

PARP1-targeted boron-10 delivery by an olaparib analogue induces DNA damage via neutron capture while enhancing radiosensitivity in glioma cells

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Abstract

Neutron capture therapy requires agents that deliver high-neutron-cross-section isotopes to tumour cells while retaining favourable biological activity. Here we report the synthesis and *in vitro* evaluation of [^{10}B]-4-phenylboronic acid olaparib ([^{10}B]-4-PBA olaparib), a derivative of the PARP inhibitor olaparib designed to couple boron delivery with PARP inhibition in a single compound. Matched ^{10}B and ^{11}B -enriched isotopologues were prepared from enriched boric acid and evaluated for PARP1 inhibition, neutron-induced chemical degradation, cellular boron uptake, γH2AX responses and radiosensitisation in T98G glioblastoma cells. Under prolonged neutron irradiation, [^{10}B]-4-PBA olaparib was converted to 4-phenol olaparib, whereas the [^{11}B] analogue did not show a comparable time-dependent increase in this product. Both 4-PBA olaparib and its phenol neutron capture product exhibited low-nanomolar PARP1 inhibitory activity and positive radiosensitisation. Neutron irradiation of T98G cells pre-incubated with [^{10}B]-4-PBA olaparib was associated with an increased γH2AX response relative to untreated controls, while the matched [^{10}B]/[^{11}B] comparison showed a non-significant trend consistent with a ^{10}B -specific contribution. Monte Carlo microdosimetry modelling supported substantially higher specific energy deposition in boron-loaded cells under the experimental neutron field. Together, these data establish [^{10}B]-4-PBA olaparib as a feasible proof-of-concept scaffold for combining PARP inhibition with boron delivery and support further optimisation of PARP inhibitor-derived neutron capture agents.

INTRODUCTION

Neutron capture agents (NCAs) are therapeutic compounds utilised in neutron capture therapy (NCT) that incorporate stable isotopes with large neutron capture cross-sections, including ^{10}B or ^{157}Gd , that undergo characteristic nuclear reactions under neutron irradiation. In the case of ^{10}B , neutron capture generates short-range, densely ionising α and ^7Li particles, together with a 478 keV photon in most events, while ^{157}Gd neutron capture produces a cascade of short-range electrons accompanied by prompt photons^[1–4]. Because the highest linear energy transfer (LET) components have nano to micrometre-scale range, their biological effects are strongly dependent on subcellular localisation of the NCA. Clinically, BNCT has most commonly relied on the amino acid analogue [^{10}B]-4-boronophenylalanine (BPA) in combination with an external epithermal neutron beam for tumours such as head and neck, malignant glioma and cutaneous melanoma^[5, 6]. However, the types of cancer that can be treated *via* NCT is limited to superficial tumours due to the limited penetration of thermal neutrons into tissue^[7].

Beyond conventional BNCT with BPA, internally generated neutrons during proton and heavy ion therapy may create future opportunities for neutron capture-based dose amplification^[8, 9]. However, a central challenge across all neutron capture approaches remains the development of small molecules that deliver sufficient ^{10}B to relevant intracellular sites while retaining favourable pharmacology. Therefore, we proposed that a clinically established DNA repair inhibitor could be adapted into a boron delivery

agent. Furthermore, recent studies have shown that olaparib can potentiate BPA-mediated BNCT and that BNCT formulations including PARP inhibitors can enhance DNA damage responses^[10, 11].

PARP inhibitors such as olaparib (**1**, Fig. 1) are used clinically in the treatment of cancers with homologous recombination defects, including BRCA1/2-altered breast and ovarian cancers^[12]. PARP1 detects DNA strand breaks and catalyses poly(ADP-ribose) synthesis to coordinate recruitment of repair factors. PARP inhibitors suppress catalytic activity and, depending on structure and context, can also trap PARP1 on DNA, thereby exacerbating replication-associated DNA damage^[13, 14]. We hypothesised that PARP inhibitors containing ¹⁰B could combine cell-specific boron delivery with inhibition of DNA repair within a single compound.

Here we report the synthesis and *in vitro* biological evaluation of 4-phenylboronic acid olaparib (4-PBA olaparib, **2**), a NCA derivative of olaparib (**1**). Using matched ¹⁰B- and ¹¹B-labelled isotopologues, we examined retained PARP1 inhibitory activity, neutron-induced degradation, cellular boron uptake, γH2AX responses after neutron irradiation, and supporting Monte Carlo microdosimetry.

METHODS

General chemistry

All commercially available starting materials, reagents and solvents were used as supplied without further purification. Olaparib (**1**) and 4-(4-fluoro-3-(piperazine-1-carbonyl)benzyl)phthalazin-1(2H)-one (**7**) were purchased from Ambeed. Thin layer chromatography (TLC) was performed on aluminium-backed 20 µm silica gel plates. Visualisation was accomplished with UV light. Flash chromatography was performed under positive air pressure using Silica Gel 60 of 230–400 mesh (40–63 µm). NMR spectra were obtained using BRÜKER Avance Neo 500 and Ascend 400 MHz NMR instruments, with ¹H spectra obtained at 500 and 400 MHz, respectively, ¹⁰B spectra obtained at 128 and 54 MHz, ¹¹B spectra obtained at 160 MHz, and ¹³C spectra obtained at 125 and 100 MHz, respectively. Low resolution mass spectrometry (MS) was performed on a Shimadzu LC-2010 Electrospray Ionization (ESI) Mass Spectrometer. High resolution mass spectrometry (HRMS) was performed on a Waters Quadrupole-Time of Flight (QTOF) Xevo Spectrometer via ESI with Leucine–Enkephalin or Fluoranthene as an internal standard. LCMS analysis was performed using ESI on a Shimadzu LCMS-9050 Q-TOF mass spectrometer. Analytical RP-HPLC was conducted using a WATERS quality control high-performance liquid chromatography (QC-HPLC) system. The system consisted of a W600 quaternary gradient solvent pump, W2717 autosampler, and W2996 photodiode array UV detector set at λ = 254 nm and injected on a Phenomenex Synergi (C18, 4 µm, 4.6 x 250 mm) column. Preparatory RP HPLC (prep RP-HPLC) was conducted using the WATERS prep-HPLC system consisting of a W2X5X5Q quaternary gradient solvent pump, W2707 autosampler, W2489 UV/Visible detector set at λ = 254 nm. The compounds were purified on an Atlantis T3 OBD C18 150 x 30 mm, 10 µm column, with elution conditions noted for each final

compound. All tested compounds were > 95% pure by HPLC analysis (See supplementary information, Figures S1-S3).

Preparation of intermediates

Beginning from boric acid (**3**), intermediate compounds **4** and **5** were prepared via the method of Demory *et al.*^[15], with some minor modifications (see supplementary information).

[¹⁰B]4-(4,4,6-trimethyl-1,3,2-dioxaborinan-2-yl-2-)benzoic acid ([¹⁰B]**6**)

[¹⁰B]Methyl 4-(4,4,6-trimethyl-1,3,2-dioxaborinan-2-yl-2-)benzoate ([¹⁰B]**5**, 984.4 mg, 3.77 mmol) was taken up in 5 mL methanol and 5 mL 4M aqueous sodium hydroxide was added. The reaction mixture was then stirred at reflux overnight. After completion (confirmed by TLC) the reaction mixture was acidified with 10 mL 1M HCl_(aq) and then extracted into ethyl acetate. The combined organic layers were then dried with MgSO₄ and evaporated under reduced pressure, and used in subsequent steps without further purification (810.7 mg, 87%). ¹H NMR (400 MHz, CDCl₃) δ 8.06 (d, *J* = 8.3 Hz, 2H), 7.90 (d, *J* = 8.2 Hz, 2H), 4.37 (dq, *J* = 12.3, 6.2, 2.9 Hz, 1H), 1.89 (dd, *J* = 13.9, 2.9 Hz, 1H), 1.61 (dd, *J* = 13.9, 11.5 Hz, 1H), 1.39 (s, 3H), 1.38 (s, 3H), 1.36 (d, *J* = 6.2 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 172.90, 139.96, 133.92, 130.77, 129.08, 71.51, 65.37, 46.05, 31.29, 28.26, 23.22; ¹⁰B NMR (54 MHz, CDCl₃) δ 30.0; HRMS (ESI) *m/z* calcd. for C₁₃H₁₆¹⁰BO₄ [M-H]⁻: 246.10488; found 246.10464

[¹¹B]4-(4,4,6-trimethyl-1,3,2-dioxaborinan-2-yl-2-)benzoic acid ([¹¹B]**6**)

