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Original article

In vivo studies of the PARP inhibitor, AZD-2281, in combination with fractionated radiotherapy: An exploration of the therapeutic ratio

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ABSTRACT

Background and purpose: Pre-clinical data have shown that PARP inhibitors (PARPi) may increase the efficacy of radiotherapy in prostate cancer. However, it is uncertain as to whether PARPi lead to clonogenic kill when combined with radiotherapy (RT).

Material and methods: We tested the PARP inhibitor AZD-2281 as a radiosensitizing agent under oxic and hypoxic conditions for clonogenic survival in vitro and in vivo using the human prostate cancer cell line, 22Rv1. In addition, the effects of PARPi + RT on normal tissue were investigated using a crypt clonogenic assay.

Results: AZD-2281 inhibited cellular PARP activity under both oxic and hypoxic conditions. The addition of AZD-2281 radiosensitized 22Rv1 cells under oxia, acute hypoxia and chronic hypoxia in vitro. The combination of AZD-2281 with fractionated radiotherapy resulted in a significant growth delay and clonogenic kill in vivo. No increased gut toxicity was observed using this combined PARPi + radiotherapy regimen.

Conclusions: This is the first preclinical study to demonstrate direct clonogenic kill in vivo by the addition of AZD-2281 to radiotherapy. As we did not observe gut toxicity, the use of PARPi in the context of prostate cancer radiotherapy warrants further investigation in clinical trials.

Prostate cancer is the most common non-cutaneous malignancy in men. While low-risk cancers are associated with an excellent prognosis, 25–50% of patients with intermediate- or high-risk disease will fail treatment despite surgery and/or fractionated radiotherapy (RT) [1,2]. In order to improve outcomes after radiotherapy two major strategies have been shown to decrease biochemical failure rates: (1) the use of neoadjuvant and/or concomitant androgen deprivation therapy (ADT); and (2) the use of dose escalation and improved image-guidance [3,4].

Tumor hypoxia is a major mechanism of radioresistance and has been shown to be associated with poor clinical outcome in a variety of diseases, including prostate cancer [5]. ADT may improve tumor oxygenation and decrease DNA repair gene expression as a mechanism for increased clinical efficacy when external beam radiotherapy (EBRT) and ADT are combined [6–8]. Therapeutic approaches that directly target DNA repair may be clinically advantageous if the therapeutic ratio is maintained. Maintenance of the therapeutic ratio could be achieved by any of the following: (1) documentation of defects in DNA damage sensing and repair in malignant cells; (2) preferential use of certain DNA repair pathways (e.g. base excision repair or homologous recombination) in malignant tissues compared with normal tissues; (3) targeting of repair defects in hypoxic cells; and (4) optimal scheduling of a DNA repair inhibitor in the neoadjuvant, concurrent or adjuvant combined treatment settings [9,10].

Poly(ADP-ribose) polymerase (PARP)-1 is a 113-kD nuclear protein that binds to both single- and double-strand DNA breaks. It is actively involved in DNA single-strand break repair via base excision repair. DNA bound activated PARP-1 utilizes NAD+ to synthesize long polymer chains (poly (ADP ribosyl)ation) on a variety of nuclear target proteins, including topoisomerases, histones and PARP-1 itself [11]. AZD2281 is a compound that is an inhibitor of the mammalian PARP-1 enzyme. It has been shown to kill selected tumor cell lines in vitro and inhibit xenograft growth in vivo either

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as a stand-alone treatment in tumors that have germline or somatic defects in DNA repair genes or in combination with chemotherapy or radiotherapy [12–14]. In addition to inherent genetics, tumor cells may also be preferentially sensitized by PARP inhibitors (PARPi) to radiotherapy by targeting of the endothelium and tumor vasculature, and increased sensitivity of repair-deficient hypoxic cells (reviewed in [10]). With respect to the latter concept, we have previously shown that hypoxia can also decrease HR protein expression and this renders cells sensitive to inhibitors of PARP-1 via "contextual" synthetic lethality [15].

Despite previous reports showing an increase in growth delay with combined RT-PARPi treatment in situ, no study to date has shown a direct kill of tumor clonogens in vivo. This is an important concept given the fact that RT-PARPi conceptually sensitizes both tumor-associated endothelium and tumor cells and in the absence of RT tumor control (e.g. TCD50), a decrease in ex vivo clonogenic survival using combined RT-PARPi would support this combined therapy regimen. In the present work, we show that the PARP inhibitor AZD-2281 directly radiosensitizes prostate cancer clonogens as assessed by in vitro and ex vivo clonogenic assays. This increased cell kill did not lead to increased cell kill of gut cell clonogens using the same fractionation scheme. RT-PARPi is therefore a promising approach to eventually improve clinical outcomes in the curative setting.

Methods

DNA repair inhibitors

The PARP inhibitor (PARPi) AZD-2281 was dissolved in 100% DMSO and stored at −20 °C in single use aliquots for in vitro experiments. A final concentration of 1 μM AZD-2281 was used for in vitro experiments. Inhibitors were added to cell cultures such that the final DMSO concentrations were kept constant at 0.1% (v/v). For in vivo experiments, AZD-2281 was first dissolved in 100% DMSO and then diluted 1/10 in 10% HPBCD/PBS to a maximal concentration of 5 mg/ml. AZD-2281 was administered via intraperitoneal injection at a 100 mg/kg dose. AZD-2281 was obtained from KuDOS Pharmaceuticals/Astra-Zeneca (Cambridge, United Kingdom).

