SUPPORTING INFORMATION

G-Quadruplex DNA as a Molecular Target for Induced Synthetic Lethality in Cancer Cells

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Table S1. Physical Data for PDS Ligands

Compound	CLogP	Mass	FRET DT_m at 1 μM, K	FRET DT_m at 1 μM, K	FRET DT_m at 1 μM, K	FRET DT_m at 1 μM, K	GI_{50} (HT1080) μM	GI_{50} (HT1080) μM
			H-Telo	C-MYC	K-RAS	Ds-DNA	Previous study	This study
PDS	2.1	596	34.8±0.6	16.8±0.8	20.2±0.8	0.5±0.2	0.6±0.1	0.9±0.4
PDSI	3.3	571	36.5±0.9	17.1±0.8	25.7±0.7	0.0±0.0	1.8±0.2	0.4±0.1
PDSK	6.9	758	35.5±0.8	16.0±1.0	25.7±0.7	0.9±0.2	2.2±0.2	3.2±0.6
NU7441	5.4	413	n/a	n/a	n/a	n/a	n/a	2.6±0.7

Previous study; FRET/GI_{50} ([19])

Table S2. GI_{50} values for binary PDSI and NU7441 treatments in HCT116 WT and BRCA2$^{-/-}$ cells (μM ± Standard error).

Cell Line	GI_{50} PDSI	GI_{50} NU7441	GI_{50} Binary	I-value
HCT116 WT	3.8 ± 0.7	4.8 ± 0.2	1.7 ± 0.3	0.66 ± 0.02
BRCA2$^{-/-}$	1.8 ± 0.3	2.23 ± 0.14	0.28 ± 0.07	0.60 ± 0.01

Table S3. Statistical analysis details for nuclear 53BP1 and nuclear RAD51 levels, data from Figure 3B, 3C, 3G and 3H. Mean significance quoted between pairwise columns as *P<0.05, **P<0.01, ***P<0.001 for each cell line tested (HT1080, HCT116 WT or BRCA2$^{-/-}$) using unpaired student t-test (two tail). ns denotes 'no significant difference'

HT1080	**Nuclear 53BP1**			**Nuclear RAD51**		
	PDSI	**NU7441**	**Binary**	**PDSI**	**NU7441**	**Binary**
Control	***	***	***	***	ns	***
PDSI		***	***		***	***
NU7441			***			***

HCT116	**Nuclear 53BP1**			**Nuclear RAD51**		
	PDSI	**NU7441**	**Binary**	**PDSI**	**NU7441**	**Binary**
Control	***	***	***	ns	ns	ns
PDSI		ns	*		ns	ns
NU7441			*			ns

BRCA2$^{-/-}$	**Nuclear 53BP1**			**Nuclear RAD51**		
	PDSI	**NU7441**	**Binary**	**PDSI**	**NU7441**	**Binary**
Control	***	***	***	***	***	***
PDSI		***	ns		***	**
NU7441			***			***

