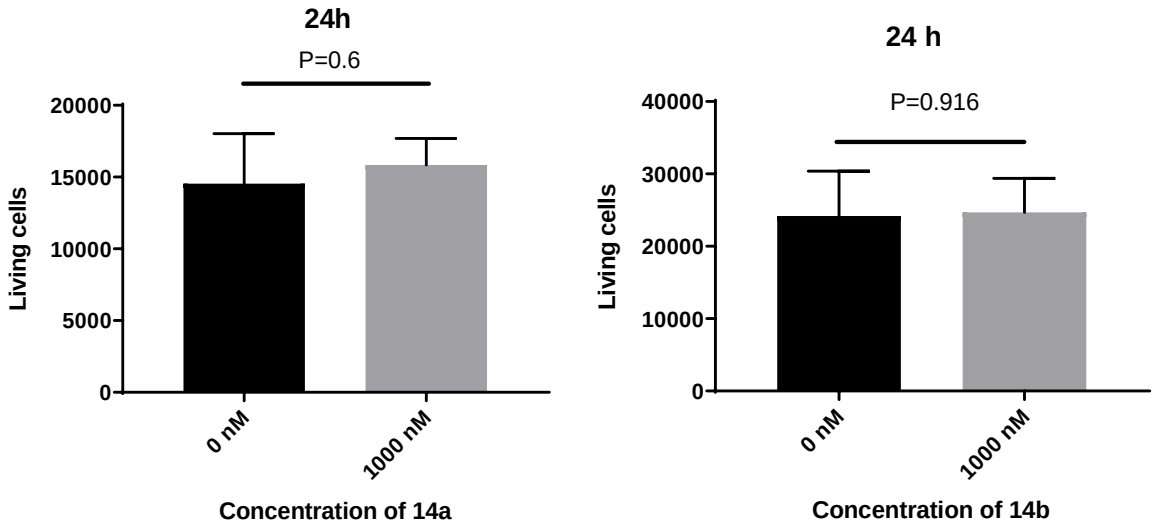


Figure S5. A549 cells were incubated in the absence or presence of 1000 nM of **14a** or **14b** for 24h.

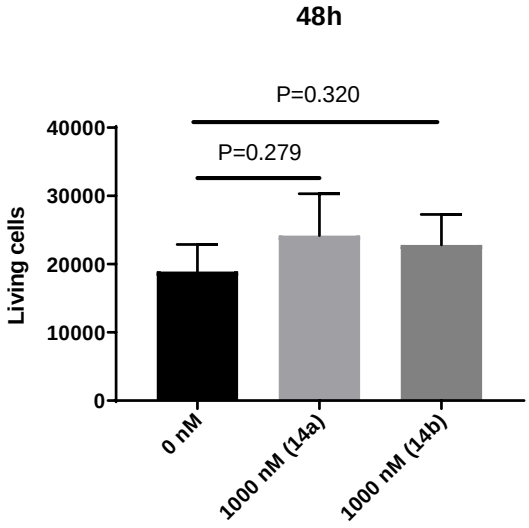


Figure S6. A549 cells were incubated with and without 1000 nM of **14a** or **14b** for 48h.

The effects of **14a** and **14b** on cell viability were furthermore investigated using a cell counting kit-8 assay (CCK8, dojindo, https://www.dojindo.com//). Incubation of lung cancer cells A549 with 1000 nM of **14a** and **14b**, respectively, for 24h did not affect cell viability compared to controls (Figure S7).

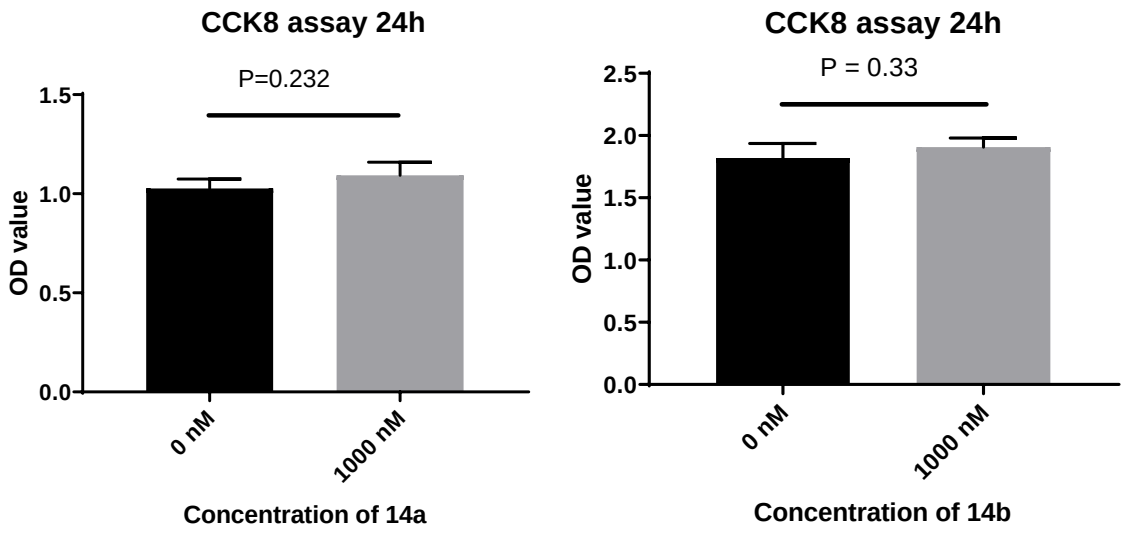


Figure S7. A549 cells were incubated in the absence and in the presence of 1000 nM of **14a** or **14b** for 24h before investigating cell viability using the cell counting kit-8 assay.

In order to confirm these results in an additional cell line, the cytotoxic potential of **14a** and **14b** was evaluated at triple-negative breast cancer cells (MDA-MB-231) which natively express high levels of CD73. After incubating MDA-MB-231 cells for 24h in the absence or presence of increasing concentrations of **14a** and **14b** (100 nM, 1000 nM, 3000 nM and 10000 nM), the number of living cells was determined using fluorescence-activated cell sorting (FACS). Incubation with either compound for 24h did not show any effect on cell number, even at very high concentrations of 10 µM. Thus, both compounds were devoid of cytotoxic effects.

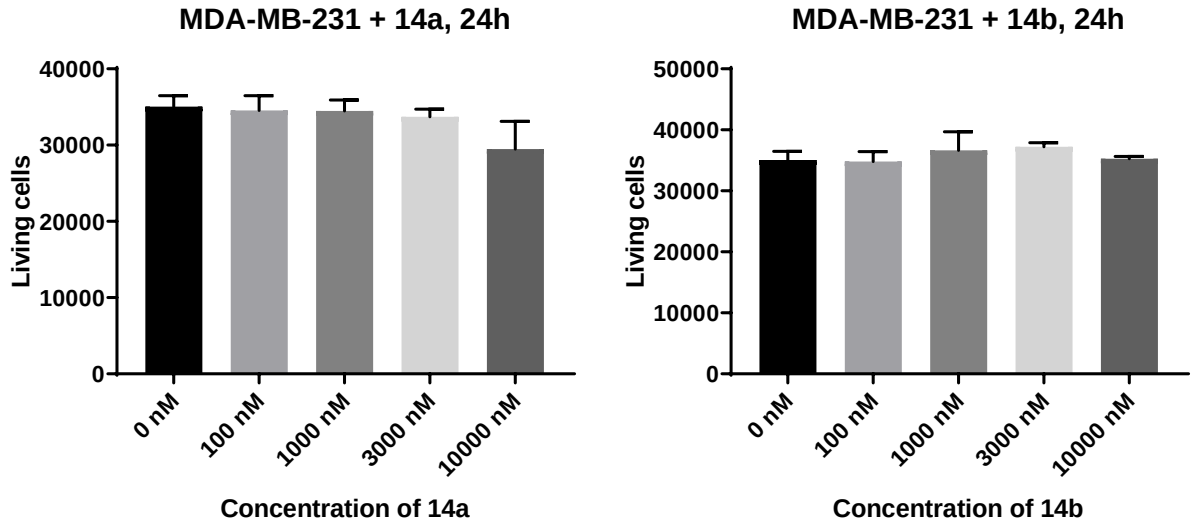


Figure S8. MDA-MB-231 cells were incubated in the absence or presence of 100 nM, 1000 nM, 3000 nM or 10000 nM of **14a** or **14b**.

