The PARP inhibitor AZD2461 is differentiated from olaparib for both PARP3 activity and drug resistance

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Abstract

AZD2461 is a poly (ADP) ribose polymerase (PARP) inhibitor developed as a follow-up to olaparib, the first PARP inhibitor approved for cancer therapy. Resistance to olaparib in BRCA knock-out models predominantly involves over-expression of P-glycoprotein. AZD2461 was selected as a poor substrate for drug transporters and we demonstrate efficacy *in vivo* against olaparib-resistant tumors that over-express P-glycoprotein. In addition, AZD2461 appeared better tolerated in combination with chemotherapy than olaparib, suggesting AZD2461 could have significant advantages in the clinic. However, the superior toxicity profile in mice does not extend to rats. As explanation, we show both differential PARP3 activity for olaparib and AZD2461 and a significantly higher PARP3 level in mouse bone marrow cells compared with rats and humans. These findings have implications for the use of mouse models to assess bone marrow toxicity for DNA-damaging agents and inhibitors of the DNA damage response. Structural modeling of the PARP3 active site with different PARP inhibitors also highlights the potential to develop compounds with different PARP family member specificity profiles designed for optimal anti-tumor activity and tolerability.

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Introduction

Inhibitors of the DNA damage response (DDR) offer an exciting opportunity to identify targeted cancer therapies (1-3). In addition to enhancing the effectiveness of DNA-damaging chemotherapies and ionizing radiation (IR) treatment, DDR-inhibitors have the potential for single-agent activity in specific tumor genetic backgrounds based on the principle of synthetic lethality (4). This was first exemplified by inhibitors of the DDR protein poly(ADP-ribose) polymerase (PARP) in Breast Cancer Associated (*BRCA*)-deficient genetic backgrounds that are associated with a high lifetime risk of breast and ovarian cancer (5, 6).

The mechanism for this single-agent activity has been linked to the role of PARP in the repair of DNA single-strand breaks (SSBs) (7, 8) and DNA double-strand breaks (DSBs) (9, 10). Following inhibitor treatment, PARP is trapped onto the unrepaired SSB, resulting in a protein-DNA adduct (11) that impedes replication fork progression, leading to replication fork collapse and the generation of the more genotoxic DSBs. These DSBs would normally be repaired by the homologous recombination repair (HRR) pathway (12), in which both *BRCA1* and *BRCA2* genes play pivotal roles (13). However, in tumors with HRR-defective backgrounds (e.g. because of BRCA deficiency), error-prone DNA repair pathways are utilized instead (9, 10, 14), resulting in the accumulation of genomic instability, chromosomal aberrations, and subsequently, cancer cell death. As a patient's normal tissue ordinarily has a functional HRR pathway, this results in tumor-specific cell killing.

Olaparib (AZD2281) is an oral and potent inhibitor of PARP activity (15) and the first PARP inhibitor to gain regulatory approval (16), (17). Olaparib demonstrates low nanomolar activity against PARP1 and PARP2 enzymes and only weak enzyme activity against tankyrase-1 (15). The vast majority of poly(ADP) ribosylation that can be detected in

mammalian cells is attributed to PARP1 (18), the key PARP protein involved in SSB repair, with PARP2 recognizing gaps and flap structures (19).

Olaparib has demonstrated single-agent anti-tumor activity in patients with both *BRCA*-mutant ovarian (20) and breast cancer (21), as well as in the broader serous ovarian cancer patient population, where patients who do not harbor *BRCA* mutations have also gained clinical benefit from single-agent olaparib treatment (22, 23). In this latter study, olaparib demonstrated a significant increase in progression-free survival (PFS) compared with placebo in patients with high-grade serous ovarian cancer in a maintenance setting. Olaparib was very well tolerated as a single agent and cessation of treatment was due primarily to tumor progression, most likely as a consequence of emerging tumor resistance.

Currently, there is a paucity of data around resistance mechanisms associated with olaparib in the clinical setting. Preclinically, resistance mechanisms that have been identified in *BRCA*-deficient tumors include the reactivation of *BRCA2* gene reading frames by secondary mutations (24); the loss of 53BP1 (25, 26) or REV7 (27) in *BRCA1*;p53-deficient cancer cells, and the over-expression of the *Abcb1a* and *Abcb1b* genes encoding the mouse drug efflux transporter P-glycoprotein (P-gp), for which olaparib is a substrate. This last mechanism has been described in both *BRCA1*;p53-deficient (28) and *BRCA2*;p53-deficient (29) mouse mammary tumors. While the clinical significance of P-gp-based resistance for olaparib is still not clear, it may be of relevance in some cancers that are more commonly associated with high P-gp levels, e.g. colorectal cancers and acute leukemias.