SUPPLEMENTARY FIGURES

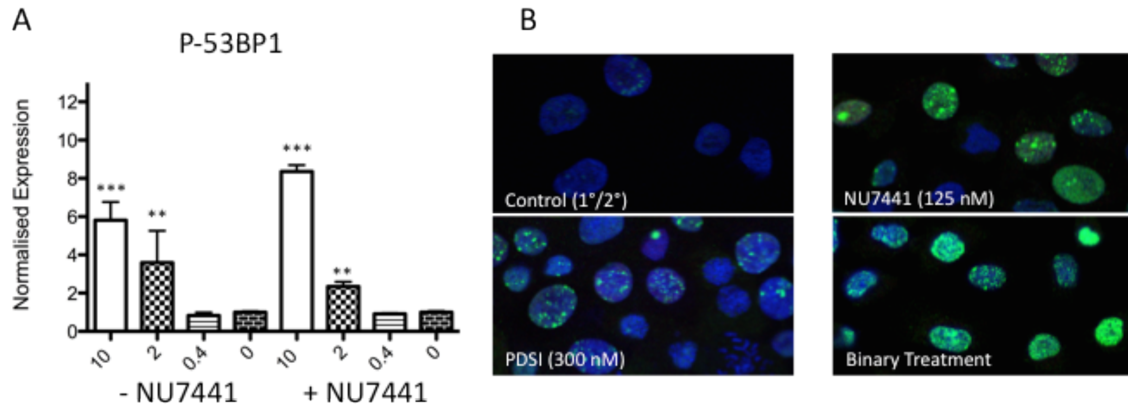


Figure S1. Induction of DNA damage response in HT1080 cells. DNA damage marker phospho-53BP1 (nuclear) **(A)** protein levels as measured using in-cell western blotting for HT1080 cells treated with PDSI (0-10 μM) and PDSI + NU7441 (1 μM). All wells were normalised to *ß*-actin (ACTB1) levels and different treatments normalized to non-treated control. *P<0.05, **P<0.01, ***P<0.001. **(B)** Visualisation of 53BP1 DNA DSB damage marker nuclear foci (green) in HT1080 cells treated with PDSI (0.3 μM) and NU7441 (0.125 μM). Microscopy was performed using a Leica Tandem confocal with a 63x oil phase objective. Cell nuclei are stained with Hoechst 33342.

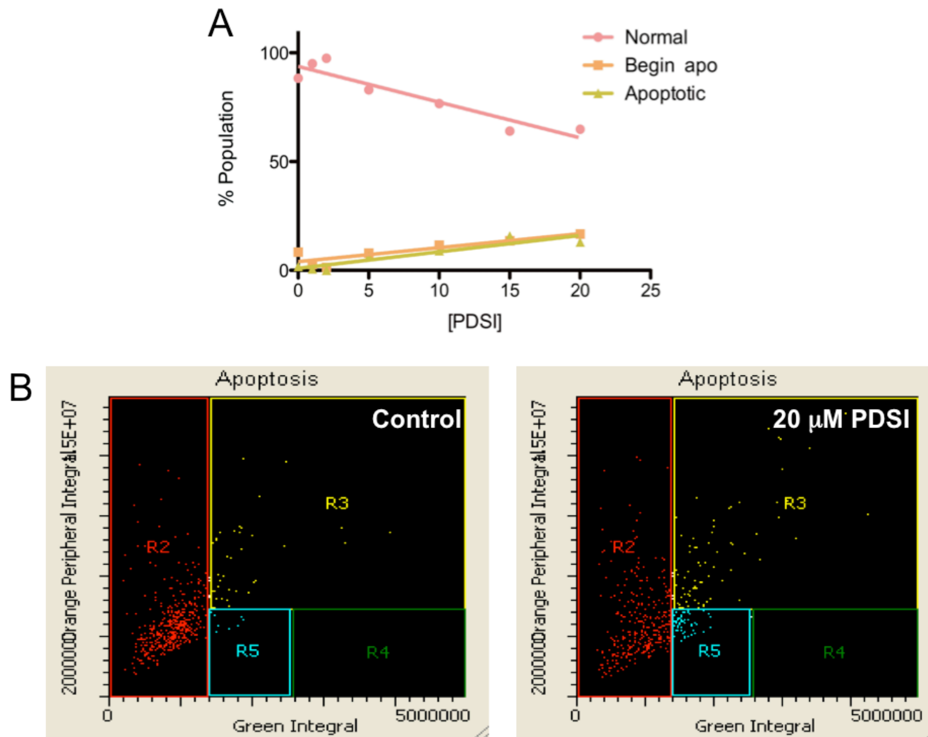


Figure S2. Induction of apoptosis with increasing dose of PDSI in HT1080 cells as measured by laser scanning cytometry. **A** illustrates proportion of normal, early and late apoptotic cells as dose increases. **B** illustrates a scatter plot output for control (left) or 20 μM PDSI (right). Where: R2 corresponds to normal cells with intact plasma membranes, many with cytoplasmic Mitoshift staining; R3, early apoptotic cells which become permeable to Yo-Pro-1; R4, loss of mitochondrial staining potential (Mitoshift); and R5, highly permeable cells which lose Yo-Pro1 staining (and also stain with propidium iodide).