This compound was synthesised in an identical manner to its ¹⁰B-enriched equivalent ([¹⁰B]**6**), from [¹¹B]methyl 4-(4,4,6-trimethyl-1,3,2-dioxaborinan-2-yl-2-)benzoate ([¹¹B]**5**, 260.0 mg, 0.991 mmol), yielding the final product as a white powder (244.9 mg, quant. yield). ¹H, and ¹³C NMR data matched that reported for [¹⁰B]**6**. ¹¹B NMR (160 MHz, CDCl₃) δ 27.2. LRMS (ESI) *m/z* = 247 [M-H]⁻ C₁₃H₁₆¹¹BO₄

Synthesis of tested compounds

[¹⁰B]4-(4-(4-(2-Fluoro-5-((4-oxo-3,4-dihydrophthalazin-1-yl)methyl)benzoyl)piperazine-1-carbonyl)phenyl)boronic acid ([¹⁰B]**2**)

[¹⁰B]4-(4,4,6-trimethyl-1,3,2-dioxaborinan-2-yl-2-)benzoic acid [¹⁰B]**6** (400.0 mg, 1.618 mmol), EDCI·HCl (372.2 mg, 1.942 mmol), and HOBT (262.3 mg, 1.942 mmol) were weighed into a dry round bottom flask and stirred in 5 mL DMF for an hour. 4-(4-Fluoro-3-(piperazine-1-carbonyl)benzyl)phthalazin-1(2H)-one (**7**, 711.4 mg, 1.942 mmol) was then added to the reaction mixture which was then stirred overnight at room temperature. Upon completion (by TLC), the reaction mixture was acidified with 4M HCl_(aq) and extracted with CH₂Cl₂. The combined organic extracts were dried with MgSO₄ and evaporated under reduced

pressure. The isolated intermediate was then stirred in a 1:1 mixture of acetonitrile and 1M HCl_(aq) for 4 hours at reflux. Upon completion, the reaction mixture was extracted into EtOAc, and the combined organic extracts evaporated under reduced pressure and purified by RP-HPLC to give the final product as a white powder after lyophilisation (531.4 mg, 64%). HPLC (prep. RP-C18, 36% ACN): t_R : 4.2 min. **¹H NMR (400 MHz, DMSO-d₆)** δ 8.25 (d, J = 7.8 Hz, 1H), 8.18 (s, 2H), 7.99–7.75 (m, 3H), 7.85 (d, J = 7.7 Hz, 2H), 7.49–7.16 (m, 5H), 4.33 (s, 2H), 3.73–3.10 (m, 8H). **¹³C NMR (100 MHz, DMSO-d₆)** δ 169.38, 164.08, 159.40, 156.38 (d, J = 244.6 Hz), 144.84, 136.94, 135.75, 134.83 (d, J = 3.3 Hz), 134.06, 133.47, 131.78 (d, J = 7.9 Hz), 131.54, 129.08, 128.91, 127.90, 126.08, 125.93, 125.47, 123.51 (d, J = 18.4 Hz), 115.96 (d, J = 21.5 Hz), 46.39, 41.25, 36.45; **¹⁰B NMR (128 MHz, DMSO-d₆)** δ 32.7. **HRMS (ESI)** m/z calcd. for C₂₇H₂₃¹⁰BFN₄O₅ [M-H][−]: 512.17764; found 512.17882

[¹¹B](4-(4-(2-Fluoro-5-((4-oxo-3,4-dihydrophthalazin-1-yl)methyl)benzoyl)piperazine-1-carbonyl)phenyl)boronic acid ([¹¹B]**2**)

The title compound was synthesised in an identical manner to its ¹⁰B-enriched equivalent ([¹⁰B]**2**), from [¹¹B]4-(4,4,6-trimethyl-1,3,2-dioxaborinan-2-yl-2-)benzoic acid ([¹¹B]**6**, 244.9 mg, 986 μ mol), yielding the final product as a white powder (233.9 mg, 46%). HPLC, ¹H, and ¹³C NMR data matched that reported for [¹⁰B]**2**. **¹¹B NMR (160 MHz, DMSO-d₆)** δ 28.8. **HRMS (ESI)** m/z calcd. for C₂₇H₂₃¹¹BFN₄O₅ [M-H][−]: 513.17400; found 513.17542

4-(4-Fluoro-3-(4-(4-hydroxybenzoyl)piperazine-1-carbonyl)benzyl)phthalazin-1(2H)-one (**8**)

Synthesised via the same amide coupling method as **2**, from 4-hydroxybenzoic acid (Merck, 133.1 mg, 963.7 μ mol). The crude product was purified by RP-HPLC to give the final product as a white powder after lyophilisation (178 mg, 38%). HPLC (prep. RP-C18, 30–70% ACN in 12 mins): t_R : 6.1 min. **¹H NMR (500 MHz, DMSO-d₆)** δ 9.88 (s, 1H), 8.25 (dd, J = 7.9, 1.4 Hz, 1H), 7.96 (d, J = 8.1 Hz, 1H), 7.88 (t, J = 7.7 Hz, 1H), 7.81 (t, J = 7.6 Hz, 1H), 7.44 (ddd, J = 8.0, 5.0, 2.3 Hz, 1H), 7.36 (dd, J = 6.4, 2.3 Hz, 1H), 7.27 (d, J = 8.5 Hz, 1H), 7.23 (t, J = 9.0 Hz, 1H), 6.80 (d, J = 8.6 Hz, 1H), 4.33 (s, 2H), 3.65 (s, 2H), 3.54 (s, 2H), 3.40 (s, 2H), 3.22 (s, 2H); **¹³C NMR (125 MHz, DMSO-d₆)** δ 169.97, 164.52, 159.85, 159.38, 156.85 (d, ¹ $J_{C,F}$ = 245.3 Hz), 145.31, 135.30 (d, ⁴ $J_{C,F}$ = 3.1 Hz), 133.95, 132.23 (d, ³ $J_{C,F}$ = 8.1 Hz), 132.02, 129.86, 129.55, 129.40 (d, ³ $J_{C,F}$ = 4.1 Hz), 128.37, 126.55, 126.12, 125.95, 124.02 (d, ² $J_{C,F}$ = 19.0 Hz), 116.42 (d, ² $J_{C,F}$ = 22.1 Hz), 115.38, 46.95, 41.89, 40.59, 40.50, 40.42, 40.33, 40.26, 40.17, 40.09, 40.00, 39.92, 39.83, 39.66, 39.50, 36.90; **HRMS (ESI)** m/z calcd. for C₂₇H₂₄FN₄O₄ [M + H]⁺: 487.17818; found 487.17873

Neutron irradiation conditions and Monte Carlo dosimetric calculations

Samples were exposed to the unfiltered high-intensity 20 cm x 20 cm beam configuration at DINGO, which is a predominantly thermal neutron beam (approx. 59%), but also contains epithermal (21%) and

fast (20%) neutron components. For flux calibration, pre-weighed gold wires were placed adjacent to the sample positions and irradiated under the same DINGO configuration described previously^[16]. ^{198}Au activity was quantified using HPGe γ -spectroscopy. Previously published thermal-to-epithermal neutron ratios for this DINGO configuration were used to correct the bare-gold activation measurements and estimate the thermal neutron flux^[17, 18].

To support the DNA damage analysis, in-depth understanding of the neutron field characteristics and estimation of the physical dose that the cells received is required. We implemented a Monte Carlo microdosimetry study using Geant4, version 11.3.p02 (with QGSP_BIC_AllHP physics list and Thermal Neutron Scattering Libraries) and the validated *in silico* model of the DINGO beamline^[17, 18].

The sample geometry was reproduced in Geant4 to simulate the local spectrum of all primary and secondary particles at a 20 μm layer at the bottom of the eight media wells as illustrated in Supplementary Fig. S4 (referred to as scoring volume hereafter). This was achieved by registering the kinetic energy of the neutrons together with their step length (distance travelled between subsequent interactions within the scoring volume or reaching its boundaries). The step length was then summed for all neutrons falling within the individual energy bins and divided by the scoring volume, resulting in particle spectral fluence - this can be summarised as “particle traffic grouped by their kinetic energy at the time of the interaction within the volume of interest”.

The local particle spectral fluence is normalised to the experimental thermal neutron flux of $2.6 \pm 0.3 \times 10^8 \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ and a specified experimental exposure time to calculate the equivalent number of each particle species that would be generated during irradiation at the DINGO beamline. Figure 2 illustrates the validated incident neutron spectrum on the sample and the local spectrum simulated with the presence of the media in the beam moderating and scattering neutrons. Although the volume of a single well is only approximately 0.6 mm^3 , its thickness from the beam's eye view is nearly 10 mm of mostly water and polystyrene, which can effectively moderate and scatter neutrons. In particular it increases the number of neutrons with near-thermal energy that may interact with the cells and due to the high neutron capture cross-section in ^{10}B , can considerably contribute to the total dose. It is therefore crucial to consider the neutron spectra at the cell level.

