Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

DNA Polymerase-catalyzed incorporation of Nucleotides modified with a G-quadruplex-Derived DNAzyme

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Material

5-Iodo-2´-deoxyuridine was purchased from *Carbosynth*. 2,2,2-trifluoro-N-(pent-4-ynyl)acetamide[1], and 5-(aminopentynyl)-2'-deoxyuridinetriphosphate[2] were prepared according to literature. DNA synthesis columns purchased from *ABI Applied Biosystems*. All reagents are commercially available and were used without further purification. Dry solvents were obtained from *Sigma-Aldrich* and used without further purification. All synthetic reactions were performed under an inert atmosphere. Flash chromatography was performed using *Merck* silica gel G60 (230–400 mesh) and *Merck* precoated plates (silica gel 60 F254) were used for TLC. Anion-exchange chromatography was performed on an ÄktaPurifier (GE Healthcare) with a DEAE Sephadex™ A-25 (GEHealthcare Bio-SciencesAB) column using a linear gradient (0.1 M – 1.0 M) of triethylammonium bicarbonate buffer (TEAB, pH 7.5). Reversed phase high pressure liquid chromatography (RP-HPLC) for the purification of compounds was performed using a Shimadzu system having LC8a pumps and a Dynamax UV-1 detector. A VP 250/16 NUCLEODUR C18 HTec, 5µm (Macherey-Nagel) column and a gradient of acetonitrile in 50 mM TEAA buffer (pH 7.0) were used. All compounds purified by RP-HPLC were obtained as their triethylammonium salts after repeated freeze-drying. Analytical RP-HPLC was performed using a Shimadzu Prominence system. A VP 250/8 NUCLEODUR C18 HTec, 5µm (Macherey-Nagel) column and a gradient of acetonitrile in 50 mM TEAA buffer (pH 7.0) were used. NMR spectra were recorded on *Bruker* Avance 400 (^{1}H: 400 MHz, ^{13}C: 101 MHz, ^{31}P: 162 MHz) spectrometer. The solvent signals were used as references and the chemical shifts converted to the TMS scale and are given in ppm (δ). HR-ESI-MS spectra were recorded on a Bruker Daltronics microTOF II and ESI-MS spectra were recorded on a Bruker Daltronics amaZonSL.

KF (Klenow Fragment) *exo*$^{-}$ DNA polymerase and the respective reaction buffer were purchased from *Thermo Scientific*. *KlenTaq* DNA polymerase was expressed and purified as described before.[3] T4 polynucleotide kinase PNK was purchased from *New England BioLabs*. Oligonucleotides not synthesized in our lab were purchased from *Metabion* and *Biomers.net*. [γ-^{32}P]ATP was purchased from *Hartmann Analytics* and natural dNTPs from *Thermo Scientific*.

Streptavidin sepharose high performance was purchased from *GE Healthcare* (Matrix: highly cross-linked agarose, 6%; binding capacity/ml: >300 nmol biotin/ml medium; average particle size: 34 µm). Hemin and 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) were purchased from *Sigma-Aldrich*.

Buffers and solutions

- 1 M TEAA buffer (1 M acetic acid, 1 M triethylamine, (pH 7))
- 1 M TEAB buffer (1 M triethylamine, saturated with CO_2, (pH 7.5))
- 10 x *KlenTaq* reaction buffer (500 mM Tris-HCl (pH 9.2), 160 mM $(NH_4)_2SO_4$, 25 mM $MgCl_2$, 1% Tween 20)
- 10 x *KF exo*$^-$ reaction buffer (500 mM Tris-HCl (pH 8.0 at 25°C), 50mM $MgCl_2$, 10mM DTT) (*Thermo Scientific*)
- PAGE gel loading buffer (80% formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanole FF)
- Lithium cacodylate buffer 200 mM.[4] To prepare 20 mL volume of 200 mM lithium cacodylate at pH 7.2, dissolve 0.552 g of cacodylic acid in 10 mL of milliQ water. Adjust the pH of the solution using 1 M LiOH. Adjust the volume to 20 mL with milliQ water, filter the solution, and store up to several weeks in a glass bottle at 4°C.
- Caco.KTD buffer (20 mM lithium cacodylate buffer (pH 7.2) plus 20 mM KCl/180 mM LiCl, 0.1% Triton X-100 and 0.2% DMSO). It is prepared by mixing 10 mL of 200 mM lithium cacodylate buffer (pH 7.2), 10 mL of KCl/LiCl solution (200 mM KCl/ 1.8 M LiCl in water), 200 µL DMSO, 100 µL Triton X-100 and 80 mL water.[5]
- 1 x Binding buffer (200 mM Na_3PO_4, 1.5 M NaCl (pH 7.5))
- Hemin (100 µM solution in DMSO)
- ABTS (20 mM solution in water)
- H_2O_2 (60 mM solution in water)