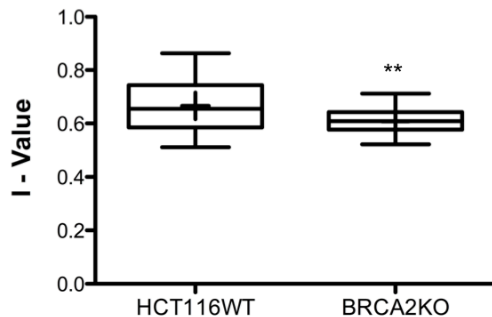
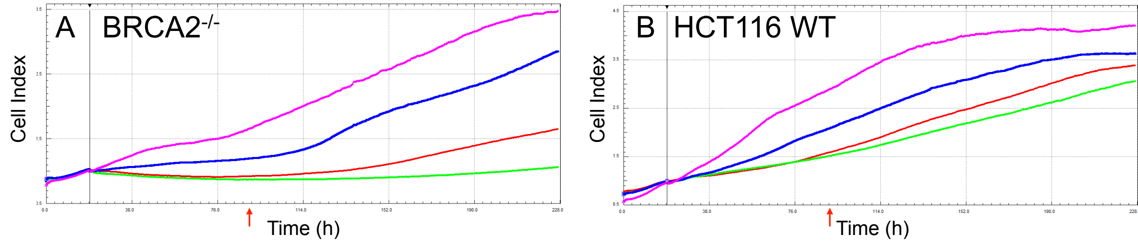
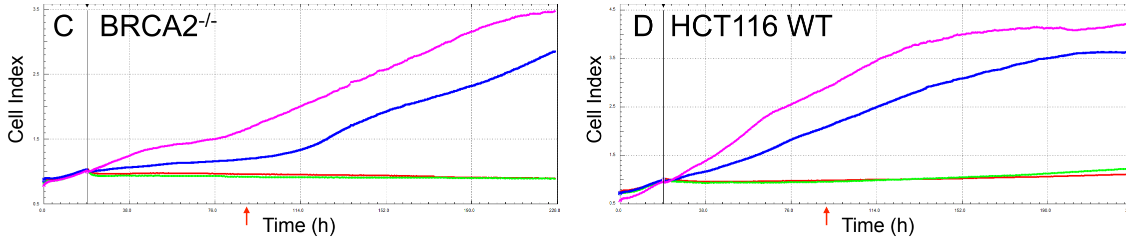


Figure S3. Spread of I-Values calculated from GI_{50} values in HCT116 WT cells and $BRCA2^{-/-}$ cells. Concentration of NU7441 was 1 μM for combination treatments. Mean values were 0.66 for HCT116 WT and 0.6 for $BRCA2^{-/-}$ cells (Table S2). **P<0.01, student-T-test, 1 or 2 tail.



Key: Pink – Control; Blue – NU7441 (1 μM); Red – PDSI (2 μM); Green – PDSI (2 μM) + NU7441 (1 μM)



Key: Pink – Control; Blue – NU7441 (1 μM); Red – PDSI (10 μM); Green – PDSI (10 μM) + NU7441 (1 μM)

Figure S4. Illustrative xCELLigence RTCA-SP plots monitoring continuous cell growth in real-time for BRCA2$^{-/-}$ (A, C) and HCT116 WT cells (B,D) after treatment with PDSI and/or NU7441. A and B illustrate the response with 2 μM PDSI treatments. C and D illustrate the response with 10 μM PDSI. NU7441 dose was 1 μM in all panels. Key is in the figure. The vertical line at approx 20 h is when drug is added, and the red arrow denotes 72 h later when GI_{50} values are calculated. Figures are extracted from xCELLigence software.