These local spectra and the relevant number of particles were then used as input in the subsequent simulations, where simple cell volumes are irradiated with the “local particle field”. Cell shapes were defined as plano-convex ellipsoids in three sizes, where the horizontal radii r_1 and r_2 , and the thickness c are: i) 7.5 μm , 7.5 μm and 5.0 μm , ii) 12.5 μm , 12.5 μm and 6.0 μm , and iii) 17.5 μm , 17.5 μm and 7.0 μm , respectively. Cells were modelled either without boron (control) or with a uniform intracellular distribution of ^{10}B corresponding to a total mass of 2.88 pg ^{10}B per cell. In this simulation, the energy deposition by each particle and the number of particles that interacted with the cell volume were registered. The energy deposition allowed calculating the specific energy z (Gy), the frequency-mean lineal energy y_F (keV/ μm) and dose-mean lineal energy y_D (keV/ μm) using the following equations:

$$z = \epsilon / m$$

1

$$\bar{y}_F = \frac{1}{N} \sum_{i=1}^N y_i$$

2

$$y = \epsilon / \bar{l}$$

3

$$\bar{y}_D = \frac{\sum_{i=1}^N y_i^2}{\sum_{i=1}^N y_i}$$

4

where ϵ is the energy imparted in the mass m , N - the number of events and l - mean chord length (average length of randomly oriented lines traversing a volume). The lineal energy quantity refers to the energy deposition of a particle in a single event. The simulated microdosimetric spectra (dose-weighted lineal energy distribution) are shown in Supplementary Fig. S5, where b) contains two additional peaks in the high lineal energy region corresponding to the α and ${}^7\text{Li}$ particles. Finally, we estimated the number of boron neutron captures, which are directly proportional to the number of generated α and ${}^7\text{Li}$ particles during the irradiation.

Neutron capture degradation analysis

To assess the impact of boron neutron capture on the remainder of the neutron capture agent molecule, 10 mM solutions of $[{}^{10}\text{B}]$ and $[{}^{11}\text{B}]$ olaparib-4-phenylboronic acid (**[${}^{10}\text{B}$]**2 and **[${}^{11}\text{B}$]**2, respectively) in pH 9–10 ammonium bicarbonate buffer was prepared in 0.5 mL Slide-a-lyzer dialysis cassettes and irradiated for 0, 6, and 12 hours in the DINGO neutron beamline (thermal neutron flux ($E_n < 0.5$ eV) = $2.6 \pm 0.3 \times 10^8$ n.cm $^{-2}$.s $^{-1}$)^[16]. The solutions were then observed via analytical RP-HPLC (10–90% ACN in H $_2$ O, 0.1% TFA) by injecting 20 μL of the undiluted solution. The samples were also analysed by LCMS with the same gradient.

PARP1 enzyme inhibition assay

PARP1 inhibitor IC $_{50}$'s were determined for all compounds using a PARP universal chemiluminescent assay kit (Bio-technique 4676-096-K). In summary, the histone coated wells were hydrated with assay buffer for ~ 30 mins. The compounds were dissolved in DMSO, serially diluted, added to the wells and

incubated with 0.5 U of PARP1 enzyme for 10 mins. The autoribosylation reaction was started by addition of the reaction cocktail, which contains biotinylated-ADP-ribose, ADP-ribose, NAD and activated DNA, and allowed to proceed for 60 mins at room temp. The wells were thoroughly washed and the amount of biotinylated poly-ADP-ribose detected by binding with strep-HRP and reacting this with a PeroxyGlow chemiluminescent HRP substrate.

Cell culture

T98G cells (ECACC 92090213) were maintained as subconfluent culture in Minimum Essential Media (MEM, Gibco 11095098), supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37°C in a 5% CO₂ atmosphere.

Cell uptake

T98G cells were seeded into 35 mL petri dishes at a concentration of 5×10^5 cells per dish and allowed to attach overnight. Compound **[¹⁰B]2** was prepared at a concentration of 100 µM in cell media. Cells were incubated in this solution for periods of 10, 30, 60 and 120 mins. At the conclusion of the incubation time the cell monolayer was washed three times in PBS before being digested in 500 µL of 70% HNO₃ (Suprapur, Merck) at room temp for 48 hours with gentle agitation. The digests were transferred to 50 mL Falcon tubes and diluted 1/50 with water to give a final concentration of 0.3 M HNO₃. Boron concentration in the final diluted digests was quantified using a quadrupole inductively-coupled mass spectrometer (ThermoFisher iCAP-Q) at the Wollongong Isotope Geochronology Laboratory (WIGL) at the University of Wollongong (UOW) as per Howell *et al.*^[9] The mass of boron per digest was used to calculate the mass of boron per cell based on the starting cell number.

Radiosensitisation

Radiosensitisation due to PARP inhibition was assessed by means of a cell proliferation assay. T98G cells were seeded into a 24 well plate at a concentration of 1500 cell/well and allowed to attach overnight. Compounds were prepared at 100 µM as per above and serially diluted to 10, 1, 0.1, 0.01 and 0.001 µM. Cells were incubated in the dilution series for 2 hours at 37°C, 5% CO₂ to achieve maximal uptake and then irradiated with either 0 or 4 Gy of X-rays (320kVp; 10mA; 1.5mm Al + 0.25mm Cu + 0.75mm Sn filtration; 45cm SSD) at a rate of 1 Gy/min with a Precision X-RAD320 irradiator. Cells were incubated for a further 22 hours with the inhibitor present at 37°C, 5% CO₂ before being washed once with PBS and the fresh media added. Cells were allowed to proliferate for 6 days (approximately 7 doubling times) before the viable cell mass was assayed by resazurin/resarufin conversion assay. A 1% stock of resazurin sodium salt was diluted 250 times in PBS and warmed to 37°C. The media was aspirated from the cells, replaced with resazurin solution and incubated for 60 mins at 37°C. The conversion to resarufin was measured using a Spectramax i3x plate reader (Molecular Devices) at

excitation 555 nm and emission 585 nm. All data was normalised and EC_{50} values of drug concentration calculated for 0 Gy and 4 Gy by fitting the log(inhibitor) vs. normalised response model from Graphpad Prism (10.1.2). The radiosensitisation factor is calculated by the following equation:

$$rsf = \frac{EC50^{0Gy}}{EC50^{4Gy}}$$

5

Boron neutron capture DNA damage

Neutron irradiation was conducted on the DINGO neutron tomography beamline^[19] on the Open Pool Australian Lightwater (OPAL) reactor following the methodology described by Howell *et al.*^[16] The day prior to irradiation 4×10^4 T98G cells were seeded into the wells of an 8 chamber Permanox plastic chamber slide (Nunc Lab Tek). Both [¹¹B]2 and [¹⁰B]2 were prepared as a boronic acid - fructose complex at 1 mM concentration in saline following the principles described in Nomoto *et al.*^[20] This is achieved by raising the pH to ~ 10 with saturated NaOH solution, mixing with a 10% molar excess of D-(-)-fructose (Merck) for 20 mins and slowly lowering the pH to 7.0 with HCl (Merck). The fructose complex was diluted 10x in growth media to produce a final concentration of 100 μ M. Cells were incubated for 60 mins at 36°C, topped up to zero headspace with PBS, sealed with adhesive film and irradiated in the neutron beam for 1 or 10 mins at a thermal neutron flux of $2.6 \pm 0.3 \times 10^8 \text{ n} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$. Immediately following irradiation the media was replaced with fresh growth medium and the cells were incubated for 40 mins at 36°C 5% CO₂, fixed in 10% neutral buffered formalin for 10 mins and stored at 4°C in PBS for further processing. Cells were permeabilized with 0.1% Triton-X for 10 min at room temperature and blocked with 5% BSA for 1 h at room temperature on a rocker. Cells were then incubated with primary antibody (γ H2AX anti-mouse (Merk Life Science, Australia) 1:1200 dilution in 1% BSA) overnight at 4°C, washed with 0.1% PBST, and incubated with secondary antibody (Alexa Fluor 488 goat anti-mouse (AbCam, Australia) 1:800 dilution in 1% BSA) for 1 h at room temperature on a rocker. After a final PBST wash, cells were first counterstained with Phalloidin iFluor-594 (1:1000, Abcam, Australia) for 1 h and DAPI (4',6-diamidino-2-phenylindole) (1 μ g/mL) for 20 min at room temperature, washed again, and mounted using FluoroShield mounting media (Sigma). Samples were left to cure overnight at room temperature (protected from light) and stored at 4°C for up to two weeks prior to analysis.

The resulting γ H2AX foci were quantified by counting the number of foci per nucleus in 55 randomly selected cells from duplicate wells, by eye, using a 100 $\times$ objective. Images of foci were also taken using a 63 $\times$ objective. Optically sectioned focal stacks of representative regions were acquired using a Zeiss Imager Z2 microscope with Apotome reconstructed using the Zen Apotome Plus package. Maximum intensity projections of the stack were prepared to illustrate the distribution of foci within the nucleus.

