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PhpC modulates G-quadruplex-RNA landscapes in human cells

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------ Experimental Procedures

I. MATERIALS

1. Chemical probes. BRACO-19 (Figures 2A and S1) was solubilized at 20 mM in DMSO (for Sulforhodamine B (SRB) cytotoxicity assay), then diluted at 2 mM in DMSO (for G4RP.v2 experiments). PhpC (Figure 2A) was solubilized at 20 mM in DNase/RNase-free UltraPure Distilled Water (Invitrogen, cat. n° 10977035; for SRB cytotoxicity assay and G4RP.v2). TASQ (template-assembled synthetic-G-quartet, Figures 2A and S1) were solubilized at 1 mM (2 mM for clickable ^{az}MultiTASQ, see below) in DNase/RNase-free UltraPure Distilled Water and stored at 4 °C. To obtain clicked ^{az}MultiTASQ (= biotinylated ^{az}MultiTASQ), SPAAC (Strain-promoted azide-alkyne cycloaddition) was performed as follows: 100 µL of a 2 mM aq. solution of clickable ^{az}MultiTASQ (BOC-deprotected) were mixed with 22 µL of 10 mM aq. solution of DBCO-PEG(4)-Biotin (Iris Biotech, cat. n° RL-2520) and 78 µL of DNase/RNase-free UltraPure Distilled Water, to have a 1:1.1 ratio (Clickable ^{az}MultiTASQ:DBCO-PEG(4)-Biotin). The coupling reaction was stirred at 37 °C for 1 h (HPLC-MS monitoring) after which the clicked ^{az}MultiTASQ was used without further purification.

2. Oligonucleotides (ONs). All oligonucleotides (ONs) used here (Table S1) were purchased from Eurogentec (Belgium), diluted in ultrapure water (18.2 MΩ.cm resistivity) at 500 µM for stock solutions, except for NRAS primers (100 µM stock solutions), and stored at -20 °C. The actual concentration of these stock solutions was determined through a dilution to 1-5 µM theoretical concentration *via* a UV spectral analysis at 260 nm with the molar extinction coefficient values provided by the manufacturer. G4-ONs for 1. qPCR Stop assays (c-Kit2 QSA, c-Myc QSA, hTelo QSA and *S. pombe* G4), fluorescence quenching assays ($^{5'}$Cy5-Myc, $^{5'}$Cy5-NRAS) and CD titrations (hTelo, NRAS), 2. fluorescence titrations (hTelo and ssDNA) and 3. (reverse) competitive FRET-melting experiments (F21T, F-NRAS-T) were prepared at 1. 50 µM, 2. 250 µM and 3. 25 µM, respectively, in a Caco.K buffer, comprised of 10 mM lithium cacodylate buffer (pH 7.2) plus 10 mM KCl/90 mM LiCl solution. As an example, the 25 µM working

solution was prepared by mixing 5 µL of the stock solution (500 µM) with 10 µL of 100 mM lithium cacodylate buffer (pH 7.2), plus 10 µL of 100 mM/900 mM KCl/LiCl solution and 75 µL of ultrapure water. The high-order structure was folded by heating the previously prepared solution at 90 °C for 5 min, cooling it on ice (several hours) and then storing it at least overnight at 4 °C. ONs for qPCR Stop assays were diluted at 0.5 µM (in 10 mM KCl solution) for templates and 6 µM (in ultrapure water) for the QSA primer. ON primers for G4RP-RT-qPCR (NRAS primers) were diluted at 10 µM in sterile DNase/RNase-free UltraPure Distilled Water and store at 4 °C.

3. Cell culture. MCF7 cells were routinely cultured in 75 cm^2 tissue culture flasks (Nunc) at 37 °C in a humidified 5% CO_2 atmosphere (in cell culture incubator) in Dulbecco's Modified Eagle Medium (DMEM, Dutscher, cat. n° L0104) supplemented with 10% (v/v) fetal bovine serum (FBS, Dutscher, cat. n° S1810) and 1% (v/v) Penicillin-Streptomycin (Pen-Strep, 106.8 U/mL Pen, 106.8 µg/mL Strep, Gibco, cat. n° 151-40-122). Cells were subcultured twice a week using standard protocols.

II. METHODS

1. qPCR stop assay. Polymerase reactions were carried out in triplicate in 96-well format using a Mx3005P qPCR machine (Agilent) equipped with FAM filters (λ_{ex} = 492 nm; λ_{em} = 516 nm) in 10 µL (final volume). To a 1.35-µL solution of 0.5 µM template oligonucleotide (in 10 mM KCl) were added 3.15 µL or 2.65 µL of 380 mM KCl for experiments without (control) or with molecules, respectively (around 100 mM KCl final concentration). Next, 0.5 µL of the 6 µM QSA primer were added (for the four qPCR stop assay templates), then 0.5 µL of 6.75 µM molecules (5 mol. equiv., in 10 mM KCl) and 5 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad). After a first denaturation step (95 °C, 5 min), a two-step qPCR comprising a hybridization step (85 °C, 10 s) and an elongation step (60 °C, 15 s) for 33 cycles was performed, and measurements were made after each cycle. Final data (Figure S2 and Table S2) were analysed with OriginPro, Version 2018 (OriginLab Corporation). The starting emission (first qPCR cycle) of SYBR Green (FI) was set to 2200 and the FI at the 33th cycle was used for calculation. One biological triplicate (n= 3) was used. For statistical hypothesis student's t-test and Welch's unequal variances t-test were used depending on variances equality. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2. Fluorescence titrations. Spectra (Figure S3) were recorded on a JASCO FP8500 spectrofluorometer in a 10 mm path-length quartz semi-micro cuvette (Starna). Fluorescence experiments were carried out in 1 mL of 10 mM lithium cacodylate buffer (pH 7.2) + 90 mM LiCl/10 mM KCl, with PhpC (2 µM). After a first fluorescence measure of PhpC alone, increasing concentrations (1-2-5 mol. equiv. compared to PhpC, *i.e.*, 2-4-10 µM, respectively) of hTelo G4 or a 14-mer ssDNA were added. Spectra (λ_{ex} = 365 nm, λ_{em} = 380-700 nm, Ex and Em slits = 2.5 nm, 1 nm pitch, 2 s response, scan speed = 500 $nm.min^{-1}$) were recorded 5 min (at 25 °C) after the addition of the (oligo)nucleotides. Fluorescence intensity (FI) at 452 nm was used for the calculation of its quenching (Table S3) after each addition of (oligo)nucleotides using the following formula: ([$FI_{452\ nm}$ after addition * 100]/$FI_{452\ nm}$ PhpC alone) - 100.