Here, we characterize a second-generation PARP inhibitor, AZD2461, derived from the same chemical series as olaparib, which retains the same level of anti-cancer cell potency (*in vitro* and *in vivo*) but is differentiated from olaparib in terms of sensitivity to drug resistance mechanisms and PARP inhibitor profile.

Materials and Methods

Olaparib and AZD2461 PARP inhibitor compounds

The PARP inhibitor olaparib (AZD2281, KU-59436) has been described previously (15). AZD2461 synthesis is described in the international patent WO2009/093032, specifically compound numbers 2b and 47. Formulation of compounds for *in vivo* studies is provided in **Suppl Materials and Methods**.

Cell line and culture methods

Human lung carcinoma A549 cells, breast cancer cell lines MDA-MB-436, T47D, BT549, MDA-MB231, SUM1315MO2, and SUM149PT, cervical cell lines HeLa, were grown in culture media conditions described in **Suppl Materials and Methods**. *BRCA1* mutation status of breast cell lines has been described previously (30).

Alkaline comet assay

A549 cells were pre-treated for 1 hour at 37^{0}C with 0.05% dimethyl sulfoxide (DMSO), olaparib, or AZD2461 (500 nM). Cells were treated with 30 Gy γ-IR (on ice) and allowed to recover at 37^{0}C for the indicated time. SSBs (tail moments) were analyzed by alkaline comet assay as described (31). Comet tail moments were scored from 100 cells per experiment using Comet Assay IV (Perceptive Instruments). Two-way analysis of variance was carried out on the mean values for each time point from replicate experiments.

Immunofluorescence

For γ-H2AX analysis, A549 cells on cover slips were pre-incubated for 1 hour with DMSO or PARP inhibitors, and then irradiated (2Gy). The cells were incubated at 37ºC for the times

stated to allow repair. Cells were then immunostained for γ-H2AX foci, which were counted microscopically. For more details see **Suppl Materials and Methods**.

PARP1, PARP2, and tankyrase isolated enzyme *in vitro* assays

The activity (IC_{50}) of PARP inhibitors was assessed against purified PARP1, PARP2, and tankyrase enzymes *in vitro* as described (15).

PARP3 Bio-NAD *in vitro* assay

PARP3 protein was prepared as described (32). A detailed protocol of the assay is described in **Suppl Materials and Methods**. In short, the ribosylation reaction mix was incubated with Bio-NAD+ (Trevigen) and Sau3A-cut peGFP-C1 plasmid (Clontech). Products were separated by SDS-PAGE, blotted onto nitrocellulose membrane, and signal detected using Streptavidin-HRRP (GE Healthcare).

Clonogenic and cell proliferation assays

Cells were detached from the flask using trypsin and washed with medium. The pellet was dislodged in growth medium into a single-cell suspension and counted in Nucleocounter. Cells were seeded in recommended dilutions (see **Suppl Material and Methods**) into 6-well plates in triplicates overnight. Olaparib and AZD2461 were prepared as 10 mM stock solution in 100% DMSO and added in final concentrations 0, 0.123, 0.37, 1.1, 3.3, and 10 μM to the cells. Plates were incubated at 37°C, 95% air, 5% CO_2 until colonies of >50 have formed (approximately 7–14 days after dosing). Colonies were visualized by Giemsa staining (see **Suppl Material and Methods**) and scored using Colcount software (Oxford Optronix). Cellular IC_{50} values were determined for each cell line using Microsoft Excel and ID-BS XLfit (v4.2.2) charting application.