SUPPLEMENTARY MATERIALS AND METHODS

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Chemical Synthesis

All the building blocks required for the chemical synthesis of PDS analogues were prepared according to previously described methods.[19] PDSA and PDSK were synthesised as previously reported.[19] PDSI was synthesised according the following procedure: Chelidamic acid hydrate (1.0 g, 5.1 mmol) was suspended in thionyl chloride (100 mL) and refluxed for 3 days. The solvent was then removed *in vacuo* and a white solid was obtained, which was dissolved in freshly distilled dichloromethane (10 mL). The solution was then cooled at 0 °C, bubbled with fresh argon and added to dry triethylamine (1 mL) with slow stirring for 1 h. 4-(2-*tert*-Butoxycarbonylamino-ethoxy)-quinolin-2-ylamine (3.10 g, 10.2 mmol), prepared according to a procedure already described[19], suspended in dry dichloromethane (5 mL) was then added and the reaction temperature was allowed to rise to 25°C. The reaction was stirred overnight, the solvent then evaporated *in vacuo* and the crude product recrystallized from dry acetonitrile (5 mL). The Boc protected product was collected by filtration as a white solid, suspended in dichloromethane (10 mL) and added to trifluoroacetic acid (2 mL) with stirring. The yellow solution was stirred for 5 h before the solvent removed *in vacuo*. Upon addition of diethyl ether to the yellow oil, PDSI (2xTFA salt) precipitated as a white powder and was collected by filtration (1.1 g, 55%). The purity was checked by LC-MS and further HPLC purification was performed if needed. Spectroscopic data were in agreement with those previously reported.[19]

Cells and cell culture

Human HT1080 fibrosarcoma cells (ATCC# CCL-121) were grown in Dulbecco's Minimum Essential Media (supplemented with 10% foetal calf serum) at 37°C in 5% CO_2 in air. HCT116 colorectal carcinoma cells (ATCC# CCL-247) were grown in McCoy's 5a Medium (supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2 in air. HCT116 WT and $BRCA2^{-/-}$ cells were provided by Professor Carlos Caldas (CRUK Cambridge Institute, Cambridge, UK).

Measurement of growth inhibition using impedance-based continuous cell monitoring

Cell growth was recorded continuously during treatment with small molecules using the xCELLigence RTCA-SP real-time cell monitoring system (ACEA). Briefly, this system uses a proprietary 96-well E-plate format where a gold-plated electrode is

embedded on the bottom surface of each tissue culture well. The measurement of impedance across the electrode in each well is dependent on the overall surface area of cellular contact. The measured impedance is monitored during the time course of cell culture and will change upon cellular insult or growth as cells adhere, replicate, change morphology or lose well contact during cell death. A 'Cell Index' measure is calculated and allows comparison between different well conditions, always normalized to media only control.[S1]

In a typical experiment cells (HT1080, HCT116 WT or HCT116 BRCA2$^{-/-}$) are seeded in 96-well E-plates (5,000 cells/well) and allowed to adhere and begin log phase growth (typically 18-20 h, 37°C in 5% CO_2 in air). The E-plate is removed at 20 h and cells treated with PDSI, PDS, PDSK and NU7441 (generally 50, 10, 2 and 0.4 μM). The plate was re-installed into the RTCA-SP device and cell growth monitored for 72 h. All growth, measured as impedance and plotted as cell index, is monitored without disturbing the E-plate for the remainder of the experiment.

Growth data were analyzed and GI_{50} curves plotted using proprietary xCELLigence software. I-values were calculated using the equation; $I = a/A + b/B$, where a is the PDSI GI_{50} in combination with NU7441 at concentration b, A is the PDSI only GI_{50} and B is the NU7441 only GI_{50}.[26] When $I < 1$, the interaction is synergistic; when $I = 1$, the interaction is additive; and when $I > 1$, there is an antagonistic interaction.

In Cell Western assay

Cells (HT1080) were seeded in 96-well clear bottomed black plates (10, 000/well; Corning) and incubated at 37°C in 5% CO_2 in air for 18 h to attach. Cells were treated with PDSI and NU7441 and incubated at 37°C in 5% CO_2 in air for 24 h. Media was removed and cells were fixed with 4% formaldehyde in neutral buffered saline (CellPath) for 20 min, washed with 0.1% Triton-X (in PBS; 5 x 5 min with gentle shaking) and incubated with Odyssey blocking buffer (Li-Cor) for 90 mins. Rabbit anti phospho-53BP1 (1:200 dilution, #2674, Cell Signalling) and mouse anti ACTB1 (1:200 dilution, ab6276, Abcam) were diluted with Odyssey blocking buffer and incubated at 4°C overnight. Unbound antibodies were removed with 0.1% Tween (in PBS; 5 x 5 min with gentle shaking). Secondary antibodies (IRDye® 800CW goat anti-rabbit and 68LT0 goat anti-mouse, Li-Cor) were diluted with Odyssey blocking buffer (1:500) and incubated for 1 h at room temperature. Unbound antibodies were removed with 0.1% Tween and plates allowed to dry before imaging using a Li-Cor Odyssey system. Background expression was subtracted (control containing no primary antibody) for each channel. For each well expression was normalized to ACTB1 and then finally all wells were normalized to non-treated control.