3. Sulforhodamine B (SRB) cytotoxicity assay. According to Ref.[1] Experiments were performed in 96-well plates (flat bottom, transparent plate, with lid; Corning-Falcon cat. n° 353072) using CLARIOstar Plus plate-reader device (BMG LABTECH). MCF7 cells were seeded in a 96 well-plate at a density of 4.10^3 cells in 160 µL (25.10^3 cells/mL) per well, keeping a column of the plate without cells (background control), and allowed to recover 24 h. Cells were treated live with various concentration of G4 ligands (40 µL of 5X G4 ligands in supplemented DMEM, see Table

S4 for 1X concentration range) or not (negative control = untreated cells; only supplemented DMEM plus the same % DMSO of G4 ligands, if applicable), in technical duplicate, for 72 h at 37 °C. Cells were fixed with an addition of 120 µL of cold 10% (w/v) trichloroacetic acid (TFA) to wells for 1 h at -4 °C, washed with water (x5), allowed to dry overnight at 25 °C (or dried with blow dryer < 1 h), stained with 0.057% (w/v) SRB (in 1% (v/v) acetic acid) for 30 min at 25 °C, then quickly washed with 1% (v/v) acetic acid (x3) and allowed to dry overnight at 25 °C (or dried with blow dryer < 1 h). After incubating the plate with cold 10 mM unbuffered Trizma base solution for 5 min at 25 °C under agitation, the Abs $_{530\ nm}$ was recorded. Final data (Figure 2B and Table S5) were analyzed with Excel (Microsoft Corp.) and OriginPro, Version 2018 (OriginLab Corporation). Mean Abs values and Normalized % cell viability (see below) were calculated. IC_{50} was calculated in performing a sigmoidal fit (Dose Response, OriginPro). Three independent experiments (n= 3) were used.

–> Normalized % cell viability [x] = [(Mean Abs $_{530\ nm}$ G4 ligand [x] – Mean Abs $_{530\ nm}$ background control) / (Mean Abs $_{530\ nm}$ negative control – Mean Abs $_{530\ nm}$ background control)] *100.

[x] corresponds to the G4 ligand concentration.

4. Optical imaging performed in MCF7 cells with N-TASQ +/- pre-incubation with PhpC. Round glass coverslips (12 mm) were sterilized with 70% ethanol at least one day before cell seeding. MCF7 cells were seeded on round glass coverslips in a 4 well-plate at a density of 8.10^4 cells (1.10^5 cells/mL) per coverslip and allowed to recover for 24 h. Cells were treated live with PhpC (20 µM in supplemented DMEM, 8 h) or not (control; only supplemented DMEM, 8 h) and live co-incubated with N-TASQ (50 µM in supplemented DMEM, 6 h). Coverslips were washed with 1X PBS (x1), fixed in ice cold methanol (-20 °C, 10 min), washed with 1X PBS (25 °C, 5 min, x3), washed with water (25 °C, 1 min, x1) and mounted with Fluoromount-G (Invitrogen, cat. n° 00-4958-02) on glass slides. Slides were imaged using a confocal microscope (Leica TCS SP8, Leica Microsystems) with a 63X objective lens and LAS X software (Leica Microsystems) to collect N-TASQ fluorescence (λ_{ex} = 405 nm; λ_{em} = 450-530 nm). Images were processed using Fiji software[2] (see "5. Method for the automated N-TASQ fluorescence quantification" above) and results obtained (Table S6) were analysed with Excel (Microsoft Corp.) and OriginPro Version 2018 (OriginLab Corporation). For statistical hypothesis tests, Student's *t*-test and Welch's unequal variances *t*-test were used depending on variances equality. This last Welch's *t*-test was precisely used for N-TASQ *foci* per cell analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Images shown in Figure 2C and Figure S4 are "stacked" images obtained with 4-12 slices (z-dimension), with a brightness decrease and contrast increase only for a better visibility of these images. The N-TASQ Fluorescence intensity per cell value (Figure S7) is a normalized value obtained after division with the mean background FI value.

5. Method for the automated N-TASQ fluorescence quantification. A home-made Java-based macro program was created with the macro editing tool on Fiji[2] in order to *i*- count the number of cells, *ii*- count the number of N-TASQ *foci* in previous cells, *iii*- recover the N-TASQ fluorescence intensity (FI) (IntDen) and *iv*- the volume (in μm^3) of previous cells and N-TASQ *foci*, for every confocal image. This N-TASQ macro program comprises steps where the user has to do manual actions and automatic steps depending on parameters chose at the beginning by the user (*e.g.*, number of images to treat to go to next image, N-TASQ threshold value to count N-TASQ *foci*). **(I)** Briefly, the selected image is copied, the image slices (z dimension) of this copy are stacked ("Z Project" method) to better see cells contours and then allow user to manually surround cells (with "polygon selections" tool) to define them digitally as informatic Regions of Interest (ROI), which are saved and numbered (Figure S5). **(II)** On the original image (three-dimensional, non-stacked), all of the previously created ROI (digital cells) are used, one by one, to

automatically select N-TASQ *foci* using a FI threshold of 45 and 120 for Control and PhpC-treated cells, respectively, due to a higher background FI in images for that second condition (see Table S6). The necessity of selecting different threshold for control *versus* PhpC-treated cells stems from a notable difference of fluorescence intensity inside/outside the cells in these two conditions. Figure S6 details the methodology followed to select the most suited threshold for each condition, and the related controls. N-TASQ *foci* were counted and numbered, taking account of the fact that one *focus* can be present on several Z-stacks to extract the FI and volume with the "3D Object Counter" method. **(III)** Also, the background FI is measured on areas without cells (Figure S6), which have to be chosen manually by the user, and a mean background FI is generated for every image to allow a normalization of cells and N-TASQ *foci* FI. **(IV)** A spreadsheet document is saved and contains *i*- the experiment information (*e.g.*, the name and analysis parameters chosen, the number of images analysed, the mean background FI), *ii*- the list of cells selected (with their FI and volume, and the image n° they come from) and *iii*- the list of N-TASQ *foci* counted (with the same previous information, plus the cell n° they come from). **(V)** For every confocal image, a "stacked" image with cells surrounded and numbered (plus a scale bar) is also generated in order to identify rapidly cells of interest after the analysis of the spreadsheet. To further demonstrate that this automated analysis is relevant, Figure S7 and Table S6 gather different metrics for control *versus* PhpC-treated cells (*i.e.*, the number of N-TASQ foci (left panel, used in the main text), the FI/cell (normalized; central panel) and the FI/foci (normalized; right panel)), which all converge towards a significant reduction of G4 objects upon PhpC treatment.

6. Fluorescence quenching assay. A diluted solution of 200 nM $^{5'}$Cy5-ON (ON: Myc or NRAS) was prepared in diluting the 50 µM stock solution with Tris HCl buffer (50 mM Tris, 150 mM KCl, 0.5% (v/v) triton, pH 7.2). Diluted solutions of G4 ligands (BRACO-19, N-TASQ and PhpC) at various concentrations (see Table S7) were prepared in diluting them with the same previous Tris HCl buffer. Experiments were performed in 96-well plates (chimney well, flat bottom, black plate; Greiner Bio-One cat. n° 655090) at 25 °C using CLARIOstar Plus plate-reader device (BMG LABTECH) equipped with Cy5 filters (λ_{ex} = 610 nm; λ_{em} = 675 nm) in 100 µL final volume. To a 10 µL of the 200 nM $^{5'}$Cy5-ON (final concentration: 20 nM) were added 90 µL of the Tris HCl buffer for experiments without G4 ligand (control) or 90 µL of various concentrations of diluted small molecule solutions (see Table S7) for experiments with small molecules, in technical triplicates. After mixing the plate at 25 °C for 1 h, protected from light, the Cy5 fluorescence emission was recorded. Final data (Figures 3A and S8 and Table S8) were analysed and the $^{app}K_D$ was calculated using a sigmoidal fit (Dose Response) with GraphPad Prism version 9.5.1 for Mac OS (GraphPad software). Three independent experiments (n= 3) were used.