5'-Radioactive labeling of ODNs

DNA oligonucleotide primers were radioactively labeled at the 5' terminus with a ^{32}P containing phosphate group using T4 PNK and [γ-^{32}P]ATP. The reaction contained primer (0.4 µM), PNK reaction buffer (1 x), [γ-^{32}P]ATP (0.8 µCi/µL) and T4 PNK (0.4 U/µL) in a total volume of 50 µL and was incubated for 1 h at 37 °C. The reaction was stopped by denaturing the T4 PNK for 2 min at 95 °C and buffers and excess [γ-^{32}P]ATP were removed by gel filtration (MicroSpin

Sephadex G-25). Addition of unlabeled primer (20 µL, 10 µM) led to a final concentration of 3 µM of diluted radioactive labeled primer.

Gel electrophoresis

Denaturing polyacrylamide gels (12 %) were prepared by polymerization of a solution of urea (8.3 M) and bisacrylamide/acrylamide (12 %) in TBE buffer using ammonium peroxodisulfate (APS, 0.08 %) and *N*,*N*,*N'*,*N'*-tetramethylethylene-diamine (TEMED, 0.04 %). Immediately after addition of APS and TEMED, the solution was filled in a sequencing gel chamber (*Bio-Rad*) and left for polymerization for at least 45 min. After addition of TBE buffer (1 x) to the electrophoresis unit, gels were pre-warmed by electrophoresis at 100 W for 30 min and samples were added and separated during electrophoresis (100 W) for approx. 1.5 h. The gel was transferred to *Whatman* filter paper, dried at 80 °C *in vacuo* using a gel dryer (model 583, *Bio-Rad*) and exposed to an imager screen. Readout was performed with a molecular imager (FX, *Bio-Rad*).

Synthesis of activated oligonucleotides

The activated oligonucleotide was synthesized as described by Baccaro *et al*.[2] 200 nmol scale coupling and oxidation was performed on a DNA synthesizer (model 392, *Applied Biosystems*) using standard parameters for solid phase oligodeoxynucleotide synthesis. The 5'-carboxy-modifier C10 was dissolved in anhydrous acetonitrile to a concentration of 0.1 M and coupled to the solid support employing a DNA synthesizer. The column was dried *in vacuo* and stored at -25°C until use.

Synthesis and quantification of DNAzyme-modified dTTP

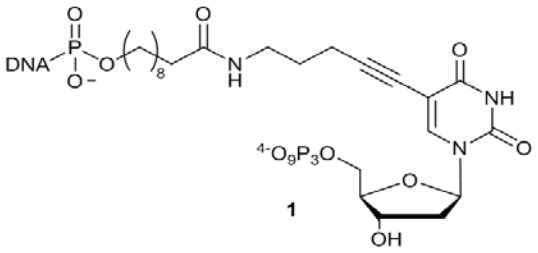

Fig. S1. Depiction of the G4-modified dTTP.

For a 200 nmol scale synthesis, the nucleotide (5 equiv, 1 µmol) was dissolved in 10 µL MeOH. The solution was diluted to 1 mL using a mixture of anhydrous DMF/acetonitrile (1:1) with 10% dry Et_3N. The conjugation of the dissolved triphosphate and the activated ODN was performed using the manual two syringe technique (18 h). After the reaction, the triphosphate solution was drawn into one syringe and the solid support was washed with 2 x 10 mL DMF and 20 mL acetonitrile, followed by drying of the column *in vacuo*. The modified oligonucleotide was deprotected and cleaved from the solid support by ammonium hydroxide (33%) (55°C, 9 h). The crude material was purified twice by reverse phase HPLC (VP 250/8 NUCLEODUR C18 HTec, 5µm (Macherey-Nagel) column and a gradient 5-70% acetonitrile/50 mM TEAA buffer (pH 7.0)). The product was lyophilized five times and analyzed by ESI-IT mass spectrometry.