Imaging 53BP1 foci by confocal microscopy

Cells (HT1080) were seeded in 8-well slides (10,000 cells/well, IBIDI) and incubated at 37°C in 5% CO_2 in air for 18 h to attach. Cells were then treated with a

binary matrix of PDSI and NU7441 depending on the experiment and incubated at 37°C in 5% CO_2 in air for 72 h. Cells were fixed with 4% formaldehyde in neutral buffered saline (CellPath; 20 mins), washed with 0.1% Triton-X in PBS (5 x 5 min with gentle shaking) and incubated with Odyssey blocking buffer (Licor) for 90 mins. Rabbit anti 53BP1 (1:200 dilution, ab36823, Abcam) primary antibody (diluted with Odyssey blocking buffer), was incubated with the cells overnight at 4°C. Unbound primary antibody was removed with 0.1% Tween in PBS (5 x 5 min with gentle shaking). Secondary antibodies, AlexaFluor 488 goat anti-rabbit IgG (1:500 dilution, Invitrogen) and AlexaFluor 633 goat anti-mouse IgG (1:500 dilution, Invitrogen) were incubated for 90 mins at room temperature with gentle shaking. Unbound secondary antibodies were removed with 0.1% Tween in PBS (5 x 5 min with gentle shaking). Cell nuclei were stained with Hoechst 33342 (1 μg/ml, Invitrogen) for 1 min and then cells washed with PBS (3 x 5 min). Cells were protected with 3-4 drops of IBIDI mounting medium (IBIDI) and kept at 4°C until imaged on a Leica tandem confocal microscope (Leica Microsystems GmbH).

Measurement of apoptotic status using laser-scanning cytometry

Cells (HT1080) were seeded in 8-well slides (80,000/well, IBIDI) and incubated at 37°C in 5% CO_2 in air for 18 h to attach. Cells were then treated with a range of PDSI (1.25-20 μM) and incubated at 37°C in 5% CO_2 in air for 24 h. Cells were incubated with a cocktail of Hoechst 33342 (10 μg/ml, Invitrogen), propidium iodide (1 μg/ml, Invitrogen), Yo-Pro1 (10 μg/ml, Invitrogen) and Mitoshift (0.1 μM, Trevigen Inc) for 30 mins prior to imaging.

Fluorescence was determined by quantitative imaging cytometry using an iCys Research Imaging Cytometer (CompuCyte) with iNovator software (CompuCyte). A sequential scanning protocol for quantification was configured with four channels. The first scan used 488 nm argon laser excitation with green channel detection (500 nm-560 nm) for YoPro, orange channel detection for Mitoshift (580 nm-620 nm) and long red channel detection (650 nm LP) for propidium iodide. In the second scan, 405nm diode laser excitation was used for blue channel detection (445 nm-485 nm) for Hoechst. High-resolution scans were acquired using the 40x objective and 0.5 mm x-step size. Cells were contoured on the blue channel to identify events (>2000 cells per well) and to measure DNA content, with a peripheral contour set 2-28 pixel width to quantify Mitoshift (mitochondrial potential) in the orange channel, the green channel to quantify YoPro (apoptosis via membrane potential) and the long red channel to quantify Propidium Iodide (cell death via nuclear membrane permeability). Fluorescence was quantified and plotted as shown in Figure S2. A full protocol for this method can be requested from CompuCyte (www.compucyte.com).