Cell lines and tissue culture reagents

22Rv1 prostate cancer cells (gift from Dr. Yoni Pinthus) were cultured in RPMI 1640 – phenol red supplemented with 10% FCS and broad spectrum antibiotics (100 mg/L penicillin V and 100 mg/L streptomycin). Cell line was re-authenticated by STR analyses performed by The Centre for Applied Genomics, The Hospital for Sick Children (Toronto, Canada) and was confirmed mycoplasma-free, using the LookOut® Mycoplasma PCR Detection Kit (MP0035, Sigma–Aldrich). Cells were initially serially passaged through CD1 nude mice (Charles River, Wilmington, MA) and seeded into T75 flasks. Stocks were frozen down and cells were grown up and used in vitro or in vivo as needed.

RNA extraction and real-time quantitative PCR

Total RNA was isolated using Trizol Reagent (Invitrogen) as previously described [16]. Sample RNA or human reference RNA (Stratagene) was treated with DNase I (Roche Diagnostics). Reverse transcription PCR (RT-PCR) was performed using the TaqMan Reverse Transcription kit (Applied Biosystems). cDNA was then amplified for hypoxia-mediated target gene VEGF using ABI TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems); HPRT was used as an endogenous control. PCR was run using an ABI real-time quantitative PCR machine (Applied Biosystems). The fluorescence intensity threshold was set at 0.2 and the reaction cycle-threshold was obtained. Relative gene expression was calculated using the comparative CT method.

Western blotting

Western blotting was completed as follows: 50 μg of lysate was initially denatured in SDS sample buffer (58 mM Tris–HCl, pH 6.8, 1.71% SDS (w/v), 0.83% (w/v) b-mercaptoethanol, glycerol 6% (v/v), 0.002% (w/v) bromophenol blue) at 100 °C for 5 min and loaded into SDS–PAGE wells on 10% tris–glycine gels. Gels were wet transferred to PVDF for 1 h and blocked with LI-COR blocking buffer (LI-COR Biosciences, NE, diluted 1:1 in PBS for 1 h at room temperature). The blots were incubated with appropriate primary antibodies diluted in LI-COR blocking buffer (1:1 in PBS) overnight at 4 °C, followed by five washes with TBS-T at room temperature. Primary antibodies were used at the following dilutions: anti-actin mouse monoclonal antibody (A5316, Sigma–Aldrich, WI) 1:50,000, anti-PARP mouse monoclonal clone C-2-10 antibody (4338-MC, TACS/Trevigen, MD) 1:1000, anti-RAD51 (H-92) rabbit polyclonal antibody (sc-8349, Santa Cruz Biotechnology Inc., CA) 1:1000, anti-Ku70 (A-9) mouse monoclonal antibody (sc-5309, Santa Cruz Biotechnology, Inc., CA) 1:1000, anti-HIF-1a rabbit monoclonal antibody (2015-1, Epitomics, CA) 1:1000. The washed blot was incubated with appropriate secondary antibodies conjugated to IRDye 680 or IRDye 800 (LI-COR Biosciences, NE) diluted 1:20,000 in LI-COR blocking buffer (diluted 1:1 in PBS) for 30 min at room temperature, followed by five washes with TBS-T at room temperature. Western blots were imaged and analyzed using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences, NE).

In vitro clonogenic survival assay

Logarithmically growing 22Rv1 cells were exposed to oxic conditions (21% O_2) in the incubator. Acute hypoxic (6 h) and chronic hypoxic (72 h) conditions were obtained using a HypOxystation H35 workstation (HypOxygen, MD) at 0.2% O_2 with 5% CO_2 and balanced N_2. Cells were irradiated (2, 5, or 10 Gy) with 137Cs rays using a MDS Nordion ionizing gamma irradiator (Gammacell 40 Exactor) under oxia or hypoxia.

Clonogenic survival was assayed as previously described [17]. After exposure to gassing conditions, drug treatment, and/or irradiation, cells were trypsinized from T75 flasks and seeded in triplicate in 6-well tissue culture dishes containing media supplemented with DMSO, or 1.0 μM PARP inhibitor (AZD-2281). The cells were returned to the tissue culture incubator for 16–20 h. Drug media were then replaced with fresh inhibitor-free media and cells were returned to the incubator and left to grow. After 16 days, surviving colonies (defined as >50 cells) were fixed and stained with 1% (w/v) methylene blue in 50% (v/v) ethanol. Clonogenic survival fraction (SF) was expressed as the relative plating efficiencies of the irradiated to control cells. Five independent experiments were completed.

For radiation dose–response curves, the data were fitted to the linear–quadratic (LQ) equation $S = e^{-\alpha D-\beta D2}$ using GraphPad Prism 5.0 (GraphPad Software, CA), where S is the surviving fraction, α and β are inactivation constants and D is the dose in Gy. The oxygen enhancement ratio (OER) was calculated based on the relative doses under hypoxia versus oxia at a survival fraction of 0.1 [15]. The area under the curve (AUC) which represents the mean inactivation dose (MID) was also calculated using GraphPad. The parameters SF2 (survival fraction at 2 Gy), α, and MID have been used to intercompare radiation survival curves [18]. The radiation Sensitizer enhancement ratios (SER) for AZD-2281, was calculated by dividing MID of Control by MID with AZD-2281.

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