Quantification of ODN was conducted by measuring absorbance at 260 nm in water. In accordance to Lambert Beer's law, the concentration of the ssDNA was calculated using sequence specific extinction coefficients. For quantification and concentration measurements the following was used:

$$c = A(260\ nm)/\varepsilon \cdot d$$

Calculation was done using the online oligonucleotide properties calculator: OligoCalc.[6] To predict the absorbance of the ODN functionalized nucleotides, the ODN and the respective nucleotide was calculated by OligoCalc. The absorbance of the solution was measured using a NanoDrop ND-1000 Spectrometer (*ThermoScientific*) and the concentration was then calculated by Lambert Beer's law.

	DNA Sequence	mass calc.	mass found	yield%
mod. dTTP	dTTP*-d(TTT TTG GGT AGG GCG GGT TGG GAA A)-3´	8645.6	8644.4	3.8%

DNA Sequences

radioactive-labeled primer **2**: 5´-d(GAC CCA CTC CAT CGA GAT TTC)

template for incorporation of dTTP **3**: 5´-d(TGC CTG GTG TTT GGG AGA AAT CTC GAT GGA GTG GGT C)

immobilized primer **4**: 5´-biotin-d(TTT TTT TTT TTT TTT TTT TGA CCC ACT CCA TCG AGA TTT C)

canonical template **5**: 5´-d(GGT CTA GCT ACA G**A**G AAA TCT CGA TGG AGT GGG TC)

non-canonical template **6**: 5´-d(GGT CTA GCT ACA G**T**G AAA TCT CGA TGG AGT GGG TC)

G4-sequence **7**:[7] 5´-d(TTT TTG GGT AGG GCG GGT TGG GAA A)

Primer extension reaction

A typical primer extension reaction (20 µL) employing *KF exo*$^-$ DNA polymerase contained 1 x reaction buffer, 150 nM ^{32}P-labeled primer **2**, 200 nM template **3**, 10 µM G4-modified dTTP **1**, 10 µM each of the 3 absent natural dNTPs, and 2 U *KF exo*$^-$ DNA polymerase. A typical primer extension reaction (20 µL) employing *KlenTaq* DNA polymerase contained 1 x *KlenTaq* reaction buffer, 150 nM ^{32}P-labeled primer **2**, 200 nM template **3**, 10 µM G4-modified dTTP **1**, 10 µM each of the 3 absent natural dNTPs and 135 nM *KlenTaq* DNA polymerase. First, primer and template were annealed. Afterwards, the primer template complex, nucleotides and DNA polymerase were incubated (55°C, *KlenTaq* DNA polymerase; 37°C, *KF exo*$^-$ DNA polymerase), and the reaction stopped after: 1, 5, 10, and 30 min. The reactions were quenched by addition of 20 µL PAGE gel loading buffer and the product mixtures were analyzed by 12% denaturing polyacrylamide gel and subjected to autoradiography.

Reaction on streptavidin sepharose beads

Determination of Lower Detection Limit

10 µL of a streptavidin-sepharose suspension mixed with 10 µL of a blank sepharose beads suspension were spun down (2400 x g) and the supernatant was carefully removed. The residue was washed two times with 80 µL 1 x binding buffer. 20 µL 1 x binding buffer and 0.5 µL primer **4** (100 µM, 50 pmol) were added to the beads. After some mixing during 10 min of incubation at room temperature, the suspension was spun down and the supernatant removed. The residue was washed with 80 µL 1 x binding buffer and two times with 80 µL 1 x *KF exo*$^-$ reaction