RESULTS AND DISCUSSION

Chemistry

4-PBA olaparib (**2**) was synthesised from either ^{10}B - or ^{11}B -enriched boric acid (**3**, ^{10}B enrichment 95%, ^{11}B enrichment $\geq 99\%$) as a starting material (Fig. 3). We proposed the synthesis of a simple synthon for coupling ^{10}B to existing structures, based on the work of Demory *et al.*^[15] which described the synthesis of 2-isopropoxy-4,4,6-trimethyl-1,3,2-dioxaborinane (**4**), followed by addition to methyl 4-iodobenzoate to yield 4-(methylcarboxy)phenylboronic acid hexylene glycol ester (**5**). Saponification of (**5**) gave protected 4-carboxyphenylboronic acid hexylene glycol ester (**6**) in 87% yield. This synthon was then utilised for the synthesis of 4-PBA olaparib (**2**) via amide coupling with 4-(4-fluoro-3-(piperazine-1-carbonyl)benzyl)phthalazin-1(2H)-one (**7**), utilising EDCI and HOBT, and subsequent acid deprotection of the boronic ester to give the final compound 4-PBA olaparib (**2**) in 64% yield after RP-HPLC purification. The corresponding ^{11}B isotopologue (**[^{11}B]**2****) was prepared analogously to provide the matched isotope-control compound for neutron capture experiments.

4-PBA olaparib (**2**) is poorly soluble in water at neutral pH (cLogP = 1.69). To solubilise the compound, we used a method used for preparing BPA at the high concentrations needed for clinical administration, exploiting the formation of a complex between the boronic acid and fructose at high pH, before slowly lowering the pH to 7 (Fig. 4)^[20, 21]. Alternatively, we observed that the compound can be solubilised directly into cell media over time with sonication, whereby it is assumed to form a comparable complex with glucose present in high concentrations in the media formulation.

Monte Carlo microdosimetry simulation results

Using our validated Geant4 model of the DINGO beamline, we performed a Monte Carlo study to simulate the neutron irradiation conditions of the neutron capture experiments, and the resulting nuclear reactions that occurred within the samples. The model estimates the characteristics of the resulting local radiation field incident on individual cells and simulates the average microdosimetric specific energy (Gy, stochastic quantity that is effectively an analogue of the physical dose delivered to a microscopic volume)^[17, 18].

The simulation results (Table 1) indicate a large, approximately 40-fold increase in the specific energy deposition in the cells due to the presence of ^{10}B (12.5 Gy), compared to the control group (0.3 Gy). The main component of the radiation dose to the control group was found to be protons (83.9% - largely due to the recoil proton generated by elastic scattering of epithermal and fast neutrons from hydrogen). In contrast, the main components of the dose to the cells containing ^{10}B was α (54.0%) and ^7Li (43.1%), due to the $^{10}\text{B}(n,\alpha)^7\text{Li}$ reaction (Fig. 5). The estimated proton contribution to the dose in the ^{10}B containing cells was only 2.4%. Because these quantities scale linearly with fluence over the irradiation times considered in this work, the corresponding estimates for the 1 min γH2AX quantification experiment are approximately $z = 1.25$ Gy for ^{10}B -treated cells, $z = 0.03$ Gy for controls, and ~ 6.5 neutron capture events per cell.

Table 1

Mean microdosimetric values for the average cell considered in the study; the total ^{10}B mass was fixed at 2.88 pg per cell across the three cell-size models and was assumed to be uniformly distributed within the cell volume. The average (based on three cell sizes) mean chord length^a was 6.72 μm . The results are reported for a 10 minute exposure.

Cell type	z (Gy)	Mean total energy deposition (MeV)	y_F (keV/ μm)	y_D (keV/ μm)	Average # of neutron capture events
^{10}B treated ^b	12.5 ± 0.3	104.6 ± 3.4	16.5 ± 0.9	123.0 ± 1.5	65
Control	0.3 ± 0.1	4.6 ± 0.2	0.6 ± 0.1	22.4 ± 1.7	-
Δ	12.2	100.0	16.0	100.6	65

^aChord length refers to a random straight line that can connect two points on the surface of a volume representing potential particle track path; the mean chord length can be calculated using Cauchy's formula $l = 4(V/S)$, where V is the volume and S the surface area.

^bCells containing 2.88 pg ^{10}B per cell

Neutron capture degradation analysis

To assess the impact of boron neutron capture on the neutron capture agent, 10 mM solutions of [^{10}B] and [^{11}B]4-PBA olaparib ([^{10}B]2 and [^{11}B]2, respectively - Fig. 6A) in pH 9–10 ammonium bicarbonate buffer were prepared in 0.5 mL dialysis cassettes and irradiated for 0, 6, and 12 hours in the DINGO neutron beamline (Fig. 6B). The thermal neutron flux ($E_n < 0.5$ eV) during this experiment was measured to be $2.6 \pm 0.3 \times 10^8 \text{ cm}^{-2} \cdot \text{s}^{-1}$ using gold neutron activation methodology described in our previous work^[16–18]. Dialysis cassettes were used for this experiment due to their geometry, which gave a 1.25 cm x 1.25 cm “window” with a depth of ~ 0.3 cm, that maximized sample exposure to the neutron beam by increasing the surface to volume ratio. After irradiation, the solutions were characterised by LCMS to identify and characterise any major neutron capture degradation products.

A single major radiolysis product was observed by LCMS in the [^{10}B]2 sample (Fig. 6C), and identified to be the 4-phenol olaparib compound (**8**, Fig. 6A), which is the most likely product from both radiolysis of the parent phenylboronic acid, and from oxidation arising from reactive oxygen species produced by activation of the solution by neutron irradiation^[22]. This major product was confirmed from both the mass observed ($m/z = 487$ [$\text{M} + \text{H}$]⁺, Fig. 6C) upon LCMS analysis of the samples irradiated at DINGO, and synthesising the suspected degradant *via* the same method as 4-PBA olaparib (**2**), but using 4-hydroxybenzoic acid in place of 4-carboxyphenylboronic acid (**6**). A statistically significant increase in the amount of 4-phenol olaparib (**8**) was observed between the 0 and 12 hour [^{10}B]2 samples (Fig. 6D), while no time dependent increase was detected in the [^{11}B]2 samples (Supplementary Fig. S6B). This indicates that the formation of 4-phenol olaparib (**8**) is due to neutron capture by ^{10}B in the sample and

not solely from oxidation by reactive oxygen species generated by the neutron radiolysis of water in each cassette.

PARP inhibition and radiosensitisation

To compare the effect of the phenylboronic acid and the phenol groups on the PARP inhibitory activity of the olaparib backbone, their inhibition activity against the PARP subtype 1 (i.e. PARP1) and radiosensitisation profiles were assessed (Table 2). Olaparib (PARP1 IC₅₀ = 5 nM)^[23] was used as a positive control and returned an IC₅₀ value in this assay of 1.7 ± 0.3 nM (Table 2). Both 4-PBA olaparib (**2**, IC₅₀ = 1.1 ± 0.2 nM) and its phenol derivative (**8**, IC₅₀ = 0.7 ± 0.3 nM) exhibited comparable inhibitory activity to olaparib as determined by this assay.

Radiosensitisation of T98G cells to 320keV X-rays by PARP1 inhibition was determined for each compound by measuring cellular proliferative capacity following irradiation with 4 Gy (1Gy/min), and without irradiation to calculate an EC₅₀ for each compound's effect on cell proliferation. A radiosensitisation factor (RSF) was calculated as a ratio between the EC₅₀ of the compound alone and after irradiation, where a higher RSF equates to a greater radiosensitisation effect. Both 4-PBA olaparib (**2**, EC₅₀ at 4 Gy = 6 ± 5 µM) and 4-phenol olaparib (**8**, EC₅₀ at 4 Gy = 3 ± 1 µM) showed comparable activity to olaparib (**1**, EC₅₀ at 4 Gy = 2.05 µM) after ionising radiation, while also exhibiting a little less potency without radiation (**2**, EC₅₀ at 0 Gy = 18 ± 10 µM; **8**, EC₅₀ at 0 Gy = 40 ± 30 µM). All compounds exhibited RSFs > 1, ranging from 2.2 to 13.3, indicating they have retained their PARP1 specific inhibition of repair pathways.

Table 2

Biological evaluation of the clinically relevant PARP inhibitor olaparib (**1**), compared to the synthesised boron containing PARP inhibitor 4-PBA olaparib (**2**), along with the phenol neutron capture degradation product (**8**).

Compound Name	PARP1 IC ₅₀ (nM)	Radiosensitisation of T98G Glioblastoma cells		
		EC ₅₀ at 0 Gy (µM)	EC ₅₀ at 4 Gy (µM)	RSF ^a
olaparib (1)	1.7 ± 0.3	4.56	2.05	2.2
4-phenylboronic acid olaparib (2)	1.1 ± 0.2	18 ± 10	6 ± 5	3
4-phenol olaparib (8)	0.7 ± 0.3	40 ± 30	3 ± 1	13.3

^aRadiosensitisation factor (RSF) calculated as the ratio between the EC₅₀ of the compound with 0 Gy ionising radiation dose and 4 Gy ionising radiation dose.