Experimental Section

General

All reagents were commercially obtained from various producers (Acros, Carbosynth, Fluorochem, Merck, Santa Cruz, Sigma Aldrich, or TCI, respectively) and used without further purification. Commercial solvents of specific reagent grades were used without additional purification or drying. 2,6-Dichloro-9-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)purine was synthesized according to a published procedure.[4] The reactions were monitored by TLC using Merck silica gel 60 F_{254} aluminum sheets and dichloromethane (DCM)/methanol (9:1 or 3:1) as mobile phase. The TLC plates were analyzed by ultraviolet (UV) light irradiation at a wavelength (λ) of 254 nm. Column chromatography was carried out on silica gel 0.040-0.060 mm, pore diameter ca. 6 nm. Mass spectra were recorded on an API 2000 mass spectrometer (Applied Biosystems, Darmstadt, Germany) with a turbo ion spray ion source coupled with an Agilent 1100 HPLC system (Agilent, Böblingen, Germany) using an EC50/2 Nucleodur C18 Gravity 3 μm column (Macherey-Nagel, Düren, Germany), or on a micrOTOF-Q mass spectrometer (Bruker, Köln, Germany) with an ESI-source coupled with an HPLC Dionex Ultimate 3000 (Thermo Scientific, Braunschweig, Germany) using an EC50/2 Nucleodur C18 Gravity 3 μm column (Macherey-Nagel, Düren, Germany). The LC-MS samples were prepared by dissolving 1 mg/ml of compound in H_2O/CH_3OH (1:1) containing 2 mM ammonium acetate. A sample of 10 μL, or 1 μL, respectively, was injected into the HPLC instrument, and elution was performed with a gradient of water/methanol (containing 2 mM ammonium acetate) from 90:10 to 0:100 for 20 min at a flow rate of 250 μL/min, or with a gradient of water/acetonitrile (containing 2 mM ammonium acetate) from 90:10 to 0:100 for 9 min at a flow rate of 0.3 ml/min. UV absorption was detected from 220 to 400 nm using a diode array detector (DAD). NMR spectra were recorded on Bruker Avance 500 MHz and Bruker Avance III HD 600 MHz spectrometers. DMSO-d_6 or D_2O was used as solvent. ^{31}P-NMR spectra were recorded at 25°C; phosphoric acid was used as external standard. For spectra recorded in D_2O, 3-(trimethylsilyl)propionic-2,2,3,3 acid sodium salt-d_4 was used as external standard. When DMSO-d_6 was used as a solvent, spectra were recorded at 30°C. Shifts are given in ppm relative to the external standard (for ^{31}P-NMR spectra) or relative to the remaining protons of the deuterated solvents used as internal standards (^{1}H, ^{13}C-NMR spectra). Coupling constants are given in Hertz (Hz). The designation used to assign the peaks in the spectra is as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br). Melting points were determined on a Büchi 530

melting point apparatus and are uncorrected. Absorption spectra were recorded on a Varian Cary 50 Bio (Agilent Technologies, USA). Absorbance was measured compared to a control without compound from 300 to 800 nm. Stock solutions (10 mM) were prepared in water. Fluorescence spectra were recorded on a FLX (Safas, Monaco) spectrofluorometer. Adjusted band widths were 5 nm for excitation and emission wavelength, and the emission was recorded from 300 to 800 nm. The excitation wavelength was set corresponding to the absorbance maxima of the corresponding compound. Absorption and fluorescence spectra were recorded in H_2O, PBS, and sodium acetate buffer pH 4. The final concentrations were 100 μM and 10 μM for the recording of absorption and emission spectra, respectively.

Purification of nucleotides

Semi-preparative HPLC was performed on a Knauer Smartline 1050 HPLC system equipped with a Eurospher-100 C18 column, 250 mm x 20 mm, particle size 10 μm. The UV absorption was detected at 254 nm. Fractions were collected, and appropriate fractions were pooled, diluted with water, and lyophilized several times, using a CHRIST ALPHA 1-4 LSC freeze dryer, to remove the NH_4HCO_3 buffer, yielding the nucleotides as white powders. The structures of the synthesized nucleosides and nucleotides were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy, in addition to LC/ESI-MS performed in both positive and negative mode. The nucleotides were additionally investigated by ^{31}P-NMR spectroscopy.

4-(((2-Chloro-9-β-D-ribofuranosyl-9*H*-purin-6-yl)amino)methyl)benzoic acid (2)

2,6-Dichloro-9-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)purine (**1**, 1.0 g, 2.2 mmol, 1.0 eq) was suspended in absolute ethanol. To the suspension, triethylamine (0.6 ml. 4.4 mmol, 2.0 eq) and 4-(aminobenzyl)benzoic acid (0.7 g, 4.5 mmol, 2.0 eq) were added and the reaction mixture was refluxed overnight.[5] The solvent was evaporated followed by purification by silica gel column chromatography (CH_3OH/DCM 1:4) yielding the protected intermediate. To the intermediate product in methanol (5 ml), sodium methoxide (0.05 g) was added.[6] After 18h at room temperature, the solution was evaporated and the crude product was purified by silica gel column chromatography (CH_3OH/DCM 1:3 plus some acetic acid) (0.9 g, 100%). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.86 (br s, 1H, N<u>H</u>CH_2) 8.40 (s, 1H, N=C<u>H</u>N) 7.79 (d, 2H, J =7.7 Hz, aryl) 7.23 (d, 2H, J = 7.7 Hz, aryl) 5.81 (d, 1H, J = 5.7 Hz, C<u>H</u>N) 4.64 (br s, 1H, NC<u>H</u>2) 4.48 (br s, 1H, C<u>H</u>OH) 4.13 (br s, 1H, C<u>H</u>OH) 3.93 (br s, 1H, C<u>H</u>CH_2) 3.64 (m overlapping with H_2O, 2H, CHC<u>H</u>$_2$). ^{13}C-

NMR (151 MHz, DMSO-d_6) δ 169.58, 155.15, 153.34, 149.85, 140.23, 140.16, 138.25, 129.26, 126.32, 118.76, 87.77, 85.91, 74.00, 70.49, 61.53, 43.24. LC-MS (m/z): positive mode 436.0 $[M+H]^+$ (calcd. for $C_{18}H_{19}ClN_5O_6$ 436.1). Purity determined by HPLC-UV (254 nm)-ESI-MS: 95.3%. Mp. 219°C.

Preparation of triethylammonium tydrogen carbonate buffer (TEAC)

A 1 M solution of TEAC was prepared by slowly adding dry ice to a 1 M triethylamine solution in water for several hours until a pH of approximately 7.4−7.6 was measured using a pH meter.[5]

4-(((2-Chloro-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(((hydroxy(phosphonomethyl)phosphoryl)-oxy)methyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)methyl)benzoic acid (3)

A solution of methylenebis(phosphonic dichloride) (0.6 g, 2.3 mmol, 5.0 eq) in trimethyl phosphate (7 ml), cooled to 0-4°C was added to a suspension of **2** (0.2 g, 0.46 mmol, 1.0 eq) in trimethyl phosphate (3 ml) at 0-4°C. The reaction mixture was stirred at 0-4°C and samples were withdrawn at 15 min interval for TLC to check the disappearance of nucleosides. After 40 min, on disappearance of nucleoside, cold 0.5M aqueous TEAC solution (pH 7.4-7.6, 20 ml) was added. It was stirred at 0°C for 15 min followed by stirring at room temperature for 1 h. Trimethyl phosphate was extracted using (2 x 100 ml) of *tert*-butylmethylether and the aqueous layer was lyophilized. This procedure was carried out in analogy to previously reported methods.[7, 8] The crude product was then purified by RP-HPLC (0-30% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization to get final product (0.08 g, 29%). ^{1}H-NMR (600 MHz, D_2O) δ 8.45 (s, 1H, N=C<u>H</u>N) 7.81 (d, 2H, *J* = 8.1 Hz, aryl) 7.43 (d, 2H, *J* = 8.1 Hz, aryl) 6.03 (d, 1H, *J* = 5.3 Hz, C<u>H</u>N) 4.73 (t, 1H, *J* = 5.2 Hz, C<u>H</u>OH) 4.53 (t, 1H, *J* = 4.6 Hz, C<u>H</u>OH) 4.36 (d, 1H, *J* = 3.4 Hz, C<u>H</u>CH$_2$) 4.16 (m, 2H, CHC<u>H$_2$</u>) 2.13 (t, 2H, *J* = 19.7 Hz, PC<u>H$_2$</u>P). ^{13}C-NMR (126 MHz, D_2O) δ 178.24, 163.14, 158.04, 157.08, 152.12, 143.98, 142.45, 138.13, 132.03, 129.80, 89.89, 86.71, 77.14, 72.98, 66.25, 46.57, 30.77. ^{31}P-NMR (202 MHz, D_2O) δ 20.28 (s, 1P, P_β) 13.65 (s, 1P, P_α). LC-MS (m/z): positive mode 594.0 $[M+H]^+$ (calcd. for $C_{19}H_{23}ClN_5O_{11}P_2$ 594.1). Purity determined by HPLC-UV (254 nm)-ESI-MS: 100%. Mp. 205°C.