buffer. In four different test tubes: 1.2 µL (10 µM, 12 pmol), 0.3 µL (10 µM, 3 pmol), 1.5 µL (1 µM, 1.5 pmol), 0.3 µL (1 µM, 0.3 pmol) template **5**, and 3 µL 10 x *KF exo*$^-$ reaction buffer, and respectively 22.2, 23.1, 21.9 µL of water were added. After 5 min 0.6 µL *KF exo*$^-$ (5 U/ µL, 3 U) and 3 µL G4-modified dTTP **1** (10 µM, 30 pmol) were added and the reaction was incubated 30 min at 37 °C with mixing every 6 min. A control experiment was prepared in the following way: 1.2 µL (10 µM, 12 pmol) template **5**, 3 µL 10 x *KF exo*$^-$ reaction buffer, and 25.2 µL; after 5 min of incubation at room temperature 0.6 µL of *KF exo*$^-$ (5 U/ µL, 3 U) was added and the reaction was incubated 30 min at 37 °C with mixing every 6 min. The suspensions were spun down and cooled on ice. The beads from each reaction tube were collected and transferred in an empty spin column. The buffer was removed by short spin at 600 x g and the beads washed five times with 10 mM Caco.KTD buffer, every time followed by short spin at 600 x g. The beads were collected from the spin column with 10 mM Caco.KTD buffer, the suspension spun down and the supernatant removed. 15 µL 20 mM Caco.KTD buffer, 11.4 µL water, 3 µL ABTS (20 mM, 60 nmol), 0.3 µL hemin (100 µM, 30 pmol) were added and after mixing the reaction was incubated for 20 min. 0,3 µL H_2O_2 (60 mM, 18 nmol) was added and after 45 min a picture was taken with a digital camera.

Discrimination Canonical/non-Canonical template

10 µL of a streptavidin sepharose suspension mixed with 10 µL of a sepharose beads suspension were spun down (2400 x g) and the supernatant was carefully removed. The residue was washed two times with 80 µL 1 x binding buffer. 20 µL 1 x binding buffer and 0.5 µL primer **4** (100 µM, 50 pmol) were added to the beads. After some mixing during 10 min of incubation at room temperature, the suspension was spun down and the supernatant removed. The residue was washed with 80 µL 1 x binding buffer and two times with 80 µL 1 x *KF exo*$^-$ reaction buffer. Canonical template samples: in three different test tubes 1.2 µL (10 µM, 12 pmol), 0.3 µL (10 µM, 3 pmol), 1.5 µL (1 µM, 1.5 pmol) template **5**, and 3 µL 10 x *KF exo*$^-$ reaction buffer, and respectively 22.2, 23.1, 21.9 µL of water were added. Non-Canonical template: in three different test tubes 1.2 µL (10 µM, 12 pmol), 0.3 µL (10 µM, 3 pmol), 1.5 µL (1 µM, 1.5 pmol) template **6**, and 3 µL 10 x *KF exo*$^-$ reaction buffer, and respectively 22.2, 23.1, 21.9 µL of water were added. After 5 min 0.6 µL *KF exo*$^-$ (5 U/ µL, 3 U) and 3 µL G4-modified dTTP **1** (10 µM, 30 pmol) were added and the reaction was incubated 30 min at 37 °C with mixing every 6 min. The suspension was spun down and cooled on ice. The beads from each reaction tube were collected and transferred in empty spin column. The buffer was removed by short spin at 600 x g and the beads washed five times with 10 mM Caco.KTD buffer, every time followed by short spin at 600 x g. The beads were collected from the spin column with 10 mM Caco.KTD buffer, the suspension spun down and the supernatant removed. 15 µL 20 mM Caco.KTD

buffer, 11.4 µL water, 3 µL ABTS (20 mM, 60 nmol), 0.3 µL hemin (100 µM, 30 pmol) were added and after mixing the reaction was incubated for 20 min. 0.3 µL H_2O_2 (60 mM, 18 nmol) was added and after 45 min a picture was taken with a digital camera.