For sulphorhodamine B (SRB) assay, cells were seeded into 96-well plates (6000 cells/well) in 90 µL of growth medium. In triplicate, AZD2461 or olaparib were added in final concentrations of 0, 1, 3, 10, 30, and 200 nM 1 hour prior to addition of 0, 3, 5, 7, 10, 12, and 15 µg/mL of MMS. Cells were incubated for 18 hours; then medium was replaced with 200 µL fresh medium. Plates were incubated for a further 72 hours before cells were fixed (10% trichloracetic acid, 100 µL/well) for 30 minutes at 4ºC. Wells were washed with water and stained with 0.4% SRB (Sigma) in 1% acetic acid for 15 minutes at room temperature. Excess stain was removed by washing with 1% acetic acid and dry stained precipitate was dissolved with 100 µL/well 10 mM Tris buffer. Absorbance was determined at 564 nM. Data are presented as percentage cell growth relative to untreated control. Cellular PF_{50} (potentiation factor at 50% cell survival) was calculated as the ratio of the IC_{50} for MMS alone versus MMS in combination with single concentrations of PARP inhibitors.

Rodent experiments

Brca1 $^{\Delta5\text{-}13/\Delta5\text{-}13}$;*p53*$^{\Delta2\text{-}10/\Delta2\text{-}10}$ mammary tumors were generated in *K14cre*;*Brca1* $^{F5\text{-}13/F5\text{-}13}$;*p53*$^{F2\text{-}10/F2\text{-}10}$ mice and genotyped as described (33). Orthotopic transplantations of tumor fragments into syngeneic animals and caliper measurements of mammary tumors have been previously reported, along with the generation of the olaparib resistance tumor T6-28 (28). All treatments were started when tumor volume reached about 200 mm^3 ($v=length*width^2/2$). Olaparib (50 mg/kg intraperitoneally [i.p.]), tariquidar (2 mg/kg i.p.), or AZD2461 (100 mg/kg orally [p.o.]) were given daily. Tariquidar was administered 30 minutes before olaparib.

Mice (CD-1 Nude *Foxn1*^{nu}, Charles River Labs, UK) were orally dosed once daily for 5 days with the combination therapies temozolomide (50 mg/kg) plus either olaparib (10 mg/kg) or AZD2461 (10 mg/kg) followed by once-daily dosing of olaparib or AZD2461 for an

additional 2 days. Rats (HsdHan:RNU-*Foxn1*^{rnu}) were dosed orally once daily for 5 days with single agents or combination therapies (temozolomide 50 mg/kg, olaparib 10 and 20 mg/kg, or AZD2461 10 and 20 mg/kg). During the dosing phase, animals were weighed daily. For bone marrow analysis, mice were culled at day 4, 6 (n = 8 per group), and 8 (n = 3 per group). Rats (n = 8 per group) were culled on day 5 at 2 hours after the final dose. All experimental procedures were carried out according to current UK Home Office regulations.

Flow cytometry analysis of bone marrow cells

Bone marrow cells were prepared from rodent femurs as described in **Suppl Materials and Methods** and samples analyzed on a flow cytometer (FACS Canto, Becton and Dickinson, UK) using forward (FSC-H) and side scatter (SSC-H) on linear scales.

RNA isolation and RT-PCR

Total RNA was extracted from fresh bone marrow of six individual mice (CD-1 Nude *Foxn1*^{nu}), six rats (HsdHan:RNU-*Foxn1*^{rnu}), and human bone marrow mononuclear cells from six different individuals (Lonza 2M-125C) using RNeasy Mini kit (Qiagen) according to manufacturers' instructions. To remove potential DNA contamination, the samples were DNase treated (RNase-Free DNase Set, Qiagen). RNA purity and concentration were determined spectrophotometrically (260/280 >1.9). RNA integrity was assessed on an Agilent 2100 Bioanalyzer using RNA 6000 Nano Chip.

Human, rat, and mouse PARP1, 2, and 3 sequences were aligned using the Megalign module in DNAstar to facilitate design of primers and probes for both TaqMan and SYBR green qRT-PCR assays in areas of sequence identity. All sequences were also blast searched using the BLAST tool from the NCBI website to ensure target specificity. Assay efficiencies were calculated for each primer set to ensure template DNA from all species were amplified

similarly. Primers and probes were custom or catalogue ordered from Life Technologies (Invitrogen). Sequences are listed in **Suppl Materials and Methods**.

