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Radiosensitization with an inhibitor of poly(ADP-ribose) glycohydrolase: A comparison with the PARP1/2/3 inhibitor olaparib

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ABSTRACT

Upon DNA binding the poly(ADP-ribose) polymerase family of enzymes (PARPs) add multiple ADP-ribose subunits to themselves and other acceptor proteins. Inhibitors of PARPs have become an exciting and real prospect for monotherapy and as sensitizers to ionising radiation (IR). The action of PARPs are reversed by poly (ADP-ribose) glycohydrolase (PARG). Until recently studies of PARG have been limited by the lack of an inhibitor. Here, a first in class, specific, and cell permeable PARG inhibitor, PDD00017273, is shown to radiosensitize. Further, PDD00017273 is compared with the PARP1/2/3 inhibitor olaparib. Both olaparib and PDD00017273 altered the repair of IR-induced DNA damage, resulting in delayed resolution of RAD51 foci compared with control cells. However, only PARG inhibition induced a rapid increase in IR-induced activation of PRKDC (DNA-PK) and perturbed mitotic progression. This suggests that PARG has additional functions in the cell compared with inhibition of PARP1/2/3, likely via reversal of tankyrase activity and/or that inhibiting the removal of poly(ADP-ribose) (PAR) has a different consequence to inhibiting PAR addition. Overall, our data are consistent with previous genetic findings, reveal new insights into the function of PAR metabolism following IR and demonstrate for the first time the therapeutic potential of PARG inhibitors as radiosensitizing agents.

1. Introduction

The poly(ADP-ribose) polymerase (PARP) family of enzymes are recruited to, and activated at, sites of DNA damage, where they add poly(ADP-ribose) (PAR) to themselves and to other DNA repair and chromatin-remodeling factors [1,2]. Once synthesised the PAR polymer is thought to act as a signal to recruit repair factors to the damage. In this way PARP proteins are considered to play a key role in co-ordinating the repair of single [3–10] and double strand DNA breaks [11–15], and in the restart of stalled or collapsed DNA replication forks [16–18]. Given this key function in DNA repair, several inhibitors of the PARP proteins are now under development for cancer treatment, to be used either alone [19] or in combination with DNA damaging agents such as radiotherapy (reviewed in [20]). PARP1 depletion has been shown to modestly increase sensitivity to ionising radiation (IR) in mouse models [21,22]. In addition, a variety of PARP inhibitors, reportedly targeting PARPs 1, 2 and 3 to various degrees, have been demonstrated to radiosensitize a variety of human tumour cell lines [23–27] including breast cancer [28–31], and have shown success in several preclinical and clinical trials [32–43]. Radiosensitization by these inhibitors is generally considered to be a replication dependent event [44,45].

The catalytic action of all PARPs are reversed by the endo- and exoglycosidase activities of poly(ADP-ribose) glycohydrolase (PARG) [46–50], and it is proposed that following recruitment of other repair proteins to sites of damaged DNA, PAR must be removed for DNA repair to be completed [6]. Consistent with a role in DNA repair, PARG deficient cells have been reported to display reduced efficiency of double strand break (DSB) [51–53] and single strand break (SSB) repair [6], and to have difficulties during situations of replication stress [53–56]. These defects in repair/replication suggest that PARG like PARP is a possible target as a single agent in certain genetic backgrounds [53] and for sensitizing to DNA damaging agents. The reported chemosensitizing effects are variable [6,52,57–61], but gene depletion or silencing of PARG using siRNA has consistently resulted in sensitivity to ionising radiation (IR) in mouse ES cells [62,63] and human cancer cell lines

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[51,64], with accumulation of mitotic defects and death occurring by mitotic catastrophe [51,64].

Each of the radiosensitizing studies above was carried out in cells deficient in PARG, and while supportive, the investigation of the therapeutic potential of PARG has been limited by the lack of a cell permeable, specific, PARG inhibitor. Recently, we developed a novel, first in class, PARG inhibitor – PDD00017273 [65], which showed synthetic lethal killing in cells deficient in certain homologous recombination associated proteins [66]. Here we test the ability of the same agent to sensitize breast cancer cells to IR. In addition, we compare this with the radiosensitizing effects of olaparib. Olaparib has reported IC_{50} values of 5 nM, 1 nM and 4 nM for PARP1, PARP2 and PARP3 respectively [67].

2. Materials and methods

2.1. Cell culture

The MCF-7 and MDA-MB-231 breast epithelial adenocarcinoma cell lines were purchased from the American Type Culture Collection (ATCC® HTB-22™ and ATCC® HTB-26™ respectively). Cell lines were maintained in Dulbecco's modified Eagle Medium (DMEM, Gibco, ThermoFisher Scientific, MA, USA) supplemented with 1× non-essential amino acids (NEAA, Sigma-Aldrich, MO, USA) and 10% Foetal bovine serum (Gibco) at 37 °C under an atmosphere containing 5% CO_2.

2.2. Inhibitors

The PARG inhibitor, PDD00017273, [65] was resuspended in dimethyl sulfoxide (DMSO) at a concentration of 20 μM and stored at −20 °C. A final concentration of 0.3 μM was used. The PARP inhibitor, olaparib, was purchased from Cambridge Biosciences (UK) and prepared in DMSO to give a 1000× stock. A final concentration of 1 μM was used. The dual-site binding tankyrase inhibitor – 8-Methyl-2-(3-oxo-3-(4-((quinolin-8-yl)aminocarbonyl)-phenylamino)propyl)quinazolin-4-one (compound 14 in reference [68]) was prepared as a 5 mM stock in DMSO. A final concentration of 5 μM was used.