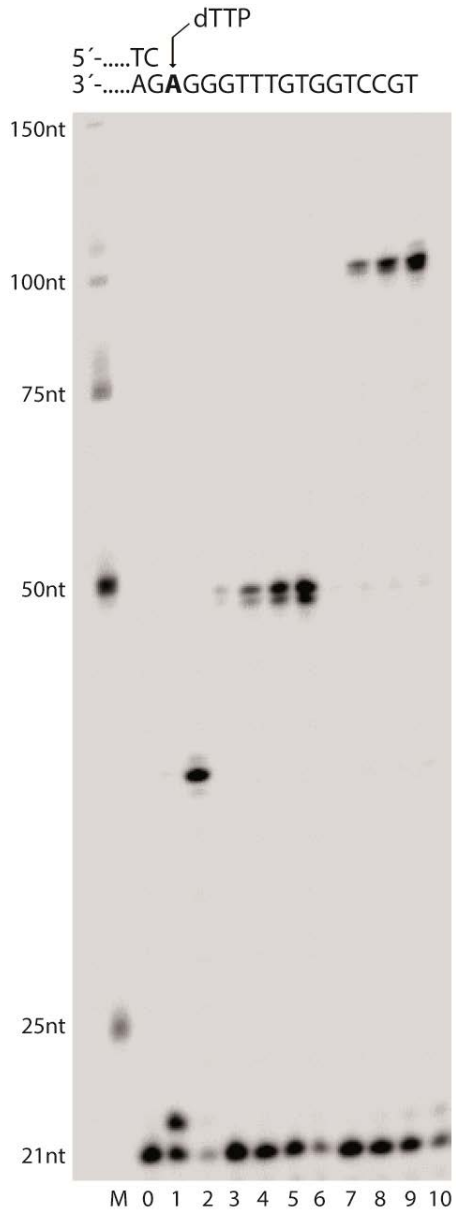

Fig. S2. Partial DNA sequences of primer and template and PAGE analysis of the primer-extension studies using *KlenTaq* DNA polymerase, a 21-nt primer **2**, a 37-nt template **3**, and 10 μM dNTPs. M: DNA marker; lane 0: 5'-^{32}P-labeled primer only; lane 1: primer extension performed in the presence of dATP, dCTP, and dGTP; lane 2: primer-extension reaction performed in the presence of dATP, dCTP, dGTP, and dTTP; lanes 3, 4, 5, 6: single-nucleotide incorporation experiments performed in the presence of G4-modified dTTP **1**, with reaction quenched after 1, 5, 10, and 30 min; lanes 7, 8, 9, 10: primer-extension reaction performed with natural dATP, dCTP, and dGTP and G4-modified dTTP, with reaction quenched after 1, 5, 10, and 30 min.