Neutron capture induced DNA damage and repair

Uptake of the [^{10}B]4-PBA olaparib compound (**[^{10}B]2**) was assessed by ICP-MS, in order to determine the kinetics of ^{10}B accumulation in T98G glioblastoma cells. Cells were incubated with 100 μM **[^{10}B]2**, washed, and digested in HNO_3 before boron measurement by ICP-MS. The intracellular ^{10}B content after incubation for 120 minutes was 2.88 pg ^{10}B per cell. A Y_{max} for the uptake of 3.061 pg ^{10}B per cell was calculated by fitting an exponential plateau model using Graphpad Prism (see Supplementary Fig. S7), which indicates the cells to be close to equilibrium at 120 min with the measured uptake being > 90% of the Y_{max} .

Neutron irradiation of T98G cells pre-incubated with 100 μM **[^{10}B]2**-fructose complex was associated with an increased γH2AX response relative to untreated controls, while the matched **[^{10}B]2**/**[^{11}B]2** comparison showed a non-significant trend consistent with a ^{10}B -specific contribution (Fig. 7). γH2AX foci are a marker of active DNA damage response that corresponds to the site that damage has occurred^[24]. The dense ionisation tracks from higher LET particles, such as result from the $^{10}\text{B}(\text{n},\alpha)^7\text{Li}$ reaction, result in clusters of foci which appear larger and more persistent compared to low LET radiation^[25]. While foci counting can be used to directly assess DNA damage with low LET radiation (such as X-rays), the relationship between foci number and quantity of damage is much more complex with high LET particles due to this clustering. Furthermore, many cancer cell lines have a high reported instance of spontaneous foci formation that can lead to elevated background levels further complicating quantification^[26]. Following 60 min compound pre-incubation and fixation at 40 min post irradiation with a thermal neutron flux of $2.6 \pm 0.3 \times 10^8 \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ for 1 minute (thermal neutron fluence: $1.6 \pm 0.2 \times 10^{10} \text{ cm}^{-2}$), the γH2AX foci were manually counted in 55 nuclei per sample from two separate samples per treatment group, giving $n = 110$ nuclei for each group: ^{10}B -olaparib (**[^{10}B]2**), ^{11}B -olaparib (**[^{11}B]2**), and untreated controls (NT). To account for the elevated spontaneous foci formation a threshold was defined as the 95th percentile of the NT distribution (28.5 foci/nucleus), and the proportion of cells exceeding this threshold was calculated for each group. Pairwise comparisons between treatment groups were performed using Fisher's exact test on 2×2 contingency tables (above/below threshold $\times$ treatment group). To estimate the uncertainty in the difference in proportions between groups, 95% bootstrap confidence intervals were computed using 10,000 resampled iterations with replacement. **[^{10}B]2** produced a significantly higher proportion of cells with elevated γH2AX foci (14.5%), compared to NT (5.5%, OR = 2.95, $p = 0.041$) with bootstrap confidence interval for the difference in the proportions of 1.18–17.3%. The **[^{11}B]2** treated cells (8.2%) did not differ significantly from NT (OR = 1.54, $p = 0.59$). Even though the ^{10}B vs ^{11}B comparison did not reach significance (OR = 1.91, $p = 0.20$) using this test, the trend remains consistent with a ^{10}B -specific contribution, but should be interpreted cautiously given the limited sample size and threshold-based analysis.

Maximum intensity projections from optically sectioned Z-stack images demonstrate that **[^{10}B]2** treated cells exhibit a sporadic increase in the nuclear distribution of foci between cells consistent with what is expected of high LET particles (Fig. 8). The pattern of this increase tends to be foci clusters rather than a uniform distribution throughout the nucleus, with some individuals approaching a pan-nuclear response,

when visually compared with [^{11}B]**2** treated and NT cells. Phalloidin staining confirmed the maintenance of cyto-architecture post irradiation, showing no evidence of acute toxicity during the 40 min post irradiation incubation period.

The enrichment of high-foci nuclei in the [^{10}B]**2** group, together with the matched [^{11}B]**2** trend, representative foci morphology and supporting microdosimetry, is consistent with neutron-capture-associated DNA damage. Together, these findings support the use of high-affinity PARP inhibitors as delivery agents for stable isotopes with high neutron-capture cross sections and demonstrate a potential therapeutic opportunity for this compound class.

CONCLUSION

In this work, we synthesised both ^{10}B - and ^{11}B -enriched derivatives of 4-phenylboronic acid olaparib (**2**) to determine the feasibility of adapting PARP inhibitors as dual modality neutron capture agents. Under neutron irradiation conditions, a primary degradation product, 4-phenol olaparib (**8**) was formed because of thermal neutron capture by [^{10}B]**2**. Both the NCA phenylboronic acid (**2**) and its phenol neutron capture degradation product (**8**) showed comparable PARP1 inhibition and radiosensitisation efficacy to olaparib itself. Neutron irradiation of [^{10}B]**2** was associated with an increased γH2AX response in T98G cells, with microdosimetry and isotopic-control trends supporting a contribution from the thermal neutron ^{10}B capture reaction. These results demonstrate that combining neutron capture and DNA repair inhibition within a single molecule may provide a synergistic strategy to enhance cancer cell death beyond either approach alone. This work also demonstrates the capabilities of computational methods to support multidisciplinary studies that involve neutron radiation delivery. Ultimately, these findings position dual-action NCAs as promising candidates for Neutron Capture Enhanced Particle Therapy (NCEPT), where internally generated neutron clouds could activate the compound targeting ionising radiation directly to diffuse micro-metastases outside the primary particle beam treatment volume.

Declarations

Competing interests

The author(s) declare no competing interests.

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Author Contribution

CD and NH contributed equally and are both corresponding authors. CD synthesised the tested compounds, and NH established and conducted all biological measurements with the assistance of GL, CH, RJ, FS, and NW. KJ and JB provided assistance with experiments at DINGO. KJ performed and analysed data from microdosimetry simulations. FD and AD performed ICP-MS analysis. AS performed neutron activation measurements. GP, BF, HY, DS and MS contributed to experimental design and supervision. CD, KJ, and NH prepared the original draft of the manuscript, which was reviewed and edited by all authors. All authors have given approval to the final version of the manuscript.

Data Availability

The datasets generated and analysed during the current study, including γ H2AX foci counts and microdosimetry summary outputs, are available from the corresponding authors on reasonable request.

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Figures

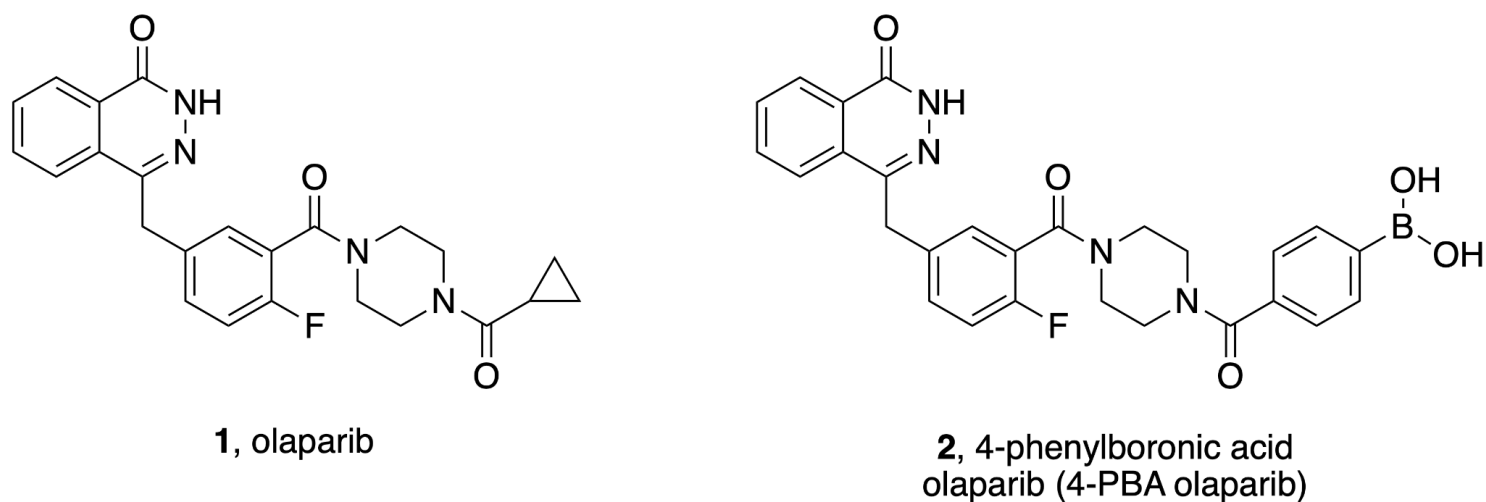


Figure 1

Structures of the PARP inhibitor olaparib (**1**), and the borylated derivative 4-phenylboronic acid olaparib (4-PBA olaparib, (**2**)).