***N*-(6-Aminohexyl)-3′,6′-dihydroxy-3-oxo-3*H*-spiro-[isobenzofuran-1,9′-xanthene]-5(6)-carboxamide (6a)**

To 5(6)-carboxyfluorescein (**5**, 0.5 g, 1. mmol, 1.0 eq) in anhydrous THF (10 ml), HOBt (0.18 g,

1.3 mmol, 1.0 eq) and DCC (0.27 g, 1.3 mmol, 1.0 eq) were added. After 20 min of activation, *N*,*N*-Boc-1,6-hexanediamine (**4a**, 0.33 g, 1.3 mmol, 1 eq) was added and the reaction was stirred overnight at rt.[9] DCU was filtered off and the filtrate was evaporated. The crude product was purified by column chromatography (CH_3OH/DCM 1:9). LC-MS (m/z): positive mode 575.5 $[M+H]^+$. Purity determined by HPLC-UV (254 nm)-ESI-MS: 93.9%. The intermediate was taken up in DCM (10 ml) and TFA (0.3 ml) and a drop of water was added. The reaction mixture was stirred at rt for 2 h followed by evaporation. ^{1}H-NMR (600 MHz, DMSO-d^6) δ 8.80+8.65 (2x t, 1H, *J* = 5.5 Hz, C=C<u>H</u>) 8.44+7.65 (2x s, 1H, C<u>H</u>=CCO or C<u>H</u>=CH) 8.22+8.15 (2x d, 1H, *J* = 8.1 Hz, C<u>H</u>=CCO) 7.68 (br s, 2H, N<u>H</u>$_2$) 6.69 (dd, 2H, *J* = 2.2, 4.7 Hz, 2x C=C<u>H</u>) 6.56 (m, 4H, 4x CHCO<u>H</u>) 3.31+3.19 (2x q, 2H, *J* = 6.7 Hz, NHC<u>H</u>$_2$) 2.76 (m, 2H, NH$_2$C<u>H</u>$_2$) 1.55 (q, 2H, *J* = 7.0 Hz, C<u>H</u>$_2$) 1.46 (m, 2H, C<u>H</u>$_2$) 1.35 (m, 2H, C<u>H</u>$_2$) 1.26 (m, 2H, C<u>H</u>$_2$). ^{13}C-NMR (151 MHz, DMSO-d$_6$) δ 168.39, 164.73, 159.83, 158.56, 158.22, 152.01, 154.78, 152.90, 140.98, 136.59, 134.81, 129.40, 126.63, 124.40, 123.38, 118.92, 116.98, 115.04, 112.94, 109.34, 102.48, 83.56, 55.22, 48.76, 39.46, 38.99, 28.99, 27.16, 26.15, 25.70. LC-MS (m/z): positive mode 475.5 $[M+H]^+$ (calcd. for $C_{27}H_{27}N_2O_6$ 475.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 95.3%. Mp. 114°C.

_N_-(2-(2-(2-Aminoethoxy)ethoxy)ethyl)-3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamide (6b)

To 5(6)-carboxyfluorescein (**5**, 0.5 g, 1.3 mmol, 1.0 eq) in anhydrous THF (10 ml), HOBt (0.18 g, 1.3 mmol, 1.0 eq) and DCC (0.27 g, 1.3 mmol, 1.0 eq) were added. After 20 min of activation, *N*-Boc-2,2′-(ethylenedioxy)-diethylamine (**4b**, 0.3 ml, 1.3 mmol, 1 eq) was added and the reaction was stirred overnight at rt.[9] DCU was filtered off and the filtrate was evaporated. The crude product was purified by column chromatography (CH_3OH/DCM 1:9). LC-MS (m/z): positive mode 607.1 $[M+H]^+$. Purity determined by HPLC-UV (254 nm)-ESI-MS: 92%. The intermediate was taken up in DCM (10 ml) and TFA (0.8 ml) and a drop of water were added. The reaction mixture was stirred at rt overnight followed by evaporation. ^{1}H-NMR (600 MHz, DMSO-d$_6$) δ 10.20 (s, 2H, 2x O<u>H</u>) 8.87+8.73 (t, 1H, *J* = 5.6 Hz, C=C<u>H</u>) 8.43+7.66 (s, 1H, N<u>H</u>CH$_2$) 8.22+8.15 (dd, 1H, *J* = 1.4, 8.0 Hz, C<u>H</u>=CO) 8.07+7.36 (d, 1H, *J* = 8.0 Hz, C<u>H</u>=CCO or C<u>H</u>=CH) 7.76 (br s, 2H, N<u>H</u>$_2$) 6.69 (dd, 2H, *J* = 2.3, 4.5 Hz, 2x C=C<u>H</u>) 6.56 (m, 4H, 4x CH=CO<u>H</u>) 3.58 (m, 4H, 2x C<u>H</u>$_2$O) 3.50 (m, 6H, 2x C<u>H</u>$_2$O+NH$_2$C<u>H</u>$_2$) 2.97+2.91 (m, 2H, NHC<u>H</u>$_2$). ^{13}C-NMR (151 MHz, DMSO-d$_6$) δ 168.39, 165.06, 159.83, 158.24, 154.93, 152.98, 152.02, 140.71, 136.37, 134.85, 129.57, 129.42, 126.67, 125.12, 124.47, 123.49, 122.43, 112.96, 109.32, 102.51, 83.51, 69.90, 69.63, 68.95, 66.88, 39.42,

38.85. LC-MS (m/z): positive mode 507.3 $[M+H]^+$ (calcd. for $C_{27}H_{27}N_2O_8$ 507.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 98.9%. Mp. 143°C.

(((((2*R*,3*S*,4*R*,5*R*)-5-(2-Chloro-6-((4-((6-(3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamido)hexyl)carbamoyl)benzyl)amino)-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)methyl)phosphonic acid (7a)

Method A[9]*:* To **3** (0.04 g, 0.07 mmol, 1.0 eq) in THF (1 ml), HOBt (9 mg, 0.07 mmol, 1.0 eq) and DCC (14 mg, 0.07 mmol, 1.0 eq) were added. After 20 min of activation, **6a** (0.03 g, 0.7 mmol, 1.0 eq) was added and the reaction was stirred overnight at rt. DCU was filtered off and the filtrate was evaporated. LC-MS analysis, however, showed that the desired reaction did not occur.

Method B[7]*:* A solution of methylenebis(phosphonic dichloride) (0.14 g, 0.6 mmol, 5.0 eq) in trimethyl phosphate (5 ml), cooled to 0-4°C was added to a suspension **8a**) (0.1 g, 0.1 mmol, 1.0 eq) in trimethyl phosphate (3 ml) at 0-4°C. The reaction mixture was stirred at 0-4°C and samples were withdrawn at 15 min interval for TLC to check the disappearance of nucleosides. After 30 min, on disappearance of nucleoside, 10 ml of cold 0.5M aqueous TEAC solution (pH 7.4-7.6) was added. It was stirred at 0-4°C for 15 min followed by stirring at room temperature for 1 h.[7] Trimethyl phosphate was extracted using (2 x 250 ml) of *tert*-butylmethylether and the aqueous layer was lyophilized. The crude product was then purified by RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization. Unfortunately, only starting material was isolated as indicated by LC-MS analysis.

Method C[9]*:* To **5** (0.013 g, 0.03 mmol, 1.0 eq) in THF (2 ml), HOBt (0.004 g, 0.03 mmol, 1.0 eq) and DCC (0.007 g, 0.03 mmol, 1.0 eq) were added. After 10 min of pre-activation (solution became clear), **10a** (0.02 g, 0.03 mmol, 1.0 eq) in water (2 ml) was added. The reaction was stirred at rt overnight followed by evaporation and lyophilization. The crude product was purified by preparative RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min). Fractions were collected and appropriate fractions pooled and lyophilized. LC/ESI-MS analysis, however, revealed, that the desired product was not formed.

(((((2*R*,3*S*,4*R*,5*R*)-5-(2-Chloro-6-((4-((2-(2-(2-(3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamido)ethoxy)ethoxy)ethyl)carbamoyl)benzyl)amino)-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)methyl)-phosphonic acid (7b)

Method A[9]*:* To **3** (0.04 g, 0.07 mmol, 1.0 eq) in THF (1 ml), HOBt (9 mg, 0.07 mmol, 1.0 eq) and DCC (14 mg, 0.07 mmol, 1.0 eq) were added. After 20 min of activation, **6b** (0.03 g, 0.7 mmol, 1.0 eq) was added and the reaction was stirred overnight at rt. DCU was filtered off and the filtrate was evaporated. LC/ESI-MS analysis, however, showed that the desired reaction did not occur.

Method B[7]*:* A solution of methylenebis(phosphonic dichloride) (0.18 g, 0.7 mmol, 5.0 eq) in trimethyl phosphate (5 ml), cooled to 0-4°C was added to a suspension **8b** (0.14 g, 0.15 mmol, 1.0 eq) in trimethyl phosphate (3 ml) at 0-4°C. The reaction mixture was stirred at 0-4°C and samples were withdrawn at 15 min interval for TLC to check the disappearance of nucleosides. After 30 min, on disappearance of nucleoside, 10 ml of cold 0.5M aqueous TEAC solution (pH 7.4-7.6) was added. It was stirred at 0-4°C for 15 min followed by stirring at room temperature for 1 h. Trimethyl phosphate was extracted using (2 x 250 ml) of *tert*-butylmethylether and the aqueous layer was lyophilized. The crude product was then purified by RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization. Unfortunately, only starting material was isolated as indicated by LC/ESI-MS analysis.

Method C[9]*:* To **5** (0.03 g, 0.08 mmol, 1.0 eq) in THF (2 ml), HOBt (0.011 g, 0.08 mmol, 1.0 eq) and DCC (0.016 g, 0.08 mmol, 1.0 eq) were added. After 10 min of pre-activation (solution became clear), **10b** (0.06 g, 0.08 mmol, 1.0 eq) in water (2 ml) was added. The reaction was stirred at rt overnight followed by evaporation and lyophilization. The crude product was purified by preparative RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min). Fractions were collected and appropriate fractions pooled and lyophilized. LC/ESI-MS analysis, however, revealed, that the desired product was not formed.