7. Competitive FRET-melting experiments. Diluted solutions of small molecules (BRACO-19, N-TASQ and PhpC) at 100 µM were prepared in diluting them with ddH_2O. Experiments were performed in 96-well plates using Mx3005P qPCR device (Agilent) equipped with FAM filters (λ_{ex} = 492 nm; λ_{em} = 516 nm). To a mixture of F-ON-T (ON: F21T or F-NRAS-T) (final concentration: 200 nM) and Caco.K buffer (Caco.K and Caco.K1 buffers for F21T and F-NRAS-T, respectively) were added 5 mol. equiv. (*i.e.*, 1 µM) of small molecule solutions in technical triplicates. For competition, to these mixtures were added 5-10-20 mol. equiv. (compared to ON, *i.e.*, 1-2-4 µM, respectively, which correspond to 1-2-4 mol. equiv. compared to G4 ligand, respectively) of competitors, in technical triplicates. After an initial increase step (from 25 to 90 °C, 30 s), a stepwise decrease (1 °C every 30 s for 67 cycles, from 90 to 25 °C) was performed followed by a stepwise increase (1 °C every 30 s for 67 cycles, from 25 to 90 °C), and measurements were made after each cycle (of the two stepwise parts). Final data of the last stepwise increase (Figures 3B and S9 and Tables S9 and S10) were analysed with Excel (Microsoft Corp.) and OriginPro Version 2018 (OriginLab Corporation). The emission of FAM was normalized (0 to 1) and the $T_{1/2}$ (°C), *i.e.,* the temperature

for which the normalized emission is 0.5, was recovered manually. The $\Delta T_{1/2}$ (°C) was calculated ($\Delta T_{1/2}$ (°C) = ($T_{1/2}$ ON ± G4 ligand ± competitor) – (Mean $T_{1/2}$ ON alone)). Three independent experiments (n = 3) were used. For statistical hypothesis student's t-test and Welch's unequal variances t-test were used depending on variances equality. This last Welch's *t*-test was precisely used for F21T (+ BRACO-19, PhpC or Clickable MultiTASQ) conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

8. Reverse competitive FRET-melting experiments. To a mixture of F-NRAS-T (final concentration: 200 nM) and Caco.K buffer were added 1-2.5-5 mol. equiv. (*i.e.*, 0.2-0.5-1 µM) of N-TASQ solution or 5 mol. equiv. (*i.e.*, 1 µM) of PhpC solution in technical triplicates. For reverse competition, to these mixtures with PhpC (at 5 mol. equiv.) were added 1-2.5-5 mol. equiv. (compared to ON, *i.e.*, 0.2-0.5-1 µM, respectively, which correspond to 0.2-0.5-1 mol. equiv. compared to PhpC, respectively) of N-TASQ competitor, in technical triplicates. After an initial increase step (from 25 to 90 °C, 30 s), a stepwise decrease (1 °C every 30 s for 67 cycles, from 90 to 25 °C) was performed followed by a stepwise increase (1 °C every 30 s for 67 cycles, from 25 to 90 °C), and measurements were made after each cycle (of the two stepwise parts). Final data of the last stepwise increase (Figure S10 and Table S11) were analyzed with Excel (Microsoft Corp.) and OriginPro Version 2018 (OriginLab Corporation). The emission of FAM was normalized (0 to 1) and the $T_{1/2}$ (°C), *i.e.,* the temperature for which the normalized emission is 0.5, was recovered manually. The $\Delta T_{1/2}$ (°C) was calculated ($\Delta T_{1/2}$ (°C) = ($T_{1/2}$ ON ± G4 ligand ± competitor) – (Mean $T_{1/2}$ ON alone)). Two independent experiments (n= 2) were used.

9. G4RP-RT-qPCR (or GARP.v2) experiments. MCF7 cells were seeded in 175 cm^2 flask at a density of 7.10^6 cells and allowed to recover overnight. Cells were treated live with BRACO-19 (8.4 µM in supplemented DMEM, 0.4% (v/v) DMSO, 48 h),[3] PhpC (90 µM in supplemented DMEM, 0.4% (v/v) DMSO, 48 h) or not (control; only supplemented DMEM, 0.4% (v/v) DMSO, 48 h). Cells were trypsinized, counted and then crosslinked using 1% (w/v) Formaldehyde/1X Fixing buffer (Table S12) for 5 min at 25 °C. The crosslink was then quenched with 0.125 M glycine for 5 min and washed with DEPC-PBS. Cells were resuspended in G4RP lysis buffer and then manually disrupted (1 mL syringe with 0.40 x 40 mm hypodermic needle, on ice). After centrifugation (13 200 rpm or 16 550 G, 10 min, 4 °C), the collected lysates (5% of which were collected as input control) were incubated with 80 µM TASQs (or 80 µM biotin as control) and 90 µg of Streptavidin MagneSphere Paramagnetic Particles (Promega, cat. n° Z5481) for 2 h at 4 °C under agitation (G4-precipitation). Magnetic beads were then washed with G4RP wash buffer (5 min at 25 °C, under agitation, x1) and DEPC-PBS (5 min at 25 °C, under agitation, x1) before being resuspended in DEPC-PBS supplemented with 0.4 U RNAse OUT (Invitrogen, cat. n° 10777019). The beads were then incubated at 70 °C for 2 h to release captured G4-forming targets from the beads (reverse crosslinking). For each tube, 1 mL of TRIzol (Invitrogen, cat. n° 15596026) and 0.2 mL of chloroform were used to extract RNAs (using manufacturer's instructions) before to be cleaned with RNA Clean-up protocol (RNA Clean & Concentrator-5, Zymo Research, cat. n° ZR1013; using manufacturer's instructions) at 25 °C. Extracted RNA was reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen, cat. n° 18080-044) and random hexamer primers (Invitrogen, cat. n° N8080127) using manufacturer's protocol to generate cDNA. cDNA was quantified using i*Taq* Universal SYBR Green Supermix (Bio-Rad, cat. n° 1725121) and the NRAS forward and reverse primers set (Table S1), in technical replicates. It was performed in 96-well plates using Mx3005P qPCR device (Agilent) equipped with SYBR Green filters (λ_{ex} = 492 nm; λ_{em} = 516 nm) in 10 µL final volume. Ct values of G4-precipitated RNA were normalized to the input control, as follows: G4RP-RT-qPCR signal (fold change) = $5*(2^{(\text{Mean Ct input}) - \text{Ct G4RP } or \text{ biotin}})$ with Mean Ct input corresponds to the mean of Ct obtained with the 5% input control, for each biological replicate;

Ct G4RP (or biotin) corresponds to the individual technical Ct obtained with samples G4-precipitated with TASQ (or precipitated with biotin), for each biological replicate. Three independent experiments (n= 3) were used. Final data (Figure 3C and Table S13) were analysed with Excel (Microsoft Corp.) and OriginPro, Version 2018 (OriginLab Corporation). For statistical hypothesis student's t-test and Welch's unequal variances t-test were used depending on variances equality. This last Welch's *t*-test was precisely used for BioTASQ (+ BRACO-19) and BioCyTASQ (+ BRACO-19) conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

------ Supplementary Tables

Table S1. List of oligonucleotides used in this study.