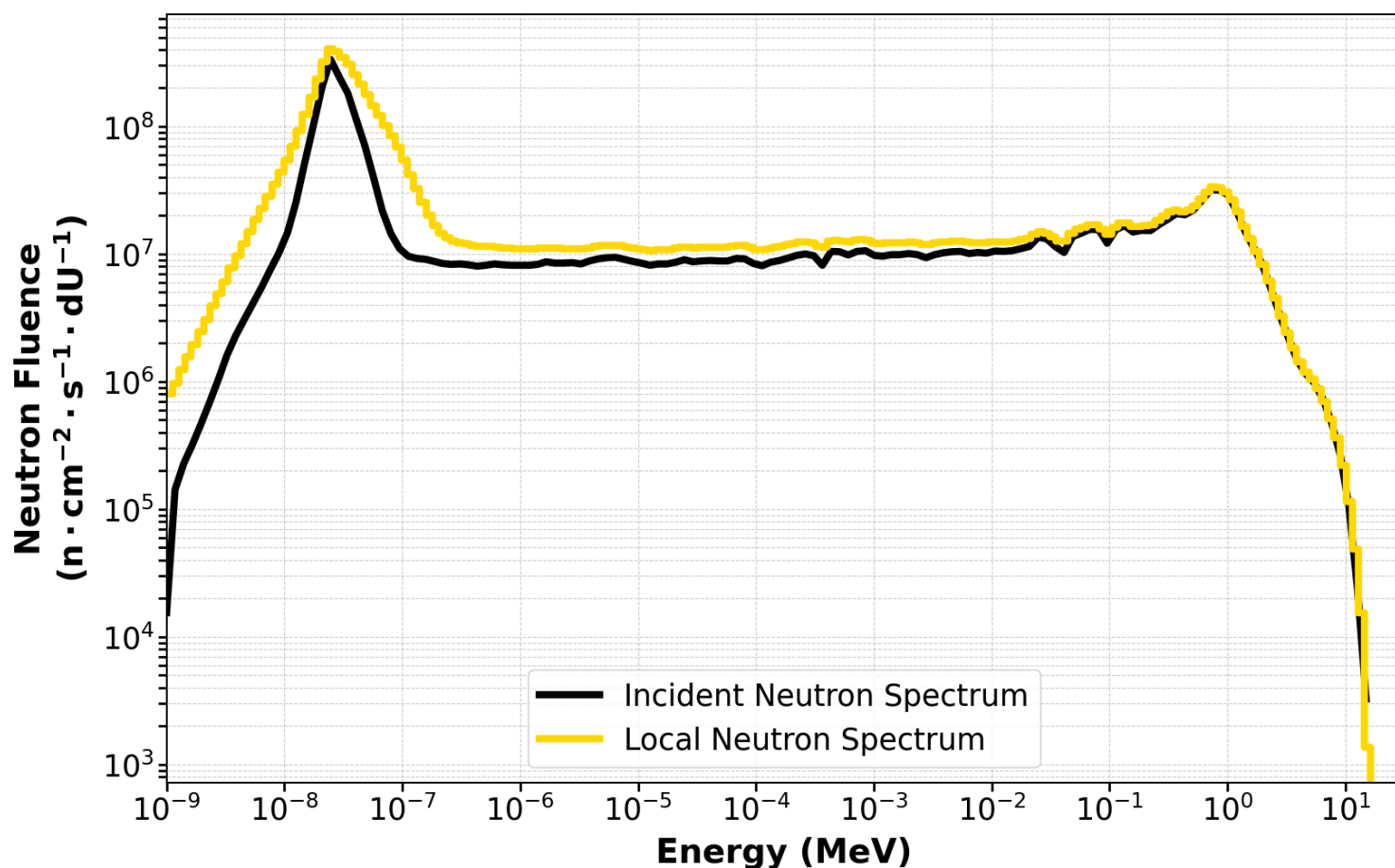


Figure 2

Neutron spectrum for the high-intensity 20 cm x 20 cm beam at DINGO, simulated in Geant4 and validated with several techniques, including absolute in-beam multifoil neutron activation analysis and out-of-beam Bonner sphere spectroscopy; black line shows the neutron spectrum incident on the sample, while the yellow line illustrates the local, attenuated neutron spectrum at the cellular layer.

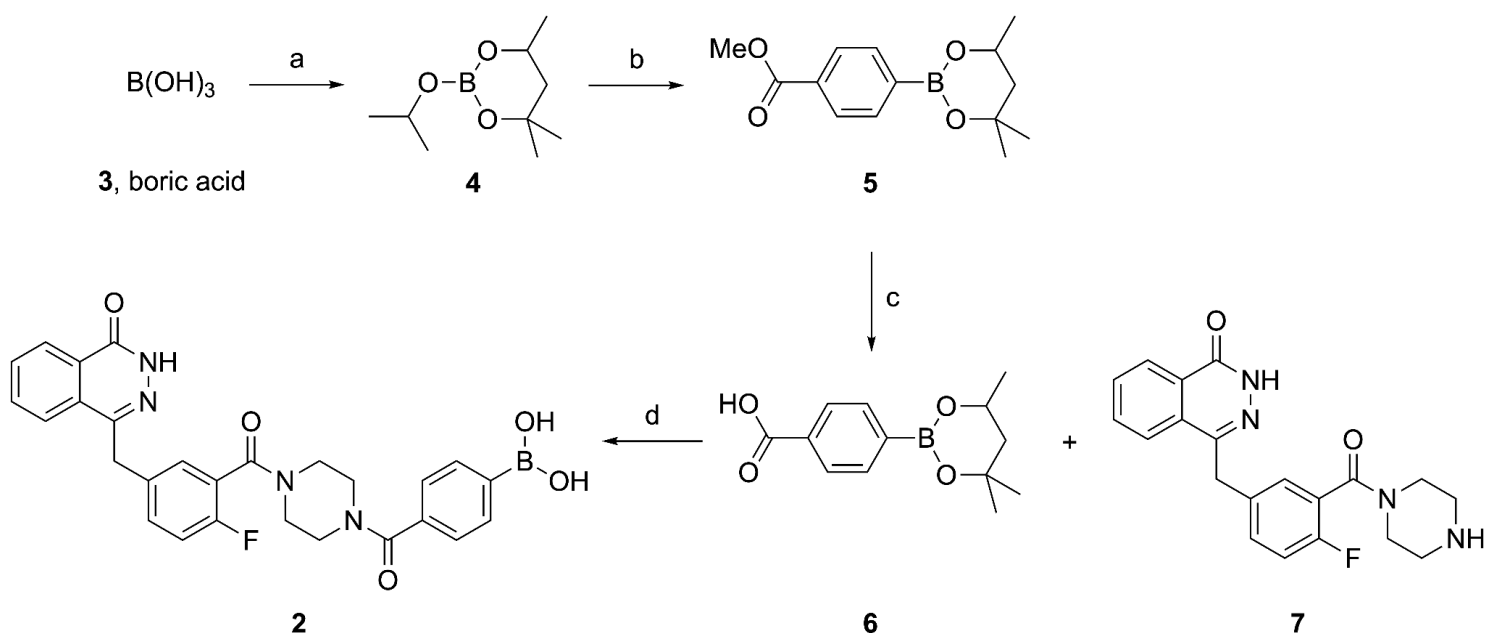


Figure 3

Synthesis of 4-PBA olaparib (**2**) from boric acid (**3**). Reagents and Conditions: (a) (i) hexylene glycol, hexane, r.t., overnight (ii) *i*PrOH, r.t., overnight, 88 % (b) *i*PrMgCl.LiCl, methyl 4-iodobenzoate, THF, -78 °C, Ar_(g), 1 h, 86 %; (c) 1:1 4M NaOH_(aq)/MeOH, r.t., overnight, 87 % (d) (i) EDCI, HOBT, DMF, r.t., overnight; (ii) 1:1 1M HCl_(aq)/ACN, reflux, 4 h, 64 %.