_N_-(6-(4-(((2-Chloro-9-((2_R_,3_R_,4_S_,5_R_)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9_H_-purin-6-yl)amino)methyl)benzamido)hexyl)-3′,6′-dihydroxy-3-oxo-3_H_-spiro[iso-benzofuran-1,9′-xanthene]-5(6)-carboxamide (8a)

To **2** (0.16 g, 0.36 mmol, 1.0 eq) in THF (2 ml), HOBt (0.05 g, 0.36 mmol, 1.0 eq) and DCC (0.07 g, 0.36 mmol, 1.0 eq) were added. After 20 min of activation, **6a** (0.17 g, 0.36 mmol, 1.0 eq) was added and the reaction was stirred overnight at rt.[9] DCU was filtered off and the filtrate was evaporated. The crude product was purified twice by preparative RP-HPLC (20-100% MeOH in H_2O in 30 min, 20 ml/min) followed by lyophilization, yielding the desired product as yellow solid (0.1 g, 30%). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.99+6.62 (m, 1H, C<u>H</u>=CCO) 8.94+8.76 (d, 1H, J = 5.6 Hz, C=C<u>H</u>) 8.43 8.41 (s, 1H, N<u>H</u>CH_2) 8.20+7.76 (d, 1H, J = 8.1 Hz, C<u>H</u>=CO or C<u>H</u>=CCO)

7.65 (s, 1H, NHC<u>H</u>$_2$) 7.36 (m, 4H, 4x CHCO<u>H</u>) 6.66 (br s, 2H, 2x C–=C<u>H</u>) 6.55 (m, 4H, aryl) 5.82 (d, 1H, *J* = 5.8 Hz, C<u>H</u>N) 5.56 (d, 2H, *J* = 7.9 Hz, NHC<u>H</u>$_2$-aryl) 5.45 (d, 1H, *J* = 6.9 Hz, O<u>H</u>) 5.18 (d, 1H, *J* = 3.7 Hz, O<u>H</u>) 5.03 (br s, 1H, O<u>H</u>) 4.68 (br s, 1H, O<u>H</u>) 4.51 (br s, 1H, O<u>H</u>) 4.43 (d, 1H, *J* = 5.9 Hz, C<u>H</u>CH$_2$) 4.12 (br s, 1H, C<u>H</u>OH), 3.93 (br s, 1H, C<u>H</u>OH) 3.65-3.54 (d m, 2H, CHC<u>H</u>$_2$) 3.21 (m, 2H, NHC<u>H</u>$_2$) 1.49 (m, 4H, (C<u>H</u>$_2$)$_2$) 1.35 (m, 2H, C<u>H</u>$_2$) 1.13 (m, 2H, C<u>H</u>$_2$). ^{13}C-NMR (151 MHz, DMSO-d$_6$) δ 168.42, 168.27, 166.19, 166.16, 164.78, 164.59, 160.08, 155.15, 154.42, 153.32, 152.14, 149.91, 142.38, 140.90, 140.36, 136.61, 134.73, 133.59, 129.52, 129.45, 129.37, 127.40, 127.17, 126.92, 125.16, 124.48, 123.52, 122.51, 118.82, 113.14, 113.05, 109.48, 109.40, 102.49, 87.67, 85.93, 73.91, 70.56, 69.98, 61.54, 54.29, 54.25, 45.88, 43.18, 29.32, 29.22, 29.14, 29.05, 26.42, 26.34, 11.31. LC/ESI-MS (m/z): positive mode 892.6 $[M+H]^+$ (calcd. for $C_{45}H_{43}ClN_7O_{11}$ 892.3). Purity determined by HPLC-UV (254 nm)-ESI-MS: 83%. Mp. 201°C.

***N*-(2-(2-(2-(4-(((2-Chloro-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-9*H*-purin-6-yl)amino)methyl)benzamido)ethoxy)ethoxy)ethyl)-3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamide (8b)**

To **2** (0.15 g, 0.35 mmol, 1.0 eq) in THF (2 ml), HOBt (0.05 g, 0.35 mmol, 1.0 eq) and DCC (0.07 g, 0.35 mmol, 1.0 eq) were added. After 20 min of activation, **6b** (0.18 g, 0.35 mmol, 1.0 eq) was added and the reaction was stirred overnight at rt.[9] DCU was filtered off and the filtrate was evaporated. The crude product was purified twice by preparative HPLC (20-100% MeOH in H_2O in 30 min, 20 ml/min) followed by lyophilization, yielding the desired product as yellow solid (0.14 g, 42%). ^{1}H-NMR (600 MHz, DMSO-d$_6$) δ 8.92 (t, 1H, *J* = 6.2 Hz, N<u>H</u>CH$_2$) 8.85 (t, 1H, *J* = 5.5 Hz, N<u>H</u>CH$_2$) 8.72 (t, 1H, *J* = 5.6 Hz, N<u>H</u>CH$_2$) 8.44+7.67 (s, 1H, C*H*=CCO or C<u>H</u>=CH) 8.41 (s, 1H, N=C<u>H</u>N) 8.22+8.14 (d, 1H, *J* = 9.2 Hz, C=C<u>H</u>) 8.05+7.35 (d, 1H, *J* = 8.0 Hz, C<u>H</u>=CO) 7.76 (t, 2H, *J* = 8.6 Hz, aryl) 7.38 (d, 2H, *J* = 6.2 Hz, aryl) 6.67 (m, 2H, 2x C=CH) 6.55
(m, 4H, 4x CH=CO<u>H</u>) 5.82 (d, 1H, *J* = 5.7 Hz, C<u>H</u>N) 5.46 (br s, 1H, O<u>H</u>) 5.19 (br s, 1H, O<u>H</u>) 5.04 (br s, 1H, O<u>H</u>) 4.67 (br s, 1H, C<u>H</u>OH) 4.51 (br s, 1H, C<u>H</u>OH) 4.11 (br s, 1H, O<u>H</u>) 3.93 (br s, 1H, O<u>H</u>) 3.64 (d, 1H, *J* = 11.5 Hz, C<u>H</u>CH2) 3.55-3.45 (m, overlapping with H_2O, 16H, CHC<u>H</u>$_2$ + NHC<u>H</u>$_2$ + 6x C<u>H</u>$_2$O). ^{13}C-NMR (151 MHz, DMSO-d$_6$) δ 168.37, 168.25, 166.37, 165.00, 164.85, 160.09, 155.15, 153.31, 152.13, 149.91, 142.55, 140.61, 140.36, 136.34, 134.73, 133.26, 129.56, 129.46, 129.38, 127.44, 127.19, 126.97, 125.18, 124.52, 123.62, 122.60, 118.83, 113.12, 113.05, 109.46, 109.39, 102.49, 102.47, 87.67, 85.93, 73.91, 70.56, 69.77, 69.68, 69.12, 69.05, 68.94,

68.82, 61.54, 43.18. LC/ESI-MS (m/z): positive mode 924.8 $[M+H]^+$ (calcd. for $C_{45}H_{43}ClN_7O_{13}$ 924.3). Purity determined by HPLC-UV (254 nm)-ESI-MS: 90%. Mp. 188°C.

_N_-(6-Aminohexyl)-4-(((2-chloro-9-((2_R_,3_R_,4_S_,5_R_)-3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-9_H_-purin-6-yl)amino)methyl)benzamide (9a)

To a solution of 2′,3′,5′-tri-*O*-acetyl protected **2** (0.6 g, 1.1 mmol, 1.0 eq) in THF (10ml), HOBt (0.15 g, 1.1 mmol, 1.0 eq) and DCC (0.23 g, 1.1 mmol, 1.0 eq) were added. After 20 min of pre-activation, *N*-Boc-1,6-hexanediamine (0.23 g, 1.1 mmol, 1.0 eq) was added and the reaction was stirred overnight at rt.[9] DCU was filtered off followed by evaporation of the filtrate. Next, the crude product was taken up in 0.5% sodium methoxide in methanol (10 ml) and the reaction was stirred at rt overnight followed by evaporation.[6] The crude product was purified by column chromatography (10% MeOH/DCM) yielding the desired intermediate as yellow oil. LC/ESI-MS (m/z): positive mode 666.7 $[M+H]^+$. Purity determined by HPLC-UV (254 nm)-ESI-MS: 92.4%. To remove the Boc group, the intermediate was taken up in 6% TFA in DCM and a drop of water was added. The reaction was stirred at rt for 24h followed by evaporation. The crude product was purified by RP-HPLC (50-100% MeOH/H_2O in 15 min, 20 ml/min) followed by lyophilization to get the desired product as white solid (0.5 g, 88%). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.41 (s, 1H, N=C<u>H</u>N) 8.36 (s, 2H, N<u>H</u>$_2$) 8.01 (s, 1H, N<u>H</u>CH_2) 7.90 (s, 1H, N<u>H</u>) 7.75 (t, 2H, J = 6.48 Hz, aryl) 7.39 (t, 2H, J = 7.84 Hz, aryl) 5.82 (d, 1H, J = 5.79 Hz, C<u>H</u>N) 4.68 (br s, 2H, 2x CHO<u>H</u>) 4.51 (s, 1H, CH_2O<u>H</u>) 4.12 (s, 1H, C<u>H</u>OH) 3.93 (s, 1H, C<u>H</u>OH) 3.75 (s, 1H, C<u>H</u>CH_2) 3.54 (overlapping with H_2O peak: C<u>H</u>$_2$OH) 3.21 (m, 2H, NH_2C<u>H</u>$_2$) 2.88 (br s, 2H, NHC$_{H2}$) 2.58 (br s, 2H, $NHCH_2$) 1.48 (br s, 2H, C<u>H</u>$_2$) 1.37 (br s, 2H, C<u>H</u>$_2$) 1.27 (br s, 4H, (C<u>H</u>$_2$)$_2$). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 166.19, 161.93, 158.17, 155.16, 153.33, 152.14, 149.93, 142.89, 140.38, 133.49, 127.35, 118.84, 87.84, 85.96, 72.94, 70.57, 61.56, 53.39, 61.56, 53.99, 42.20, 30.90, 29.91, 29.27, 26.43, 26.10. LC/ESI-MS (m/z): positive mode 534.4 $[M+H]^+$ (calcd. for $C_{24}H_{33}ClN_7O_5$ 534.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 98%. Mp. 116°C.