Name	Nature	Length (in nucleotide)	Sequence	Experiment	Reference
$^{5'}$Cy5-Myc	DNA	22	Cy5-d[$^{5'}$GAGGGTGGGGAGGGTGGGGAAG$^{3'}$]	Fluorescence quench assay	4
$^{5'}$Cy5-NRAS	RNA	18	Cy5-r[$^{5'}$GGGAGGGGCGGGUCUGGG$^{3'}$]	Fluorescence quench assay	This study
c-Kit2 QSA	DNA	100	d[$^{5'}$TAGCCATTCAGCCGTAACAGGCAGTGGAAGAGAGACAGACACGGGCGGGCGCGAGGGAGGGGCAGTACAGTAGAACCTAATGGTGTTTGATGGTATCTAA$^{3'}$]	qPCR stop assay	This study
c-Myc QSA	DNA	101	d[$^{5'}$TAGCCATTCAGCCGTAACAGGCAGTGGAAGAGAGACAGACAGAGGGTGGGGAGGGTGGGGAAGCAGTACAGTAGAACCTAATGGTGTTTGATGGTATCTAA$^{3'}$]	qPCR stop assay	This study
F21T	DNA	21	FAM-d[$^{5'}$GGGTTAGGGTTAGGGTTAGGG$^{3'}$]-TAMRA	Competitive FRET-melting	5
F-NRAS-T	RNA	18	FAM-r[$^{5'}$GGGAGGGGCGGGUCUGGG$^{3'}$]-TAMRA	Competitive FRET-melting	6
hTelo (22AG)	DNA	22	d[$^{5'}$AGGGTTAGGGTTAGGGTTAGGG$^{3'}$]	Fluorescence titration	7
hTelo QSA	DNA	100	d[$^{5'}$TAGCCATTCAGCCGTAACAGGCAGTGGAAGAGAGACAGACAGGGTTAGGGTTAGGGTTAGGGCAGTACAGTAGAACCTAATGGTGTTTGATGGTATCTAA$^{3'}$]	qPCR stop assay	This study
NRAS forward primer	DNA	23	d[$^{5'}$ATGACTGAGTACAAACTGGTGGT$^{3'}$]	G4RP-RT-qPCR	3
NRAS reverse primer	DNA	23	d[$^{5'}$CATGTATTGGTCTCTCATGGCAC$^{3'}$]	G4RP-RT-qPCR	3
QSA primer	DNA	25	d[$^{5'}$TTAGATACCATCAAACACCATTAGG$^{3'}$]	qPCR stop assay	8
S. pombe G4	DNA	97	d[$^{5'}$TAGCCATTCAGCCGTAACAGGCAGTGGAAGAGAGACAGACAGGGCAGGGCAGGGCAGGGCAGTACAGTAGAACCTAATGGTGTTTGATGGTATCTAA$^{3'}$]	qPCR stop assay	9
ssDNA	DNA	14	d[$^{5'}$CCACGCTCGTTCCG$^{3'}$]	Fluorescence titration	This study

Cy5 : Cyanine 5 (Cy5 filter on CLARIOstar Plus: λ_{ex} = 610 nm; λ_{em} = 675 nm)
FAM : 6-Carboxyfluorescein (FAM filter on Mx3005P: λ_{ex} = 492 nm; λ_{em} = 516 nm)
TAMRA: 5-Carboxytetramethylrhodamine (TAMRA filter on Mx3005P: λ_{ex} = 556 nm; λ_{em} = 580 nm)
QSA = qPCR Stop assay

Table S2. Summary of the Mean SYBR Green fluorescence intensity and ΔFI values calculated by qPCR Stop assays with several G4-DNA templates (*S. pombe* G4, *H. sapiens* hTelo G4, c-Kit2 G4 and c-Myc G4).

Mean SYBR Green fluorescence intensity (FI)				
	S.pombe G4	hTelo G4	c-Kit2 G4	c-Myc G4
Control	3122.00 ± 103.55	2967.67 ± 34.95	2952.00 ± 24.04	2441.67 ± 17.79
PhpC (5 eq.)	3295.50 ± 21.92	3089.33 ± 37.11	3030.50 ± 43.13	2496.67 ± 10.69
ΔFI				
	S.pombe G4	hTelo G4	c-Kit2 G4	c-Myc G4
PhpC (5 eq.)	173.50	121.66	78.50	55.00

Table S3. Summary of the PhpC fluorescence intensity quenching at 452 nm (%) by fluorescence titration with a 14-mer ssDNA or the hTelo G4-DNA.

PhpC fluorescence intensity (FI) at 452 nm				
	Titration with 14-mer ssDNA		Titration with hTelo G4-DNA	
	FI	Variation (%)	FI	Variation (%)
PhpC alone	609.39	/	566.88	/
1 mol. equiv.	593.79	-2.56	479.24	-15.46
2 mol. equiv.	574.71	-5.69	437.80	-22.77
5 mol. equiv.	527.40	-13.45	335.06	-40.89

Table S4. Concentration range of G4 ligands (BRACO-19, PhpC) and G4-probe (N-TASQ) used for the SRB assay.

BRACO-19 conc. (in μM)	0.05	1	5	10	15	20	30	50	100	200
N-TASQ conc. (in μM)	0.1	1	2	5	10	20	40	60	100	200
PhpC conc. (in μM)	0.1	2	12.5	25	50	100	200	400	600	1000

Table S5. Summary of the IC (inhibitory concentration) values calculated by the SRB cytotoxicity assays.

Inhibitory concentration (μM)	BRACO-19	PhpC	N-TASQ
IC_{20}	9.51 ± 2.03	148.69 ± 26.74	127.42 ± 43.10
IC_{50}	18.66 ± 2.80	387.92 ± 41.36	308.49 ± 192.23
IC_{80}	36.59 ± 4.34	1012.01 ± 156.30	746.89 ± 837.52

Table S6. Summary of the data collected and calculated after analysing confocal images with the N-TASQ macro program. Quantification was performed as described in Method (3. Method for the automated N-TASQ fluorescence quantification).

	Control	PhpC (20 µM, 8 h)
Number of images analysed	2	3
N-TASQ FI threshold	45	120
Mean background FI (IntDen)	203.14	754.27
Total number of cells analysed	84	118
Mean volume of cell (μm^3)	2 435.2	2 965.6
Mean FI of cell (IntDen)	2 963 931.5	7 601 997.1
Normalized mean FI of cell (IntDen) [a]	14 590.6	10 078.6
Total number of N-TASQ *foci* collected	7 270	3 181
Mean number of N-TASQ *foci* per cell	86.55	26,96
Mean volume of N-TASQ *foci* (μm^3)	0.63	0.59
Mean FI of N-TASQ *foci* (IntDen)	4 001.0	9 149.2
Normalized mean FI of N-TASQ *foci* (IntDen) [a]	19.70	12.13
Portion of N-TASQ *foci* volume in total cell volume (%)	0.026	0.020
Portion of N-TASQ *foci* FI in total cell FI (%)	0.13	0.12
Portion of diffuse N-TASQ FI in total cell FI (%)	99.87	99.88
Number of big N-TASQ *foci* (volume > [2 * Mean volume]) per cell	3.20	1.78

[a] Normalization of Fluorescence Intensity (FI) has been made in dividing FI values from a condition by their respective Mean background FI.