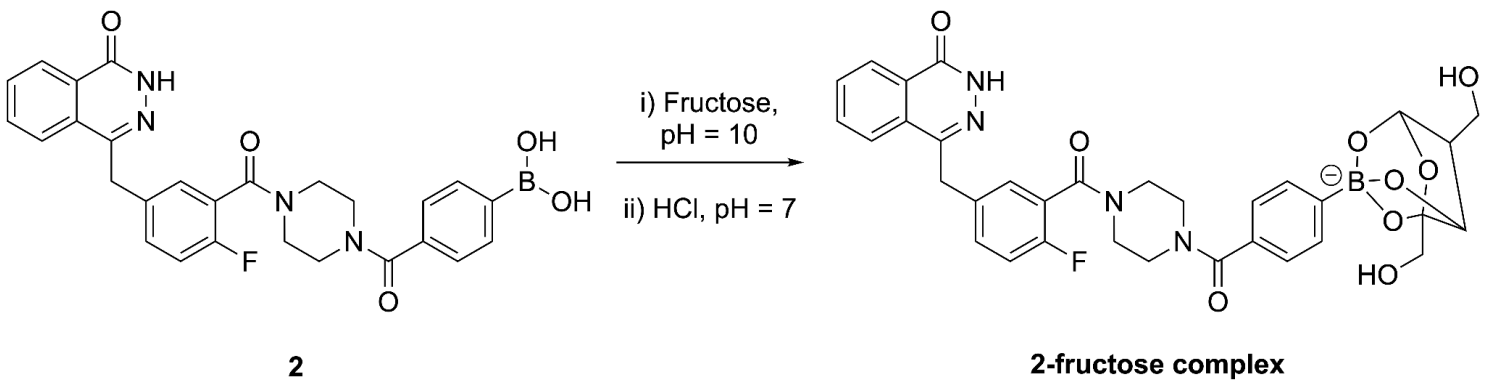


Figure 4

Formation of 4-PBA olaparib (**2**)-fructose complex.

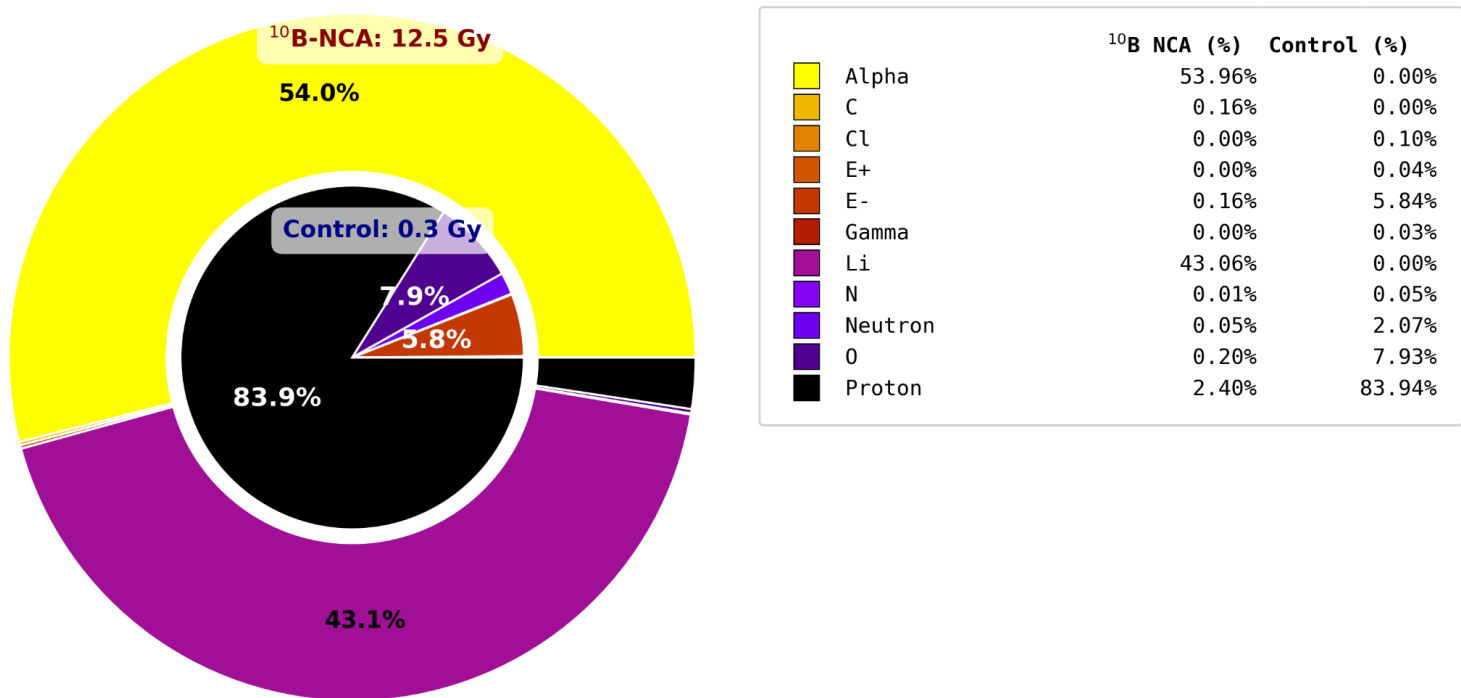


Figure 5

Simulated average dose distribution in the three cell dimensions for an equivalent of 10 minutes irradiation at the DINGO beamline using the 20 cm x 20 cm high-intensity neutron field. The inner ring

represents the control group, while the outer ring illustrates the dose distribution for the cells doped with 2.88 pg of ^{10}B per cell.

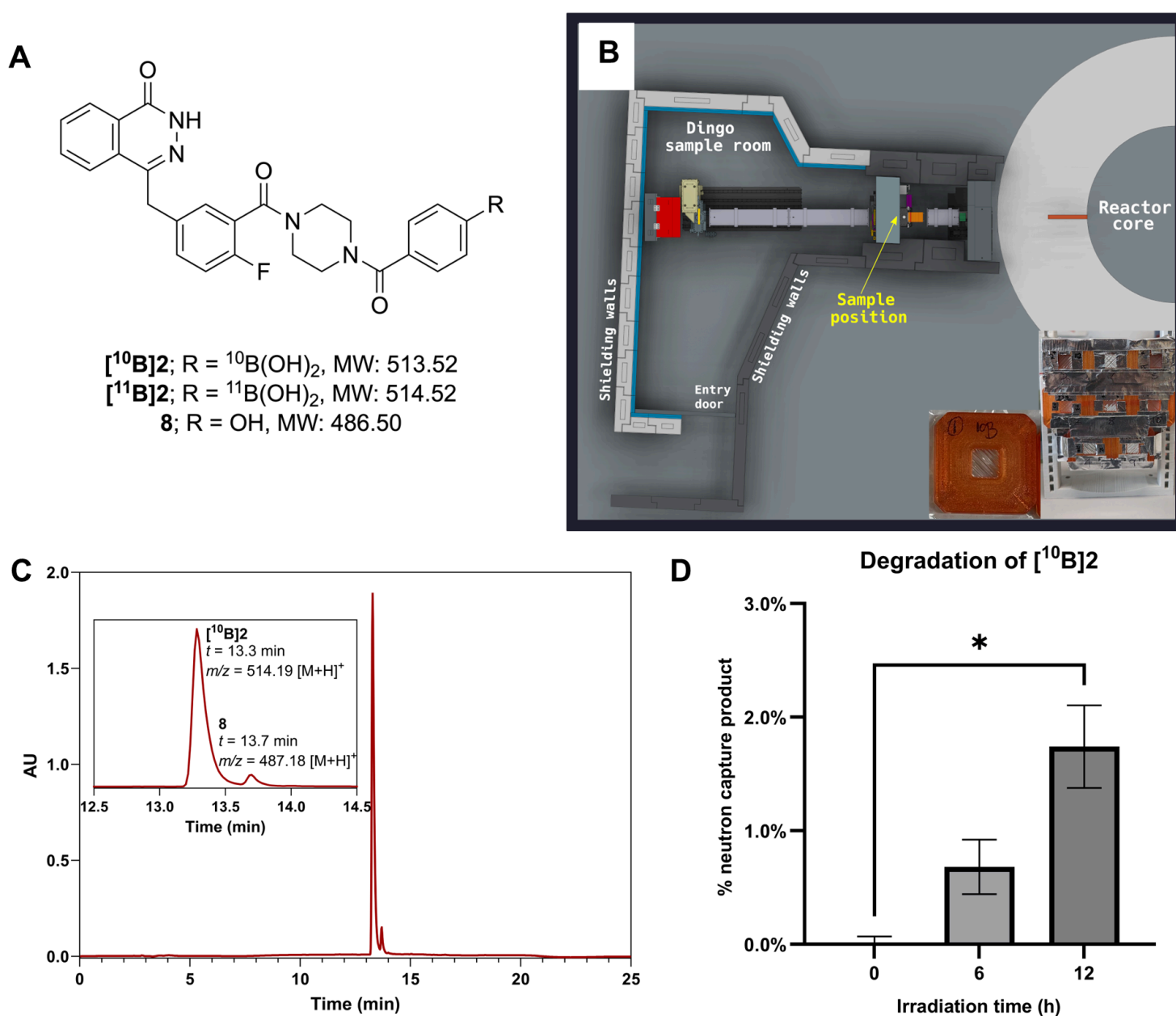


Figure 6

Results from neutron degradation analysis of 4-PBA olaparib (**2**). A) Structures of compounds synthesised from this study; B) schematic of the DINGO beamline, showing sample position relative to the reactor, including dialysis cassette arrangement. See Howell *et al.* for further information^[16]; C) HPLC trace of $[\text{}^{10}\text{B}]\text{2}$ sample after irradiation, labelled with the peaks' corresponding major m/z species from separate LCMS analysis; D) percentage of the neutron capture product 4-phenol olaparib (**8**) relative to $t = 0$ h, with increasing neutron irradiation time in the ^{10}B samples. The results between the 0 hr and 12 hr irradiations were significantly different ($* = p < 0.05$).