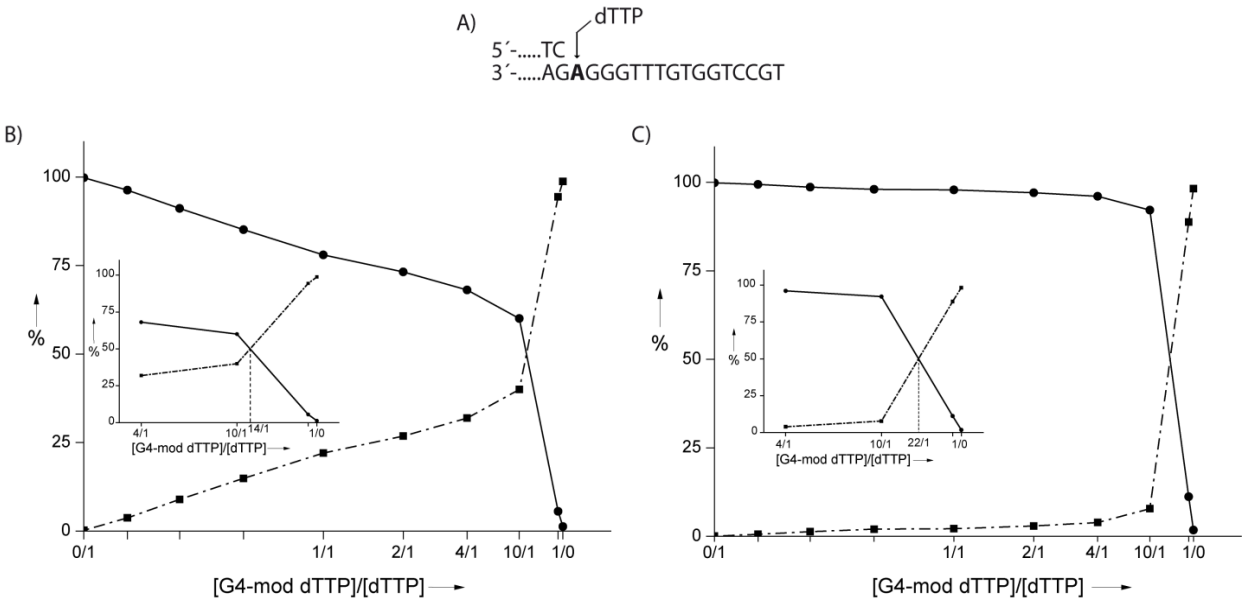

Fig. S3. Competition experiments of dTTP *versus* G4-modified dTTP. A) Partial DNA sequences of primer **2** and template **3** B) Exemplary competition experiments employing *KF exo*$^-$ DNA polymerase (30 min). The ratio of [G4-modified dTTP **1]**/[dTTP] was varied. Ratio: 0/1, 1/10, 1/4, 1/2, 1/1, 2/1, 4/1, 10/1, 100/1, and 1/0. Evaluation of the incorporation efficiency using G4-modified dTTP **1** (■, dashed line)/dTTP (●, solid line) mixtures and *KF exo*$^-$ DNA polymerase. To evaluate the incorporation efficiencies, the product bands and the background were quantified using the Bio-Rad Quantity One software. The conversion in % was plotted *versus* the concentration using the program OriginPro 8.6 32Bit. Inset: the dotted line marks the approximate ratio where both nucleotides are equally incorporated. C) Exemplary competition experiments employing *KlenTaq* DNA polymerase (30 min). The ratio of [G4-modified dTTP **1**]/[dTTP] was varied. Ratio: 0/1, 1/10, 1/4, 1/2, 1/1, 2/1, 4/1, 10/1, 100/1, and 1/0. Evaluation of the incorporation efficiency using G4-modified dTTP **1** (■, dashed line)/dTTP (●, solid line) mixtures and *KlenTaq* DNA polymerase. The conversion in % is plotted *versus* the concentration. Inset: the dotted line marks the approximate ratio where both nucleotides are equally incorporated.

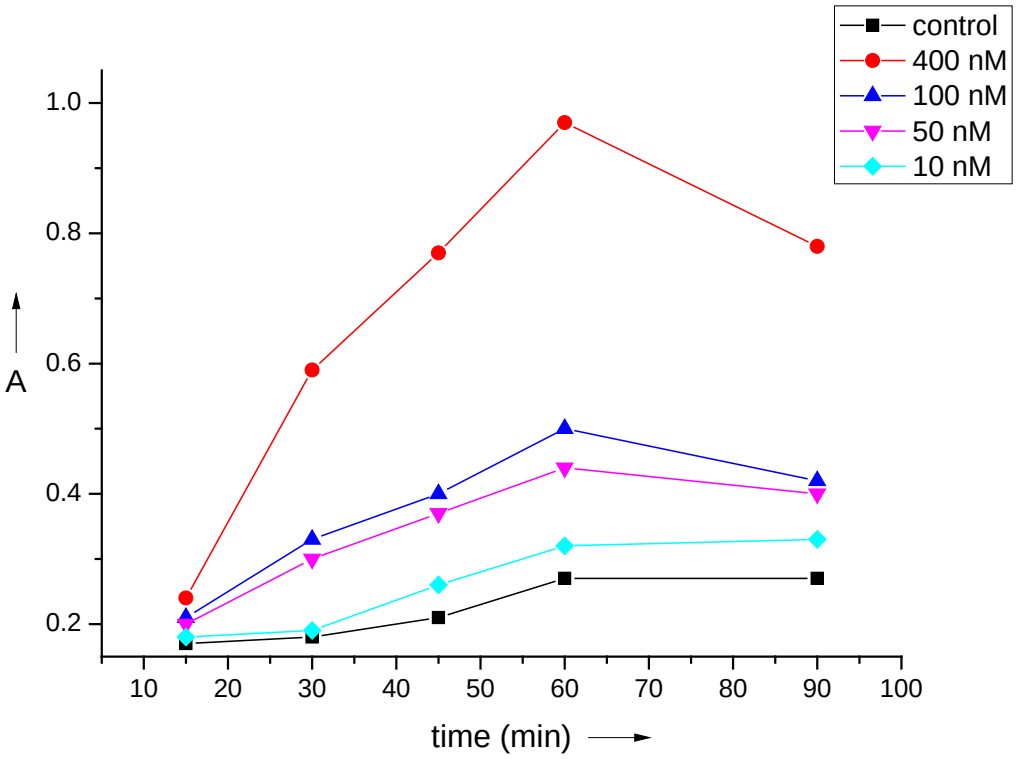

Fig. S4. $ABTS^{\bullet-}$ absorption at 420 nm recorded in function of the time after addition of hemin, $ABTS^{2-}$, and H_2O_2 in caco. KTD buffer 10 mM to the beads. The time points were taken after: 15, 30, 45, 60, and 90 min. Concentration canonical template (**5**): 400 nM (red line), 100 nM (blue line), 50 nM (magenta line), 10 nM (cyan line), and control (black line).

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