Table S7. Concentration range of G4 ligand used for the fluorescence quenching assay.

Diluted solutions conc. (in µM)	0.00675	0.0135	0.027	0.05	0.11	0.217	0.43	0.87	1.74	3.47	6.94	13.89	27.78	55.55	111.11
Final conc. (in µM)	0.006	0.012	0.024	0.049	0.098	0.195	0.391	0.781	1.563	3.125	6.25	12.5	25	50	100
mol. equiv. (compared to $^{5'}$Cy5-ON)	0.3	0.6	1.2	2.45	4.9	9.75	19.55	39.05	78.15	156.25	312.5	625	1250	2500	5000

Table S8. Summary of the $^{app}K_D$ (apparent affinity constant) values calculated by FQA assays.

$^{app}K_D$ (µM)	BRACO-19	PhpC	Clickable MultiTASQ	N-TASQ
$^{5'}$Cy5-Myc	0.37 ± 0.06	>100	0.65 ± 0.11	1.00 ± 0.03
$^{5'}$Cy5-NRAS	0.86 ± 0.26	>100	0.50 ± 0.05	0.51 ± 0.01

Table S9. Summary of the Mean $\Delta T_{1/2}$ (°C) values calculated by Competitive FRET-melting experiments with F-NRAS-T (RNA).

	Mean $\Delta T_{1/2}$ (°C)			
Ligand	No competitor	5 eq. (competitor)	10 eq. (competitor)	20 eq. (competitor)
No ligand (ON alone)	0.00 ± 2.52	/	/	/
BRACO-19	18.12 ± 0.37	/	/	/
PhpC	-3.38 ± 0.41	/	/	/
Clickable MultiTASQ	12.31 ± 0.28	12.28 ± 0.25 (PhpC)	12.43 ± 0.33 (PhpC)	12.40 ± 0.36 (PhpC)
N-TASQ	11.91 ± 0.90	11.98 ± 0.52 (PhpC)	11.13 ± 0.67 (PhpC)	11.68 ± 0.15 (PhpC)

Table S10. Summary of the Mean $\Delta T_{1/2}$ (°C) values calculated by Competitive FRET-melting experiments with F21T (DNA).

	Mean $\Delta T_{1/2}$ (°C)			
Ligand	No competitor	5 eq. (competitor)	10 eq. (competitor)	20 eq. (competitor)
No ligand (ON alone)	0.00 ± 0.16	/	/	/
BRACO-19	20.01 ± 1.27	/	/	/
PhpC	-0.36 ± 1.00	/	/	/
Clickable MultiTASQ	9.56 ± 0.86	8.63 ± 0.27 (PhpC)	8.74 ± 0.21 (PhpC)	8.96 ± 0.46 (PhpC)
N-TASQ	9.80 ± 0.74	9.71 ± 1.73 (PhpC)	9.04 ± 1.91 (PhpC)	9.32 ± 2.09 (PhpC)

Table S11. Summary of the Mean $\Delta T_{1/2}$ (°C) values calculated by reverse Competitive FRET-melting experiments with F-NRAS-T (RNA).

	Mean $\Delta T_{1/2}$ (°C)			
Ligand	No ligand	1 eq.	2.5 eq.	5 eq.
N-TASQ	0.00 ± 0.18	1.72 ± 0.21	3.79 ± 0.50	6.56 ± 0.65
Ligand	No competitor	1 eq. (competitor)	2.5 eq. (competitor)	5 eq. (competitor)
PhpC (5 eq.)	0.45 ± 0.14	1.61 ± 0.13 (N-TASQ)	3.25 ± 0.39 (N-TASQ)	5.65 ± 0.62 (N-TASQ)

Table S12. Summary of buffers and solutions used for G4RP-RT-qPCR.

Name	Composition
DEPC-PBS	0.09% (v/v) DEPC, 1X PBS, in DEPC-H_2O
DEPC-H_2O	0.1% (v/v) DEPC-treated UltraPure Distilled Water (Invitrogen, cat. n° 10977035)
5X Fixing buffer	250 mM HEPES KOH, 500 mM NaCl, 5 mM EDTA, 2.5 mM EGTA, in DEPC-H_2O
1% (w/v) Formaldehyde/1X Fixing buffer	1% (w/v) formaldehyde, 50 mM HEPES KOH, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, in DEPC-H_2O
G4RP buffer	150 mM KCl, 25 mM Tris, 5 mM EDTA, 0.5 mM DTT, 0.5% (v/v) Tergitol, pH 7.4, in DEPC-H_2O
1 M Glycine	1 M glycine, in DEPC-H_2O
G4RP lysis buffer	0.1% (w/v) SDS, 0.1 U/µL RNase OUT, in G4RP buffer
G4RP wash buffer	0.1 U/µL RNase OUT, in G4RP buffer

Table S13. Summary of the G4RP-RT-qPCR signals (fold change) calculated by G4RP-RT-qPCR (G4RP.v2) for the NRAS G4-RNA (G4-precipited and purified from cells).

G4RP-RT-qPCR signal (fold change)			
(G4-)Precipitation with	Control cells	BRACO-19-treated cells	PhpC-treated cells
Biotin (control)	0.23 ± 0.33	0.31 ± 0.23	0.23 ± 0.22
BioTASQ	5.74 ± 1.77	15.66 ± 9.59	3.48 ± 0.66
BioCyTASQ	4.66 ± 1.78	9.54 ± 5.23	2.42 ± 0.85
Clicked ^{az}MultiTASQ	4.81 ± 1.03	7.10 ± 1.74	2.28 ± 1.09