Measurement of cell survival for binary treatments using an end-point luminescence assay

An end-point luminescence system (Cell Titre Glo, Promega) was used to measure the potential synergy of binary treatments. This method is better suited for the assessment of cell survival than the xCELLigence system (see above). This assay measures the number or amount of cells by using cellular ATP, released during a lysis step, to elicit a luminescence signal in proprietary luciferase and substrate containing buffer. The cell number is directly proportional to the luminescence signal level. Readings can be normalized to untreated controls and the differences between wells gives a gauge of the cell survival for given treatment conditions. Binary survival (or death) data is plotted as a 3D response surface and compared to the calculated purely additive 'expected' response, calculated from the single compound treatments. Synergy between the combinations is expected to result in a greater than additive response.

Cells (HT1080, HCT116 WT or HCT116 BRCA2$^{-/-}$) were seeded in 96-well clear bottomed white plates (5,000 cells/well, Corning) and incubated at 37°C in 5% CO_2 in air for 18 h to attach. Cells were then treated with a binary matrix of PDSI (5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0 μM) and NU7441 (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0 μM) and incubated at 37°C in 5% CO_2 in air for 72h. Cell viability was assessed using the Cell Titre Glo system (Promega) and luminescence measured on a Pherastar plus microplate reader (BMG LABTECH GmbH). Experiments were carried out in triplicate. Cell viability data was normalized to the non-treated control and evaluated for synergy based on the Bliss independence model[22] using the program SynergySurface (www.synergysurface.com) implemented in MATLAB (R2012b, MathWorks, Inc.).

Measurement of DNA damage response (DDR) by laser-scanning cytometry

Cells (HT1080, HCT116 WT or HCT116 BRCA2$^{-/-}$) were seeded in 8-well slides (80,000/well, IBIDI) and incubated at 37°C in 5% CO_2 in air for 18 h to attach. Cells were then treated with PDSI (2 μM) and NU7441 (150 nM or 300 nM) and incubated at 37°C in 5% CO_2 in air for 72 h. Cells were fixed with 4% formaldehyde in neutral buffered saline (CellPath; 20 mins), washed with 0.1% Triton-X in PBS (5 x 5 min with gentle shaking) and incubated with Odyssey blocking buffer (Licor) for 90 mins. Rabbit anti 53BP1 (1:200 dilution, ab36823, Abcam) and mouse anti RAD51 (1:25 dilution, ab1837, Abcam) primary antibodies (diluted with Odyssey blocking buffer), were incubated with the cells overnight at 4°C. Unbound primary antibodies were removed with 0.1% Tween in PBS (5 x 5 min with gentle shaking). Secondary antibodies, AlexaFluor 488 goat anti-rabbit IgG (1:500 dilution, Invitrogen) and AlexaFluor 633 goat anti-mouse IgG (1:500 dilution, Invitrogen) were incubated for 90 mins at room temperature with gentle shaking. Unbound secondary antibodies were removed with 0.1% Tween in PBS (5 x 5 min with gentle shaking). Cell nuclei were stained with Hoechst 33342 (1 μg/ml, Invitrogen) for 1 min and then cells washed with PBS (3 x 5 min). Cells were protected with 3-4 drops of IBIDI mounting medium (IBIDI) and kept at 4°C until imaged.

Fluorescence was determined by quantitative imaging cytometry using an iCys Research Imaging Cytometer (CompuCyte) with iNovator software (CompuCyte). A scanning protocol for quantification was configured with three channels; 405 nm diode laser excitation and blue channel detection (445 nm-485 nm) for Hoechst; 488 nm argon laser excitation and green channel detection (500 nm-560 nm) for 53BPI and HeNe 633 nm laser excitation and long red channel detection (650 nm LP) for RAD51. High-resolution scans were acquired using the 60x objective and 0.5 mm x-step size. Watershed filters were applied to separate closely spaced events. Cells were contoured on the blue channel to identify events (>2000 cells per well) and to measure DNA content (for cell cycle analysis), the green channel to quantify 53BPI fluorescence and the long red channel to quantify RAD51 fluorescence. 53BPI and RAD51 fluorescence levels within the nucleus were determined and expressed as levels per cell.

Statistics

GraphPad Prism 5 was used to plot all charts and calculate statistical significance analysis using students' t-Test or ANOVA (1 or 2 way), with post-tests dependent on application. (*P<0.05, **P<0.01, ***P<0.001)

SUPPLEMENTARY REFERENCES

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