_N_-(2-(2-(2-Aminoethoxy)ethoxy)ethyl)-4-(((2-chloro-9-((2_R_,3_R_,4_S_,5_R_)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9_H_-purin-6-yl)amino)methyl)benzamide (9b)

To a solution of 2′,3′,5′-tri-*O*-acetyl protected **2** (0.6 g, 1.1 mmol, 1.0 eq) in THF (10ml), HOBt (0.15 g, 1.1 mmol, 1.0 eq) and DCC (0.23 g, 1.1 mmol, 1.0 eq) were added. After 20 min of pre-activation, *N*-Boc-2,2′-(ethylenedioxy)-diethylamine (0.26 ml, 1.1 mmol, 1.0 eq) was added and

the reaction was stirred overnight at rt.[9] DCU was filtered off followed by evaporation of the filtrate. Next, the crude product was taken up in 0.5% sodium methoxide in methanol (10 ml) and the reaction was stirred at rt overnight followed by evaporation.[6] The crude product was purified by column chromatography (10% MeOH/DCM) yielding the desired intermediate as yellow oil. LC/ESI-MS (m/z): positive mode 666.7 $[M+H]^+$. Purity determined by HPLC-UV (254 nm)-ESIMS: 92.4%. To remove the Boc group, the intermediate was taken up in 6% TFA in DCM and a drop of water was added. The reaction was stirred at rt for 24h followed by evaporation. The crude product was purified by RP-HPLC (50-100% MeOH/H_2O in 15 min, 20 ml/min) followed by lyophilization to get the desired product as white solid (0.27 g, 43%). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.92 (t, 1H, J = 6.22 Hz, N<u>H</u>CH_2) 8.42 (d, 1H, J = 5.82 Hz, N<u>H</u>CH_2) 8.41 (s, 1H, N=C<u>H</u>N) 7.76 (d, J = 8.11 Hz , 4H, aryl) 7.39 (d, 2H, J = 8.10 Hz, CH_2N<u>H</u>$_2$) 5.82 (d, 1H, J = 5.92 Hz, C<u>H</u>N) 4.68 (d, 2H, J = 5.61 Hz, 2x CHO<u>H</u>) 4.51 (t, 1H, J = 5.22 Hz, CH_2O<u>H</u>) 4.12 (m, 1H, C<u>H</u>OH) 3.93 (m, 1H, C<u>H</u>OH) 3.65 (m, 1H, C<u>H</u>CH_2) 2.55 (m, 14H, 6x C<u>H</u>$_2$ and overlapping C<u>H</u>$_2$OH) 2.93 (p, 2H, J = 5.60 Hz, NHC<u>H</u>$_2$-aryl). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 166.44, 158.23, 157.98, 155.14, 153.30, 149.91, 142.62, 140.36, 133.22, 127.42, 127.19, 118.82, 94.74, 87.64, 85.96, 73.90, 70.56, 69.89, 69.92, 69.09, 66.83, 61.55, 43.18, 39.21, 38.86. LC/ESI-MS (m/z): positive mode 566.4 $[M+H]^+$ (calcd. for $C_{24}H_{33}ClN_7O_7$ 566.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 97.8%. Mp. 81°C.

(((((2*R*,3*S*,4*R*,5*R*)-5-(6-((4-((6-Aminohexyl)-carbamoyl)benzyl)amino)-2-chloro-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)methyl)phosphonic acid (10a)

A solution of methylenebis(phosphonic dichloride) (0.22 g, 0.9 mmol, 5.0 eq) in trimethyl phosphate (5 ml), cooled to 0-4°C was added to a suspension of **9a** (0.1 g, 0.18 mmol, 1.0 eq) in trimethyl phosphate (3 ml) at 0-4°C. The reaction mixture was stirred at 0-4°C and samples were withdrawn at 15 min interval for TLC to check the disappearance of nucleosides. After 30 min, on disappearance of nucleoside, 10 ml of cold 0.5 M aqueous TEAC solution (pH 7.4-7.6) was added. It was stirred at 0° C for 15 min followed by stirring at room temperature for 1h.[7] Trimethyl phosphate was extracted using (2 x 250 ml) of *tert*-butylmethylether and the aqueous layer was lyophilized. The crude product was then purified by RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization yielding a white solid (0.02 g, 16%). ^{1}H-NMR (600 MHz, D_2O) δ 8.44 (s, 1H, N=C<u>H</u>N) 7.57 (d, 2H, J = 7.95 Hz, aryl) 7.34 (d, 2H, J = 7.91

Hz, aryl) 5.96 (d, 1H, *J* = 5.37 Hz, CHN) 4.70 (t, 1H, *J* = 5.29 Hz, CHOH) 4.52 (t, 1H, *J* = 4.55 Hz, CHOH) 4.36 (m, 1H, CHCH$_2$) 4.16 (m, 2H, CHCH$_2$) 3.83 (d, 2H, *J* = 1.44 Hz, NH$_2$CH$_2$) 3.81 (d, 2H, *J* = 1.27 Hz, NHCH$_2$) 3.27 (t, 2H, *J* = 6.86 Hz, NHCH$_2$-aryl) 2.91 (m, 2H, CH$_2$) 2.18 (t, 2H, *J* = 19.18 Hz, PCH$_2$P) 1.57 (m, 2H, CH$_2$) 1.49 (m, 2H, CH$_2$) 1.28 (m, 2H, CH$_2$). ^{13}C-NMR (126 MHz, D$_2$O) δ 172.71, 164.44, 157.77, 157.02, 152.06, 144.83, 142.48, 135.37, 130.31, 130.01, 120.88, 89.83, 86.79, 77.21, 73.07, 72.45, 66.42, 57.90, 57.53, 55.63, 46.37, 42.55, 42.20, 31.04, 29.63, 29.49, 28.41, 28.12. ^{31}P-NMR (202 MHz, D$_2$O) δ 18.92 (d, 1P, *J* = 8.13 Hz, P$_\beta$) 14.76 (d, 1P, *J* =9.32 Hz, P$_\alpha$). LC/ESI-MS (m/z): positive mode 692.5 [M+H]$^+$ (calcd. for $C_{25}H_{37}ClN_7O_{10}P_2$ 692.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 93%. Mp. 202°C.

(((((2*R*,3*S*,4*R*,5*R*)-5-(6-((4-((2-(2-(2-Aminoethoxy)ethoxy)ethyl)carbamoyl)benzyl)amino)-2-chloro-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)-methyl)phosphonic acid (10b)

A solution of methylenebis(phosphonic dichloride) (0.22 g, 0.9 mmol, 5.0 eq) in trimethyl phosphate (5 ml), cooled to 0-4°C was added to a suspension **9b** (0.1 g, 0.17 mmol, 1.0 eq) in trimethyl phosphate (3 ml) at 0-4°C. The reaction mixture was stirred at 0-4°C and samples were withdrawn at 15 min interval for TLC to check the disappearance of nucleosides. After 30 min, on disappearance of nucleoside, 10 ml of cold 0.5M aqueous TEAC solution (pH 7.4-7.6) was added. It was stirred at 0°C for 15 min followed by stirring at room temperature for 1h.[7] Trimethyl phosphate was extracted using (2x 250 ml) of *tert*-butylmethylether and the aqueous layer was lyophilized. The crude product was then purified by RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization yielding a white solid (0.06 g, 48%). ^{1}H-NMR (600 MHz, D$_2$O) δ 8.45 (s, 1H, N=CHN) 7.64 (d, 2H, *J* = 7.74 Hz, aryl) 7.38 (d, 2H, *J* = 7.88 Hz, aryl) 5.98 (d, 1H, *J* = 5.23 Hz, CHN) 4.72 (br s, 2H, CH$_2$NH$_2$) 4.62 (br s, 1H, CHOH) 4.53 (t, 1H, *J* = 4.48 Hz, CHOH) 4.36 (m, 1H, CHCH$_2$) 4.17 (m, 2H, CHCH$_2$) 3.70 (m, 4H, 2xCH$_2$O) 3.66 (q, 4H, *J* = 6.01 Hz, O(CH$_2$)$_2$) 3.56 (t, 2H, *J* = 5.19 Hz, NHCH$_2$-aryl) 3.08 (m, 2H, CH$_2$) 2.15 (m, 2H, PCH$_2$P). ^{13}C-NMR (126 MHz, D$_2$O) δ 173.07, 157.79, 156.98, 152.05, 145.06, 142.52, 135.14, 130.31, 130.15, 120.92, 89.90, 86.76, 77.21, 73.01, 72.40, 71.77, 69.32, 66.37, 57.90, 46.34, 42.30, 41.84, 30.63. ^{31}P-NMR (202 MHz, D$_2$O) δ 19.58 (d, 1P, *J* = 8.58 Hz, P$_\beta$) 14.17 (d, 1P, *J* =8.44 Hz P$_\alpha$). LC/ESI-MS (m/z): positive mode 724.5 [M+H]$^+$ (calcd. for $C_{25}H_{37}ClN_7O_{12}P_2$ 724.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 94%. Mp. 195°C.