------ Supplementary Figures

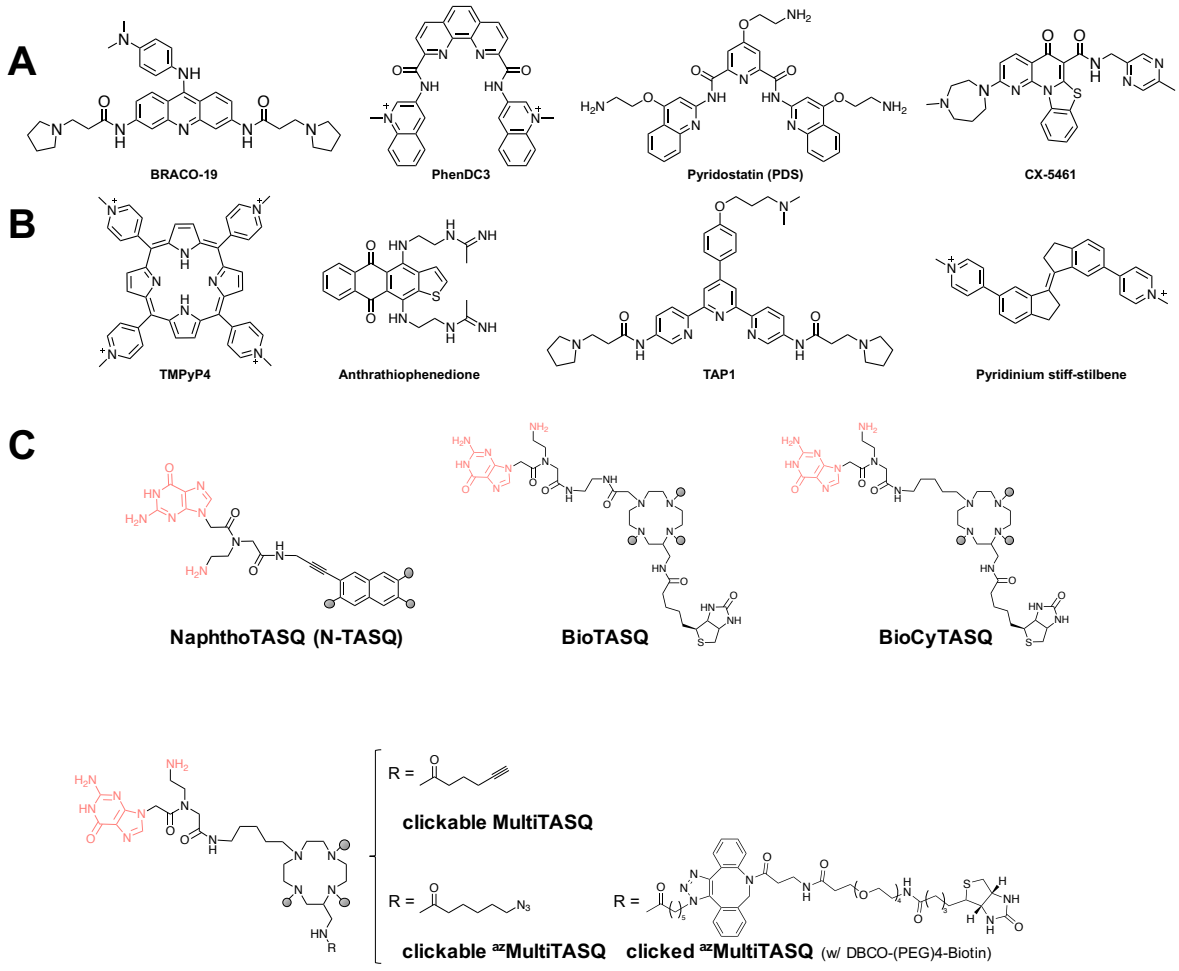

Figure S1. Chemical structures of **A**. G4 stabilizers (BRACO-19, PhenDC3, PDS and CX-5461), **B**. G4 destabilizers (TMPyP4, anthrathiophendione, TAP1 and a stiff-stilbene), and **C**. the TASQs used in this study: N-TASQ, BioTASQ, BioCyTASQ, MultiTASQ and ^{az}MultiTASQ (clickable and clicked versions).

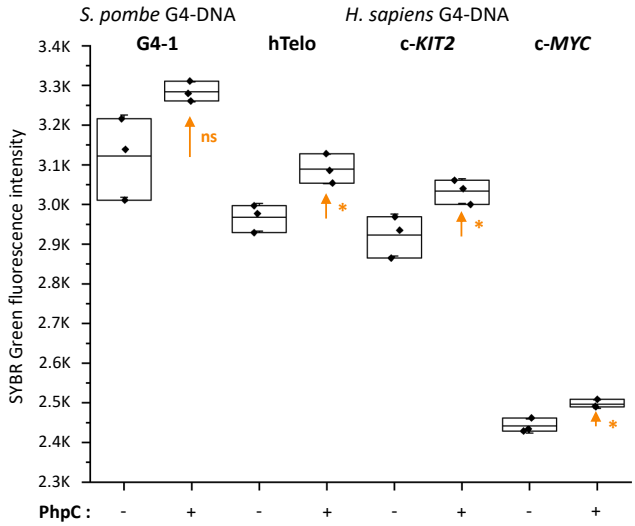

Figure S2. qPCR stop assay results (n = 3) obtained with 5 mol. equiv. of PhpC with TAG-C_2A-T_2C-AGC-CGT-A_2C-AG_2-CAG-TG_2-A_2G-AGA-GAC-AGA-CA-**X**-C-AGT-ACA-GTA-GA_2-C_2T-A_2T-G_2T-GT_2-TGA-TG_2-TAT-CTA-A where **X** is: G-GGC-AGG-GCA-GGG-CAG-GG for *S. Pombe* G4; G-GGT-TAG-GGT-TAG-GGT-TAG-GG for hTelo G4; G-ACG-GGC-GGG-CGC-GAG-GGA-GGG-G for c-Kit2 G4; and G-AGG-GTG-GGG-AGG-GTG-GGG-AAG for c-Myc G4 (two-sample t-test: * $p < 0.05$).

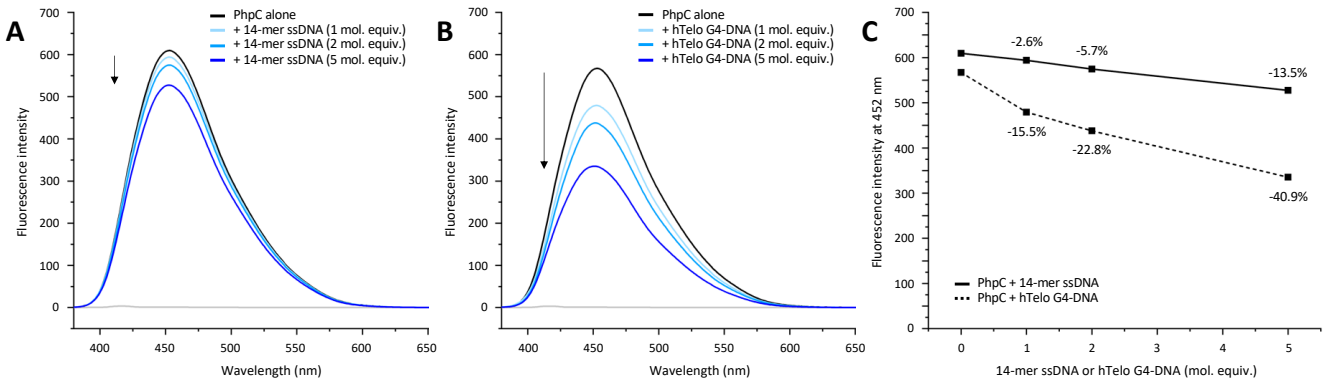

Figure S3. Fluorescence titration experiments performed with PhpC (2 μM) alone or in the presence of increasing concentrations (2-10 μM, 1-5 mol. equiv.) of either a ssDNA (**A**, d[C_2ACGCTCGT_2C_2G]) or a G4 (**B**, hTelo: d[$G_3(T_2AG_3)_3$]) carried out in 1 mL of 10 mM lithium cacodylate buffer (pH 7.2) + 90 mM LiCl/10 mM KCl, with λ_{ex} = 365 nm and λ_{em} = 380-700 nm. **C**. PhpC fluorescence intensity quenching (at 452 nm) was calculated after each addition of both ssDNA (solid line) and hTelo (hatched).