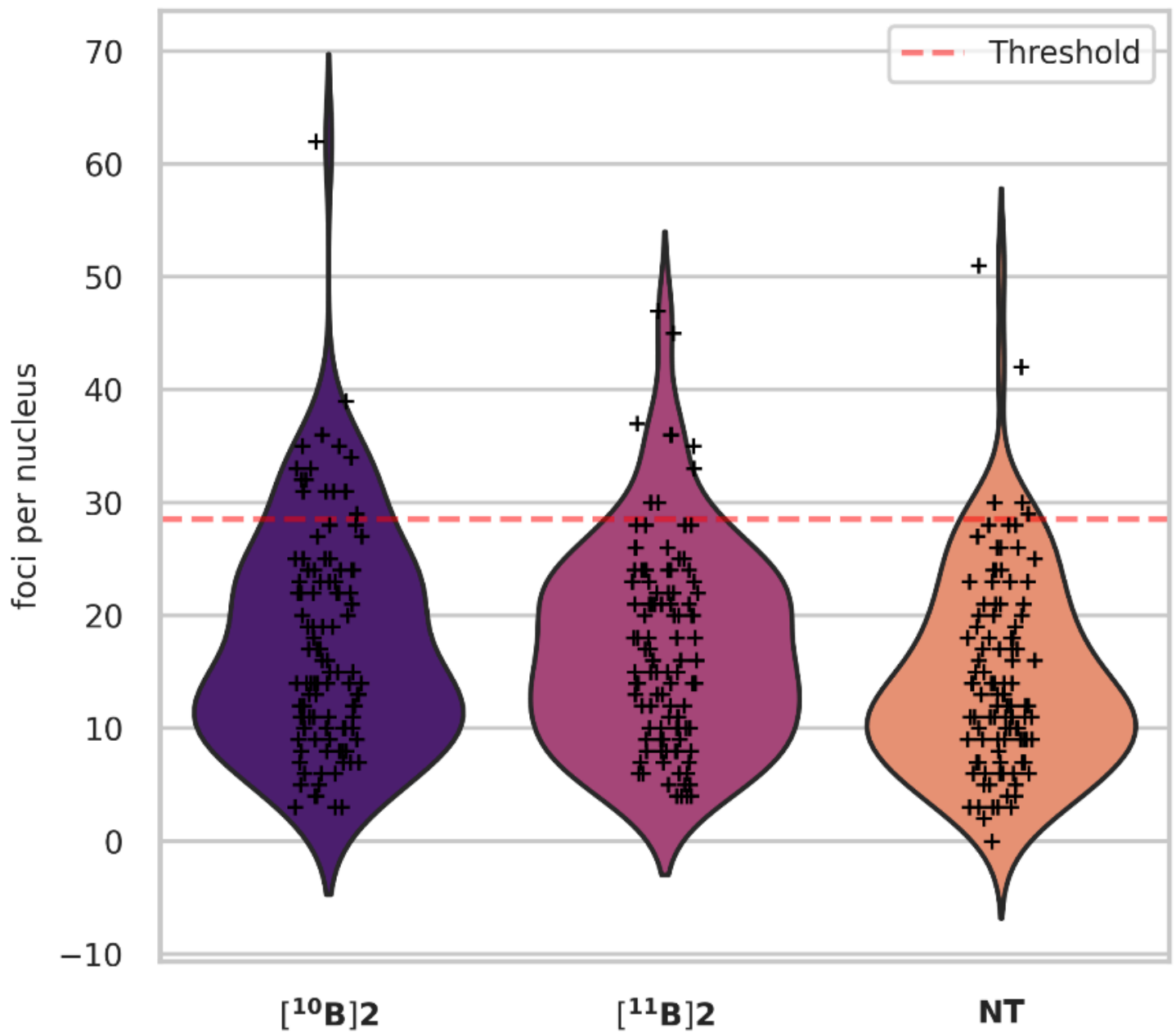


Figure 7

Distribution of γH2AX foci per nucleus in T98G cells across treatment groups, following irradiation with neutrons for 1 min, achieving a thermal neutron fluence of $\sim 1.6 \times 10^{10} \text{ cm}^{-2}$. The dashed red line indicates the 95th percentile threshold of the NT distribution (28.5 foci/nucleus).

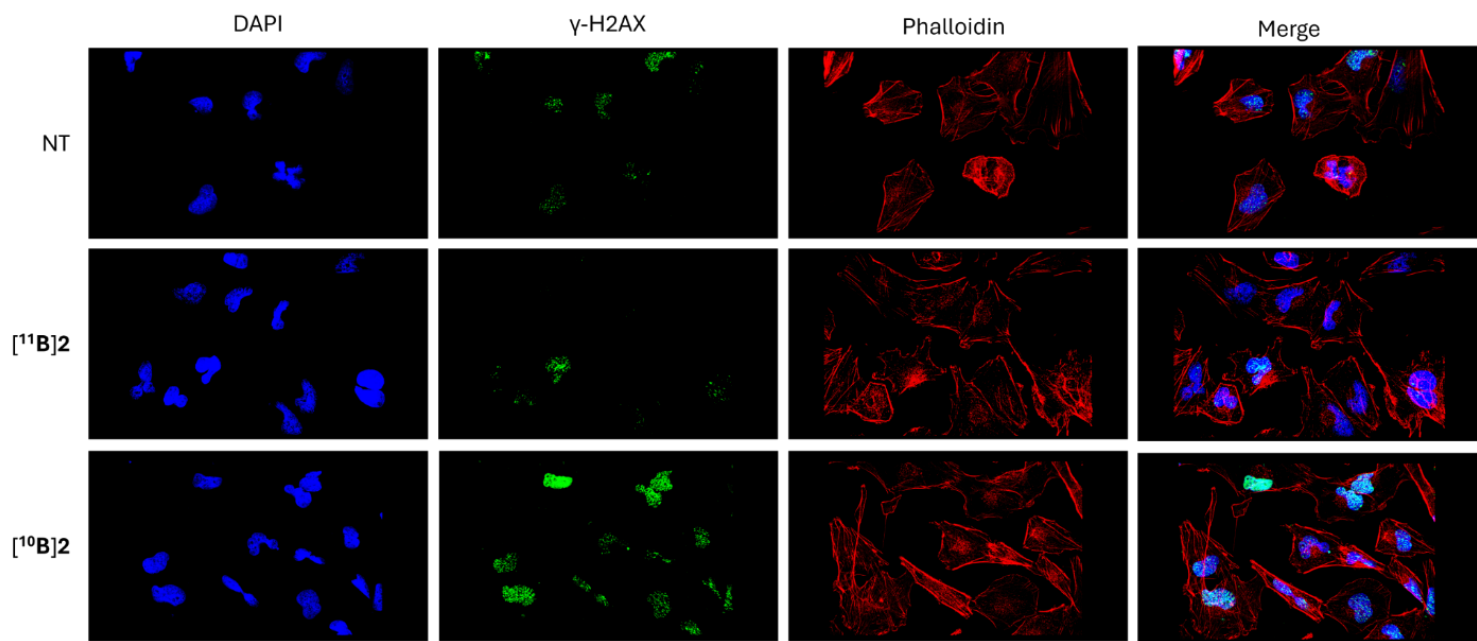


Figure 8

Representative immunofluorescence microscopy images of T98G cells showing γH2AX foci (green), nuclei (DAPI, blue) and cytoarchitecture (phalloidin, red) at 40 min post irradiation. Cells were preincubated with either $[^{10}\text{B}]\text{2}$ or $[^{11}\text{B}]\text{2}$ and irradiated with neutrons for 10 min, achieving a thermal neutron fluence of $\sim 1.6 \times 10^{11} \text{ cm}^{-2}$. Untreated cells show baseline foci formation resulting from cellular processes and neutron irradiation while cells treated with $[^{10}\text{B}]\text{2}$ show increased foci burden consistent with boron neutron capture and PARP1 inhibition. Cells treated with $[^{11}\text{B}]\text{2}$ show a slight increase in foci formation, most likely resulting from inhibition of PARP1 leading to a persistence of damage accrued through background processes. Representative images are shown from the 10 min irradiation condition and are not the dataset used for the 1 min quantitative foci analysis shown in Figure 7.

Supplementary Files

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