***N*-(6-(4-(Aminomethyl)benzamido)hexyl)-3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamide (11a)**

To 4-(Boc-aminomethyl)benzoic acid (0.2 g, 0.63 mmol, 1.0 eq) in THF (5 ml), HOBt (0.09 g, 0.63 mmol, 1.0 eq) and DCC (0.13 g, 0.63 mmol, 1.0 eq) were added. After 20 min of pre-activation, **6a** (0.3 g, 0.63 mmol, 1.0 eq) was added. The reaction was stirred at rt overnight.[9] DCU was filtered off and the filtrate was evaporated. Deprotection was achieved by treatment 6-8% TFA in DCM and drop of water for 3h at rt.[9] The mixture was evaporated and purified by RP-HPLC (20-100% methanol/water in 20 min, 20 ml/min) and lyophilization yielded the desired product (0.04 g, 6%). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.45+7.67 (br s, 1H, C<u>H</u>=CCO or C<u>H</u>=CH) 8.19+8.14 (dd, 1H, *J* = 1.4, 8.1 Hz, C=C<u>H</u>) 8.10+7.34 (d, 1H, *J* = 8.0 Hz, C<u>H</u>=CCO) 7.91 (dd, 2H, *J* = 8.3, 24.9 Hz, aryl) 7.56 (dd, 2H, *J* = 8.3, 13.8 Hz, aryl) 6.73 (m, 4H, 4x CHCO<u>H</u>) 6.60 (m, 2H, 2x C=C<u>H</u>) 4.20 (br s, 2H, NHC<u>H</u>$_2$-aryl) 3.47 (dt, 2H, *J* = 7.0, 27.5 Hz, NHC<u>H</u>$_2$) 3.38 (m, 2H, overlapping with H_2O, NH_2C<u>H</u>$_2$) 1.72 (m, 2H, C<u>H</u>$_2$) 1.63 (m, 2H, C<u>H</u>$_2$) 1.53 (m, 2H, C<u>H</u>$_2$) 1.44 (m, 2H, C<u>H</u>$_2$). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 171.39, 169.65, 169.54, 168.84, 168.58, 138.24, 138.12, 136.85, 136.78, 131.13, 130.96, 130.32, 130.28, 129.36, 129.32, 103.99, 44.19, 41.24, 41.18, 41.10, 30.65, 30.62, 30.42, 29.10, 27.90, 27.83. LC/ESI-MS (m/z): positive mode 608.2 $[M+H]^+$ (calcd. for $C_{35}H_{34}N_3O_7$ 608.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 94.5%. Mp. 215°C.

***N*-(2-(2-(2-(4-(Aminomethyl)benzamido)ethoxy)ethoxy)ethyl)-3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamide (11b)**

To 4-(Boc-aminomethyl)benzoic acid (0.2 g, 0.63 mmol, 1.0 eq) in THF (5 ml), HOBt (0.09 g, 0.63 mmol, 1.0 eq) and DCC (0.13 g, 0.63 mmol, 1.0 eq) were added. After.20 min of pre-activation, **6b** (0.3 g, 0.63 mmol, 1.0 eq) was added. The reaction was stirred at rt overnight.[9] DCU was filtered off and the filtrate was evaporated. The crude product was purified by column chromatography (CH_3OH/DCM 1:9->1:4). The desired product was obtained as pure isomer (0.067 g, 14%) and as isomer mixture (0.197 g, 42%). Deprotection was achieved by treatment 6-8% TFA in DCM and drop of water for 3h at rt. The mixture was evaporated and purified by RP-HPLC (20-100% methanol/water in 20 min, 20 ml/min). Lyophilization yielded the desired product (pure isomer: 0.033 g, 8%; isomer mix: 0.078 g, 19%). Analysis of the pure isomer: ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.72 (t, 1H, *J* = 5.59 Hz, NH) 8.48 (t, 1H, *J* = 5.57 Hz, N<u>H</u>) 8.20 (br s, 2H, NH_2) 8.15 (dd, 1H, *J* = 1.39, 8.05 Hz, C=C<u>H</u>) 8.05 (d, 1H, *J* =8.58 Hz, C<u>H</u>=CO) 7.86 (d, 2H, *J* = 8.40 Hz,

aryl) 7.67 (s, 1H, CH=CO) 7.51 (d, 2H, *J* = 8.40 Hz, aryl) 6.69 (d, 2H, *J* = 2.23 Hz, 2x C=CH) 6.56 (m, 4H, 4x CH=COH) 4.08 (d, 2H, *J* = 5.83 Hz, NHCH$_2$-aryl) 3.48 (m, 8H, 4x CH$_2$O) 3.34 (dd, 4H, *J* = 5.89, 11.82 Hz, 2x NHCH$_2$). ^{13}C-NMR (151 MHz, DMSO-d$_6$) δ 168.17, 165.88, 164.76, 159.77, 158.45, 158.16, 152.82, 151.99, 140.70, 137.03, 134.54, 129.52, 129.34, 128.73, 128.36, 127.56, 125.00, 122.42, 112.90, 109.32, 102.41, 69.64, 68.97, 68.76,
42.09. LC/ESI-MS (m/z): positive mode 640.3 $[M+H]^+$ (calcd. for $C_{35}H_{34}N_3O_9$ 640.2). Purity determined by HPLC-UV (254 nm)-ESI-MS: 99.5%. Mp. 86°C.

(((((2*R*,3*S*,4*R*,5*R*)-5-(6-Chloro-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)methyl)phosphonic acid (13)

A solution of methylenebis(phosphonic dichloride) (0.87 g, 3.5 mmol, 5.0 eq) in trimethyl phosphate (5 ml), cooled to 0-4°C was added to a suspension 6-chloropurine riboside (0.2 g, 0.7 mmol, 1.0 eq) in trimethyl phosphate (5 ml) at 0-4°C. The reaction mixture was stirred at 0-4°C and samples were withdrawn at 15 min interval for TLC to check the disappearance of nucleosides. After 30 min, on disappearance of nucleoside, 20 ml of cold 0.5M aqueous TEAC solution (pH 7.4-7.6) was added. It was stirred at 0°C for 15 min followed by stirring at room temperature for 1h.[7] Trimethyl phosphate was extracted using (2x250ml) of *tert*-butylmethylether and the aqueous layer was lyophilized. The crude product was then purified by RP-HPLC (0-30% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization to get final product as white solid (0.17 g, 55%). ^{1}H-NMR (600 MHz, D$_2$O) δ 8.95 (s, 1H, N=CHN) 8.78 (s, 1H, N=CHN) 6.27 (d, 1H, *J* = 5.00 Hz, CHN) 4.84 (t, 1H, *J* = 5.13 Hz, CHOH) 4.59 (t, 1H, *J* = 4.72 Hz, CHOH) 4.41 (d, 1H, *J* =3.65 Hz, CHCH$_2$) 4.21 (t, 2H, *J* =3.62 Hz, CHCH$_2$O) 2.15 (m, 2H, PCH$_2$P). ^{13}C-NMR (126 MHz, D$_2$O) δ 154.79, 154.23, 152.08, 148.38, 134.17, 90.87, 86.94, 77.24, 72.95, 66.17, 30.66. ^{31}P-NMR (202 MHz, D$_2$O) δ 19.73 (d, 1P, *J* = 9.47 Hz, P$_\beta$) 14.18 (d, 1P, *J* = 9.73 Hz, P$_\alpha$). LC/ESI-MS (m/z): positive mode 444.9 $[M+H]^+$ (calcd. for $C_{11}H_{16}ClN_4O_9P_2$ 445.0). Purity determined by HPLC-UV (254 nm)-ESI-MS: 94.2%. Mp. 190°C.