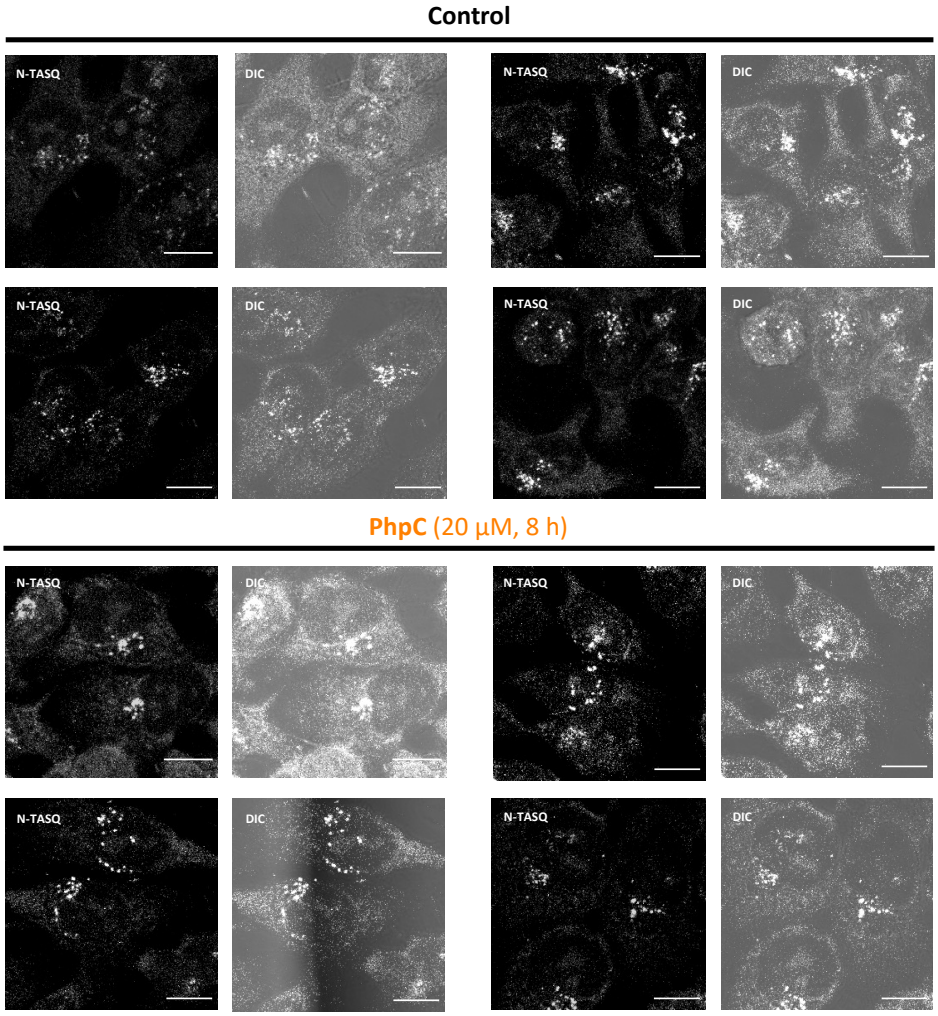

Figure S4. Additional optical images obtained with N-TASQ. Scale bars= 10 µm.

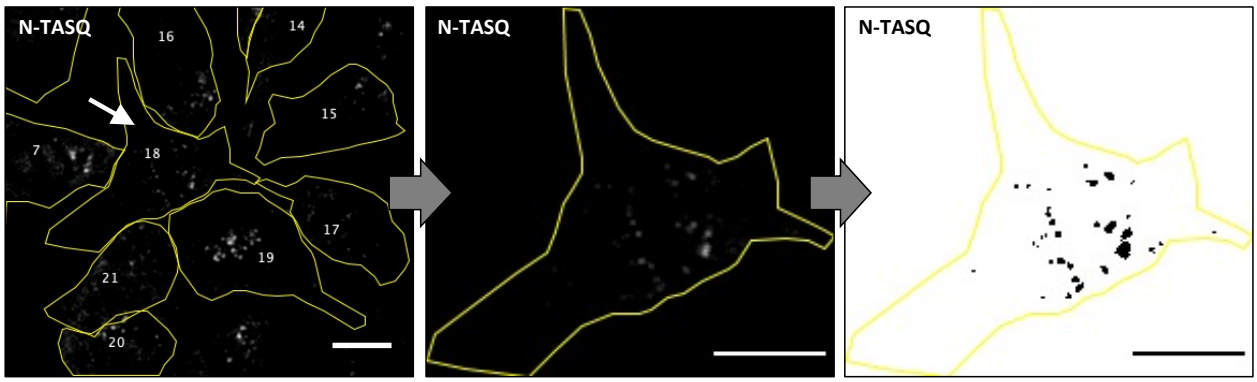

Figure S5. Representative images of the confocal images processing with the N-TASQ macro program in order to study N-TASQ *foci* (here only on one cell slice). After manual surrounding of cells (and the cell of interest, shown with the white arrow) to create Regions of Interest (ROI; surrounded with yellow line), these ROI (cells) are counted and numbered (left), then separated and treated ("Clear outside" and "Smooth" methods) (middle) and N-TASQ *foci* inside are studied ("3D Object Counter" method). Scale bars = 10 µm.

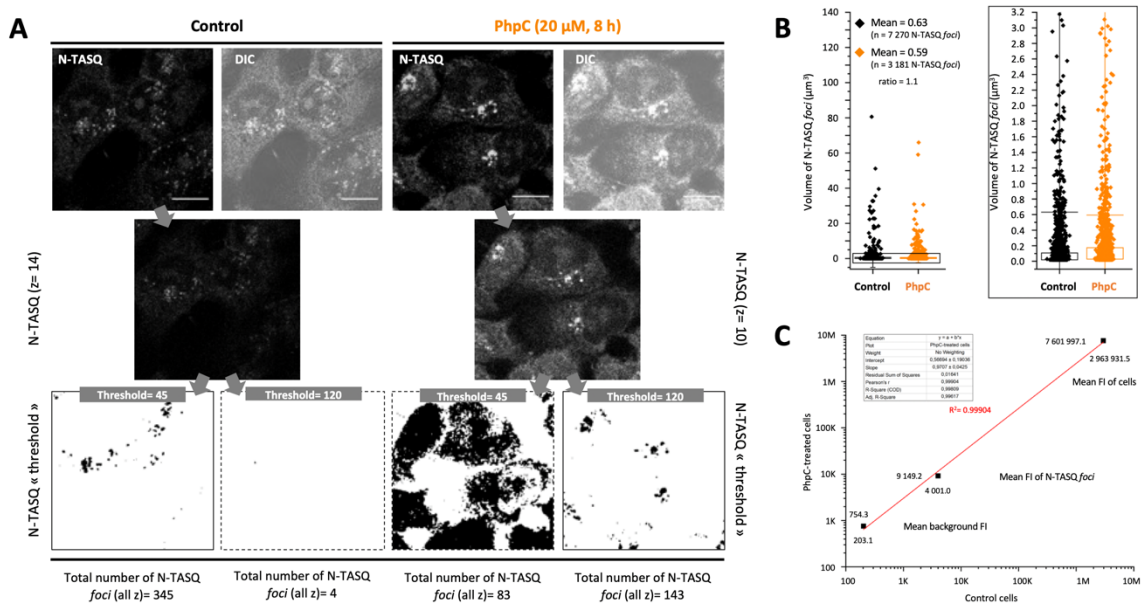

Figure S6. **A**. Methodology for the selection of the most adapted fluorescence threshold in each experimental condition. **B**. Distribution of the volume of the N-TASQ *foci* in control *versus* PhpC-treated cells obtained after the selection of the most suited threshold. **C**. Correlation between different fluorescence parameters in control and PhpC-treated cells.