2.3. SiRNA transfection

ON-TARGETplus siRNA was purchased from Dharmacon (GE Healthcare Life Sciences, CO, USA) for two individual PARG (NM_003631) siRNA oligonucleotides, PARP1 (NM_001618) and the non-targeting siRNA #1 (scramble) control. All siRNAs were resuspended at 20 μM in 1 × siRNA universal buffer (Dharmacon) and stored at −20 °C. Cells were seeded in 6-well plates and left overnight to attach. The following day, cells were transfected with 20 nM siRNA (final concentration) using Dharmafect 4 reagent (Dharmacon) following manufacturers' instructions. Knockdown was confirmed after 48 h by western blotting.

2.4. Clonogenic survival assay

Cells were plated at known densities in 90 mm dishes and left to attach for 4 h. After this time, inhibitors were added to the media at the concentrations stated above. The next day, cells were exposed to increasing doses of IR using an IBL437C Irradiator (Source 51.5TBq, Cs137) and then left for 15 days to form colonies. Colonies were stained with 4% methylene blue in 70% methanol and counted. Where siRNA knockdown was used, cells were transfected in 6-well plates and left for 48 h before replating at known densities in 90 mm dishes and exposing to increasing doses of IR.

2.5. Western blotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 1% sodium deoxycholate) in the presence of 1× protease and phosphatase inhibitor cocktails (Roche, Sigma-Aldrich, MO, USA). An aliquot of 30 μg total protein (measured by BioRad DC protein assay) was run on an SDS-PAGE gel and transferred to Hybond ECL membrane (GE Healthcare, CO, USA). This membrane was immunoblotted with antibodies against Poly(ADP-ribose) 10H (1:400, Enzo Life Sciences, NY, USA), PARG (1:500, Santa Cruz Biotechnology, TX, USA), PARP1 (1:1000, Santa Cruz Biotechnology) and TUBB (β-tubulin; 1:2000, Sigma-Aldrich), each diluted in 5% milk and incubated at 4 °C overnight. After the addition of the appropriate HRP-conjugated secondary antibody and further washes, the immunoreactive protein was visualised on Hyperfilm™ ECL (GE Healthcare) using ECL reagents (GE Healthcare) following manufacturer's instructions.

2.6. Immunofluorescence

Cells were plated on to coverslips and allowed to settle before treating with inhibitors overnight. The next day, cells were irradiated at 3 Gy and then either fixed immediately or left to repair at 37 °C for the time stated in the figures. Cells were fixed in 4% paraformaldehyde solution (Insight Biotechnology Ltd, UK) for 20 min at room temperature and then extensively washed (3 × 5 min in tris-buffered saline (TBS), 1 × 10 min in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 3 × 5 min in TBS). Coverslips were placed in 10% goat serum (ThermoFisher Scientific) in TBS for 1 h at room temperature to block followed by a further 3 × 5 min washes in TBS prior to incubation with the primary antibodies anti-γH2AX (ser139) (Cell Signaling, MA, USA), RAD51 (Santa Cruz Biotechnology), DNA-PKcs pS2056 (Abcam, UK) or poly(ADP-ribose) 10H (Enzo Life Sciences), Pericentrin (Abcam), β-Tubulin (Sigma-Aldrich) or the PAR binding reagent MABE1016 (Millipore) each diluted (1:500) in TBS containing 3% goat serum, for 16 h at 4 °C. The coverslips were subsequently washed 4 × 10 min in TBS followed by incubation with the secondary antibodies, Alexa-fluor 594 goat anti-rabbit IgG (ThermoFisher Scientific) or Alexa-fluor 488 goat anti-Mouse IgG (ThermoFisher Scientific) diluted in TBS containing 3% goat serum (1:500) for 1 h at room temperature and finally washed 3 × 5 min TBS. Coverslips were then mounted onto microscope slides with DAPI containing mountant (Vector Labs, CA, USA).

All images were obtained with a Zeiss LSM 510 inverted confocal microscope using planapochromat 63 × /NA 1.4 oil immersion objective and excitation wavelengths 488 nm, 546 nm and 630 nm. Through focus maximum projection, images were acquired from optical sections 0.5 μM apart and with a section thickness of 1.0 μm. Images were processed using Adobe Photoshop (Abacus Inc.). The frequency of cells containing foci was determined by counting at least 100 nuclei on each slide.

2.7. Identification of mitotic phenotypes

Cells were stained with antibodies against TUBB (β-Tubulin), PCNT (pericentrin) and DAPI and observed by fluorescence microscopy. Mitotic cells were classified into prophase, metaphase, anaphase and telophase stages based on DAPI staining of the DNA (example images of classification are shown in Supplementary Fig. 1). β-tubulin stained spindle formation was classed as abnormal if it was either monopolar, asymmetric or disorganized. Pericentrin was used to allow identification of multipolar, monopolar or fragmented centrosomes.

2.8. Micronuclei scoring

Micronuclei were identified by DAPI staining in cells stained for γH2AX in the samples treated with inhibitors alone and 12 h post-IR exposure. Cells with greater than five micronuclei where regarded as necrotic and therefore not included in the analysis. Micronuclei were