(((((2*R*,3*S*,4*R*,5*R*)-5-(6-((4-((6-(3′,6′-Dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamido)hexyl)carbamoyl)benzyl)amino)-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)methyl)phosphonic acid (14a)

Compounds **13** (0.02 g, 0.04 mmol, 1.0 eq) and **11a** (0.03 g, 0.04 mmol, 1.0 eq) were dissolved in absolute ethanol (5 ml). Et$_3$N (0.1 ml, 0.65 mmol, 15.0 eq) was added and the mixture was stirred

at 60°C.[5] After 18 h the solution was evaporated followed by purification by RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization to get final product (0.02 g, 50%). ^{1}H-NMR (600 MHz, CD_3OD) δ 8.61 (br s, 1H, N=C<u>H</u>N) 8.33+7.64 (s, 1H, C<u>H</u>=CCO or C<u>H</u>=CH) 8.26+8.22 (d, 1H, *J* = 5.71 Hz, C=C<u>H</u>) 8.03 (s, 1H, N=C<u>H</u>N) 7.98+7.35 (d, 1H, *J* = 7.91 Hz, C<u>H</u>=CCO) 7.75 (dd, 2H, *J* = 8.26, 24.93 Hz, aryl) 7.50 (dd, 2H, *J* = 8.31, 13.17 Hz, aryl) 7.09 (m, 2H, 2x C=C<u>H</u>) 6.60 (m, 4H, 4x CHCO<u>H</u>) 6.13 (d, 1H, *J* = 5.01 Hz, C<u>H</u>N) 4.72 (t, 1H, *J* = 5.08 Hz, C<u>H</u>OH) 4.58 (t, 1H, *J* = 4.63 Hz, C<u>H</u>OH) 4.34 (m, 1H, C<u>H</u>CH_2) 4.21 (m, 2H, CHC<u>H_2</u>O) 3.45 (m, 2H, NHC<u>H_2</u>-aryl) 3.05+2.87 (m, 4H, 2x NHC<u>H_2</u>) 2.51 (m, 1H, PC<u>H_2</u>P) 1.50 (m, 2H, C<u>H_2</u>) 1.44 (m, 2H, C<u>H_2</u>) 1.36 (m, 2H, C<u>H_2</u>) 0.97 (m, 2H, C<u>H_2</u>). ^{13}C-NMR (126 MHz, CD_3OD) δ 182.43, 174.23, 174.04, 170.89, 170.24, 169.78, 160.17, 159.73, 159.49, 154.09, 144.43, 144.06, 141.96, 141.08, 137.17, 136.48, 135.98, 134.36, 134.10, 132.44, 131.56, 130.59, 129.71, 129.20, 129.03, 128.80, 128.58, 124.44, 113.24, 104.81, 88.76, 85.41, 76.07, 71.67, 64.73, 54.11, 49.15, 47.65, 41.18, 30.16, 27.61, 27.40, 21.21. ^{31}P-NMR (243 MHz, CD_3OD) δ 20.51 (d, 1P, *J* = 6.03 Hz, P_β) 11.35 (d, 1P, *J* = 7.54 Hz, P_α). LC/ESI-MS (m/z): positive mode 1016.2627 $[M+H]^+$ (calcd. for $C_{46}H_{48}N_7O_{16}P_2$ 1016.2633) and negative mode 1014.3045 $[M-H]^-$ (calcd for $C_{46}H_{46}N_7O_{16}P_2$ 1014.2476). Purity determined by HPLC-UV (254 nm)-ESI-MS: 95.8%. Mp. decomp. > 260°C.

(((((2*R*,3*S*,4*R*,5*R*)-5-(6-((4-((2-(2-(2-(3′,6′-Di-hydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthene]-5(6)-carboxamido)ethoxy)ethoxy)ethyl)carbamoyl)benzyl)amino)-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)methyl)phosphonic acid (14b)

Compounds **13** (0.01 g, 0.05 mmol, 1.0 eq) and **11b** (0.03 g, 0.05 mmol, 1.0 eq) were dissolved in absolute ethanol (5 ml). Et_3N (0.2 ml, 0.15 mmol, 3.0 eq) was added and the mixture was stirred at 80°C.[5] After 18 h the solution was evaporated followed by purification by RP-HPLC (0-50% MeCN/50mM NH_4HCO_3 buffer in 15 min, 20 ml/min) followed by lyophilization to get final product (0.02 g, 49%). ^{1}H-NMR (600 MHz, D_2O) δ 8.30 (s, 1H, N=C<u>H</u>N) 7.88 (s, 1H, C<u>H</u>=CH) 7.60 (s, 1H, N=C<u>H</u>N) 7.52 (d, 1H, *J* = 7.37 Hz, C=C<u>H</u>) 7.48 (d, 1H, *J* = 7.75 Hz, C=CH) 7.27 (d, 1H, *J* = 7.93 Hz, CH=CO) 7.20 (d, 1H, *J* = 7.85 Hz, C<u>H</u>=CO) 7.06 (dd, 2H, *J* =2.10, 9.53 Hz, 2x CH=CO<u>H</u>) 7.03 (d, 2H, *J* = 9.26 Hz, 2x CH=CO<u>H</u>) 6.62 (m, 2H, aryl) 6.57 (m, 2H, aryl) 6.09 (d, 2H, *J* = 5.04 Hz, C<u>H</u>N) 4.73 (m, 1H, C<u>H</u>OH) 4.53 (t, 1H, *J* = 4.83 Hz, C<u>H</u>OH) 4.50 (br s, 2H,

NHC$\underline{H}_2$) 4.38 (q, 1H, *J* =3.69 Hz, C$\underline{H}$CH_2) 4.18 (d, 2H, *J* = 4.19 Hz, CHC$\underline{H}_2$O) 3.73 (t, 2H, *J* = 5.01 Hz, C$\underline{H}_2$) 3.63 (m, 2H, v) 3.60 (t, 2H, *J* = 5.01 Hz, C$\underline{H}_2$) 3.51
(t, 2H, *J* = 5.37 Hz, C$\underline{H}_2$) 3.45 (m, 2H, C$\underline{H}_2$) 3.25 (t, 2H, *J* = 5.50 Hz, C$\underline{H}_2$) 2.17 (t, 2H, *J* = 18.47 Hz, PC$\underline{H}_2$P). ^{13}C-NMR (151 MHz, D_2O) δ 176.62, 172.57, 171.74, 161.19, 160.95, 160.77, 160.23, 160.11, 156.86, 155.34, 145.48, 144.76, 142.02, 139.05, 137.31, 136.89, 136.17, 134.95, 134.30, 134.11, 131.64, 131.19, 130.47, 130.32, 129.98, 125.49, 116.04, 115.55, 106.47, 106.25, 90.06, 86.48, 77.03, 73.01, 71.85, 71.67, 71.56, 71.47, 66.39, 45.31, 42.61, 42.17, 30.37. ^{31}P-NMR (202 MHz, D_2O) δ 18.88 (d, 1P, *J* = 9.04 Hz, P_β) 14.96 (d, 1P, *J* = 9.33 Hz, P_α). LC/ESI-MS (m/z): positive mode 1048.2610 $[M+H]^+$ (calcd. for $C_{46}H_{48}N_7O_{18}P_2$ 1048.2531) and negative mode 1046.3153 $[M-H]^-$ (calcd. for $C_{46}H_{46}N_7O_{18}P_2$ 1046.2375). Purity determined by HPLC-UV (254 nm)-ESI-MS: 96.7%. mp: decomp. >190°C.

Recombinant soluble CD73

Human soluble CD73 was expressed in Spodoptera frugiperda (Sf9) insect cells and purified as previously described.[10] The cDNA encoding for the mature human CD73 (residues 27-549) fused to 6xHis-tag at the C-terminus (Genbank accession no. NM_002526) corresponding to the natural variant T376A (P21589/VAR_022091, UniProtKB/Swiss-Prot) was ligated into the pAcGP67B vector. For transfection, 1 µl of the recombinant vector (1000 ng/µl) mixed with 2.5 µl of baculovirus genomic DNA ProEasy™ (AB vector, CA, USA) was used to transfect Sf9 cells grown in Insect-XPRESS™ medium (#BE12-730Q, Lonza, Switzerland) supplemented with 10 mg/l gentamicin. The produced soluble enzyme was then concentrated by ultrafiltration with Amicon® Ulta-15 filters, 10 KDa cut-off (Merck Milipore, MA, USA), and then subjected to metal affinity chromatography (IMAC) purification with HisPur™ Ni-NTA spin columns (#88226, Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. The purified enzyme was aliquoted and stored at -80˚C until further use.
A previously published method was used to express the glutathione-S-transferase fusion protein of soluble rat CD73 in Sf9 insect cells.[11]

Cell culture

Human triple-negative breast cancer cells (MDA-MB-231), which natively express CD73, were grown in Dulbecco's Modified Eagle Medium (DMEM, #: 41966, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS, #: P30-1502, PAN Biotech, Germany)

and 100 U/ml penicillin + 100 µg/ml streptomycin (#: P06-07100, PAN Biotech, Germany). Cells were incubated at 37°C with 5% CO_2 for 72 h to reach confluence (80-90%). Confluent cells were washed with phosphate-buffered saline (PBS), then detached by a 5-min incubation with trypsin/EDTA (0.05%/0.6 mM, #P10-022100, PAN Biotech, Germany). Detached cells were re-suspended in culture media and split 1:20.

Membrane preparations

Confluent cells grown in 175 cm^2 culture flasks were detached as described above. Culture dishes (150 cm^2) were seeded with approx. 100 cells/dish and incubated at 37˚C, 5% CO_2 for 4 days. The growth medium was discarded, and the dishes were washed with 10 ml PBS and frozen at -20˚C. Frozen cells were scraped off the dishes with 1 ml of ice-cold buffer (50 mM Tris, 2 mM EDTA, pH 7.4), collected in a conical tube, then centrifuged at 1000*g* at 4˚C for 10 min. Then, the pellets were resuspended in buffer (0.5 ml/dish; 25 mM Tris, 1 mM EDTA, 320 mM sucrose, pH 7.4, 1:1000 protease inhibitor cocktail #P8340, Sigma-Aldrich, MO, USA) and homogenized three times for 30 s each (20,500 rpm, Ultraturrax, IKA-Labortechnik, Germany). The homogenate was centrifuged for 10 min at 1000*g*, 4˚C, and the supernatant was collected and centrifuged for further 30 min at 48,000*g*, 4˚C. The obtained pellets were resuspended in washing buffer (0.5 ml/dish) and centrifuged using the same conditions. After three more washing steps, the pellets were resuspended in Tris buffer 50 mM, pH 7.4 (0.1 ml/dish), aliquoted, and stored at -80°C until use.