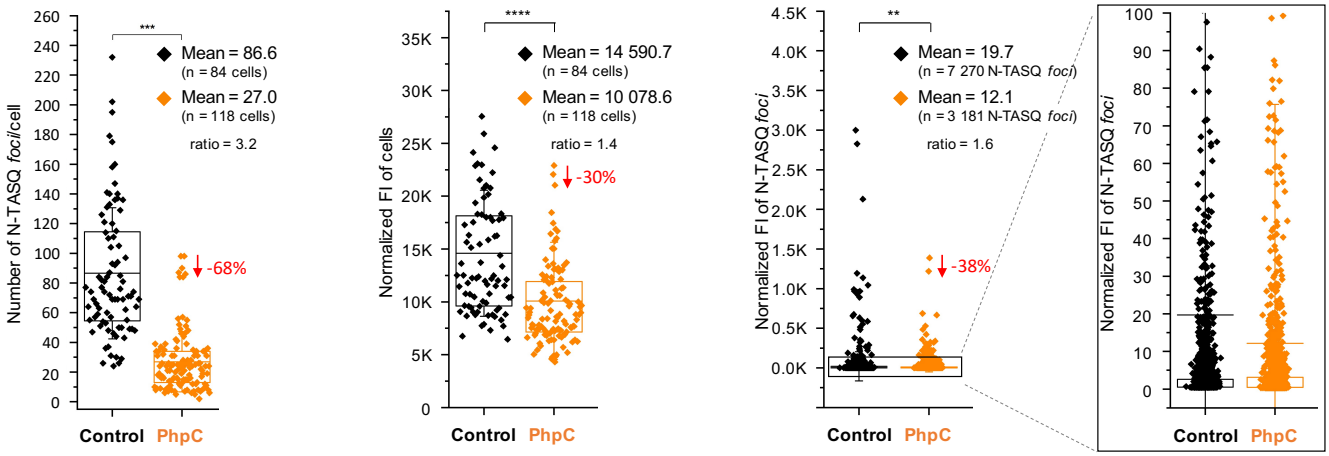

Figure S7. Different numerical exploitations of the number (left), FI/cells (normalized; center) and FI/foci (normalized; right) of N-TASQ in control (black) versus PhpC-treated cells (orange).

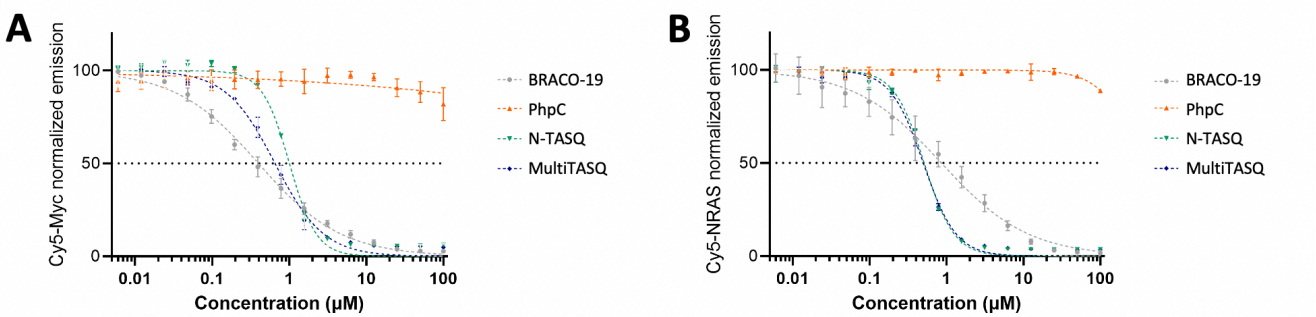

Figure S8. Fluorescence quenching assay performed with $^{5'}$Cy5-Myc (**A**) and $^{5'}$Cy5-NRAS (**B**) and BRACO-19, Clickable MultiTASQ, N-TASQ and PhpC.

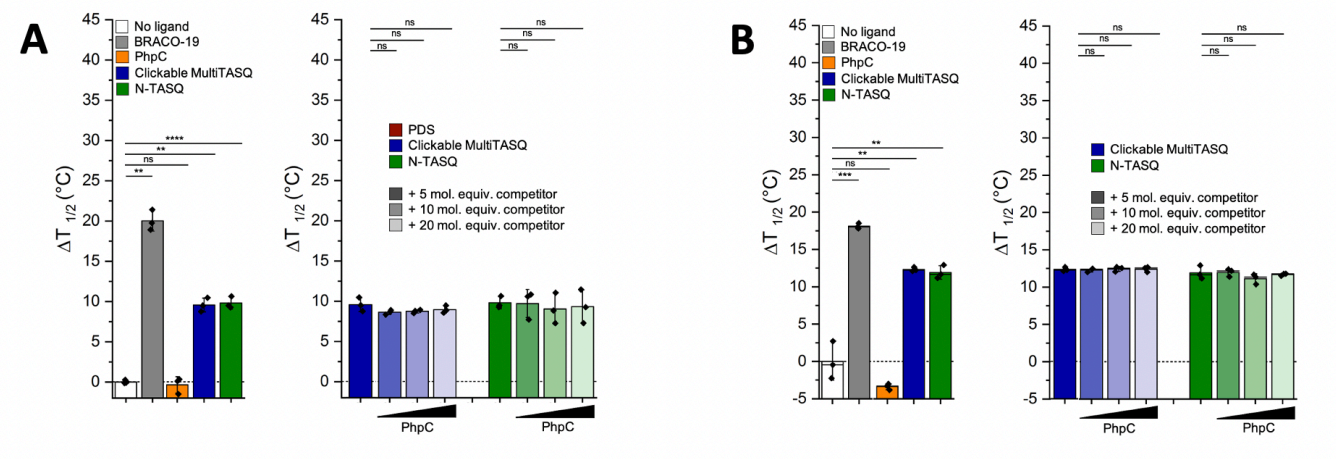

Figure S9. FRET-melting experiments performed with F21T (**A**) and F-NRAS-T (**B**) and several G4 ligands (BRACO-19, PhpC, MultiTASQ, N-TASQ) (left) and competitive FRET-melting experiments with F21T (**A**) and F-NRAS-T (**B**) and either Clickable MultiTASQ or N-TASQ *versus* an excess of PhpC (right). Error bars represent SD from the means for three independent experiments.

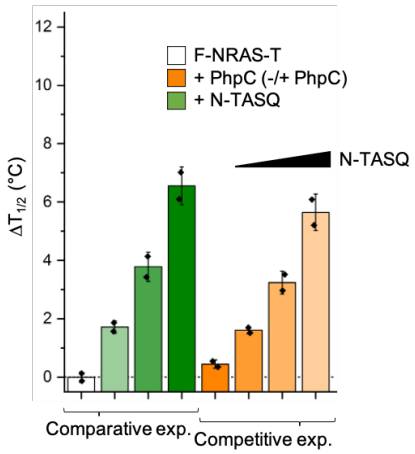

Figure S10. FRET-melting experiments with F-NRAS-T and N-TASQ (1-2.5-5 mol. equiv.) (left) and reverse competitive FRET-melting experiments with F-NRAS-T and PhpC (5 mol. equiv.) *versus* an increasing concentration of N-TASQ (1-2.5-5 mol. equiv.) (right). Error bars represent SD from the means for two independent experiments.

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