Enzyme inhibition assay

Compounds were tested using a previously described method.[12] The stock solutions were prepared in demineralized water, which were further diluted in reaction buffer (Tris 25 mM, NaCl 140 mM, sodium dihydrogen phosphate 25 mM, pH 7.4). For screening, 10 µl of each test compound were transferred into the respective test tube containing 70 µl of the reaction buffer. For determining concentration-response curves, 10 µl of different dilutions of test compounds were pipetted into the test tubes. A solution or suspension of soluble or membrane-bound CD73 (10 µl, soluble rat CD73: 1.63 ng; soluble human CD73: 0.365 ng; membrane preparation of MDA-MB-231 cells expressing CD73: 7.4 ng of protein per vial) was transferred into all test tubes except for the negative controls. The substrate [2,8-^{3}H]AMP (specific activity 7.4 x 108 Bq/mmol (20 mCi/mmol)), American Radio-labeled Chemicals, MO, USA, distributed by Hartman Analytic, Germany) was added in a volume of 10 µl (5 µM final concentration) to initiate the reaction. After 25 min of incubation at 37°C in a shaking water bath, the samples were cooled on ice, and 500 µl of precipitation buffer (lanthanum chloride, 100 mM in sodium acetate 100 mM, pH 4.0) was added to stop the reaction and enable precipitation. Samples were kept on ice for at least 30 min until complete precipitation was achieved, and then filtered through GF/B glass fiber filters using a Brandel cell harvester (M-48, Brandel, MD, USA). Reaction vials were washed three times with 400 µl of cold (4°C) demineralized water each, then 5 ml of scintillation cocktail (ULTIMA Gold XR, PerkinElmer, MA, USA) was added, and radioactivity was measured using a scintillation counter (Tri-Carb 2900TR, Packard/PerkinElmer). All experiments were performed in duplicate, baseline-corrected and normalized against negative and positive controls, respectively. Three independent experiments were conducted, and data were analyzed using Prism-GraphPad 7 (GraphPad Software, La Jolla, USA). The Cheng-Prusoff equation was used to calculate the K_i values using the following K_m values (K_m, rat CD73: 53.0 µM; K_m, human CD73: 17.0 µM; K_m, (MDA-MB-231): 14.8 µM).[13]

Fluorescence staining of the cells

The MDA-MB-231 human breast adenocarcinoma cells (ATCC, Manassas, USA), grown on 13-mm coverslips in 24-well plates were processed for (immuno)fluorescence staining, as described previously.[14] Briefly, the cells were washed with phosphate-buffered saline (PBS), and immediately fixed for 5 min with PBS containing 4% paraformaldehyde (PFA). The adherent fixed cells were incubated for 30 min in 200 µl PBS supplemented with 2% bovine serum albumin (BSA)

and 0.05% saponin (blocking buffer), and subsequently incubated for 60 min with polyclonal rabbit anti-human CD73 antibody (h5NT-1L, http://ectonucleotidases-ab.com/) diluted at 1:300 in 200 µl of blocking buffer. The cells were incubated for additional 60 min in 200 µl of blocking buffer containing Alexa Fluor® 633-conjugated goat anti-rabbit IgG (ThermoFisher Life Technologies) diluted at 1:800, and 250 nM of the tested fluorescent probes **14a** or **14b**. All stainings were performed at room temperature (RT) under 60 rpm orbital rotation, with washing of the wells after each treatment using 300 µl PBS containing 0.05% saponin. The coverslips were mounted with ProLong® Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher) and examined using a 3i spinning disk confocal microscope CSU-W1 with Photometrics Evolve EM-CCD camera, Plan-Neofluar oil 63×/1.4 objective (Carl Zeiss), and Slidebook 6.0 software (Intelligent Imaging Innovations, Inc.). Maximum intensity projection of a confocal z-stack for each channel was prepared using Imaris 8.4 software (Bitplane).

Mouse eye histochemistry

Wild-type and CD73-deficient ($CD73^{-/-}$) mice on a C57BL/6J background (kindly provided by Dr. Linda Thompson) have been described earlier.[15,16] The animals were raised under pathogen-free conditions in the Central Animal Laboratory of the University of Turku, Finland, housed at a constant temperature (22±1°C) in a light-controlled environment (lights on from 7 am to 7 pm), and provided with food and water ad libitum. The experimental procedures were reviewed by the local Ethics Committee on Animal Experimentation of the University of Turku and approved by the Provincial State Office of Western Finland with the license ID ESAVI/5762/04.10.07/2017.

The mice were killed by carbon dioxide and the eyeballs were enucleated and processed for histological analyses in the following ways. For enzyme histochemistry, the eyeballs were embedded in the cryo-mold with Tissue-Tek® O.C.T. compound (Sakura Finetek Europe B.V., the Netherlands), cut at 10 µm onto Superfrost® Plus slides (ThermoFischer Life Technologies) using a Leica CM 3050S cryostat, and stored at -80°C. Ocular CD73 activity was determined by incubating tissue cryosections for one hour at RT with 1 mM AMP in the presence of 2 mM $Pb(NO_3)_2$, followed by microscopic detection of the nucleotide-derived inorganic phosphate (P_i) as a brown precipitate.[16, 17] Multiple bright-field images of adjacent tissue areas were captured using a Pannoramic-250 Flash slide scanner (3DHistech Ltd., Budapest, Hungary), and further stitched to a larger overview using the accompanying Pannoramic Viewer 1.15.4 software.

For immunofluorescence staining, the eyes were fixed for 2 hours at RT with PBS containing 4% PFA, and embedded in the mold with a 4% solution of low melting temperature agarose (LMA, NuSieve™ GTG™ Agarose, Lonza). LMA-embedded eyes were sectioned at 100 µm thickness using a Leica VT1200S vibrating microtome and additionally fixed for 30 min with 4% PFA.
Free-floating eye sections were incubated overnight at 4°C with polyclonal rabbit anti-rat CD73 ($rNu9_L$-I5, http://ectonucleotidases-ab.com/) and chicken anti-vimentin (BioLegend Inc, San Diego, CA) antibodies, diluted at 1:500 in 300 µl of PBS containing 2% bovine serum albumin (BSA) and 0.2% Triton X-100 (blocking buffer). The samples were then washed and incubated overnight at 4°C with Alexa Fluor® 488-conjugated goat anti-chicken and Alexa Fluor® 633 goat anti-rabbit antibodies (ThermoFisher) diluted in blocking buffer at ~1:800. Fluorescent CD73 inhibitors **14a** and **14b** were added at final concentrations of 250 nM during the incubation with secondary antibodies. Stained eye sections were additionally washed with PBS, transferred onto the microscope slide, aligned using forceps under a stereomicroscope, and mounted with ProLong® medium with glass spacers inserted between the slide and the coverslip. The slides were examined using a 3i spinning disk confocal microscope CSU-W1 with C-Apochromat 40×/1.1 objective (Carl Zeiss) as described above. Z-stacks of medial retina from $CD73^{+}/^{+}$ and $CD73^{-}/^{-}$ eyes were captured at identical exposure times for each channel, and maximum intensity projections of confocal z-stacks were acquired in parallel using Imaris 8.4 software (Bitplane).

NTPDases1, -2, -3 and -8 assays

Test compounds were investigated at a concentration of 50 µM in the presence of 100 µM of ATP as a substrate. Human enzymes were recombinantly expressed in COS-7 cells, and membrane preparations were obtained. The enzyme preparations were incubated with test compound for 5 min at 37°C followed by incubation with substrate for 30 min. The reaction buffer contained 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4. The enzymatic reaction was terminated by heating at 90°C for 10 min. After cooling the samples on ice, they were investigated by capillary electrophoresis (CE) with diode array detection (DAD) at a wavelength of 260 nm essentially as described previously.[18-21] Analysis was carried out using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA). The separation was performed in a polyacrylamide-coated capillary [30 cm (10 cm effective length) × 50 µm (id), × 360 µm (od)]. Before each run, the capillary was rinsed with the background electrolyte (50 mM phosphate buffer (pH 6.5)) for 1 min at 30 psi. Samples were electrokinetically injected by applying a voltage of -6

kV for 30 s at the capillary outlet, and the nucleotides were separated by voltage application of -15 kV and 0.2 psi pressure and detectedat a wavelength of 260 nm. Data collection and peak area analysis were performed by the P/ACE MDQ software 32 KARAT obtained from Beckman Coulter (Fullerton, CA, USA). For quantification, AMP and ADP standard curves were obtained. For the inhibition analysis, at least three independent experiments were performed in triplicate (n = 3). The inhibition of the enzyme activity was calculated in relation to the positive control without inhibitor and plotted by the GraphPad Prism 8 software (GraphPad software, San Diego, CA, USA).

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