Results and Discussion

Generation of the novel PARP inhibitor AZD2461 as a follow on to olaparib

Based on emerging preclinical resistance data, we set about identifying a compound of similar efficacy to olaparib but without the potential liability associated with being a substrate for the P-gp (MDR1) transporter. Triaging of compounds following medicinal chemistry (see **Suppl Materials and Methods**) identified two series of compounds around the phthalazinone core of olaparib. AZD2461 (**Fig. 1A**) was identified as the optimal compound from the Piperidine Ether series (see international patent WO2009/093032, specifically compound number 2b and 47) and is a potent inhibitor of both PARP1 and PARP2 having enzyme IC_{50} values of 5 nM and 2 nM, respectively, comparable to that of olaparib (5 nM and 1 nM, respectively). The lack of selectivity between PARP1 and PARP2 may be considered a positive feature when attempting to inhibit SSB repair, as PARP2 also plays an important role in this pathway (34). To confirm that AZD2461 is as effective at inhibiting SSB repair as olaparib, we compared the ability of both compounds to inhibit the formation of cellular poly (ADP-ribose) (PAR) polymers following treatment with 10 mM hydrogen peroxide. AZD2461, like olaparib, was effective at inhibiting PAR formation in these assays (**Suppl Fig. 1**). To confirm that observed PAR inhibition translated into inhibition of SSB repair, we employed the alkaline COMET assay**,** where the length of the comet tail moment represents the degree of unrepaired SSBs following the induction of DNA damage by IR (**Fig. 1B**). We were also able to demonstrate that AZD2461 potentiated the anti-proliferation effect of the DNA-damaging alkylating agent methyl methanesulfonate (MMS), which induces SSBs (**Fig. 1C and Suppl Table 1**).

Activity against *BRCA*-deficient tumors through synthetic lethality is a key component of olaparib's developmental program. Thus, we sought to determine the potency of single-agent

AZD2461 in *BRCA*-deficient cancer cells. *In vitro* clonogenic assays were performed with AZD2461 and olaparib against a panel of breast cancer tumor cells with either mutant (MDA-MB-436, SUM1315MO2, and SUM149PT) or wild-type (WT) (T47D, BT549, and MDA-MB-231) *BRCA1* gene status. Both olaparib and AZD2461 exhibited similar PK and PD profiles (see **Suppl Materials and Methods** and **Suppl Fig. 2**) and significant potency as single agents in the *BRCA1*-mutant breast cancer cell lines but not in the *BRCA1* WT cell lines (**Fig. 1D**)

An assessment of *in vitro* permeability and efflux of AZD2461 was undertaken in the human intestinal-derived cell line CaCo-2 (see **Suppl Materials and Methods** and **Suppl Table 2)** and in the matched cell lines KBA1, a genetically modified version of HeLa that over-expresses high levels of P-gp (35), and KB31, which does not over-express P-gp (**Fig. 2A**). While this functional assay does not distinguish between saturation of the P-gp pumps versus low binding, it does allow discrimination between compounds that are highly effluxed and those that are not. Using these two assays, we were able to distinguish low versus highly effluxed compounds, while addition of the P-gp inhibitor verapamil provided further evidence that efflux was occurring through a P-gp mechanism. The data in **Fig. 2A** show that, in contrast to olaparib, AZD2461 has similar activity between KBA1 cells and matched WT KB31 cells. Moreover, the addition of verapamil to KBA1 cells showed very little effect on the cellular activity of AZD2461, which indicated that AZD2461 is significantly less prone to the P-gp-mediated efflux mechanisms than olaparib. Similar data supporting a lack of P-gp liability were obtained in the colorectal cancer cell line HCT-15, which is characterized by high levels of endogenous Pg-P expression (**Suppl Fig. 3 and Suppl Table 3**).

To assess AZD2461 activity in the more clinically relevant *BRCA*-mutant background and acquired resistant setting, we used *in vitro* and *in vivo* models where prolonged treatment

with olaparib had led to resistance and high levels of P-gp expression. The *BRCA2*-deficient mouse breast cancer line KB2P3.4 was generated from a *BRCA2*-deficient mouse mammary tumor that demonstrated sensitivity to olaparib treatment (36). The KB2P3.4 cell line was treated in culture with olaparib for 2 months in order to induce the olaparib-resistant line KB2P3.4R, and over-expression of Pg-P was confirmed using immunofluorescence with a P-gp antibody (**Fig. 2B**). Treatment of the parental KB2P3.4 with AZD2461 resulted in a similar response to olaparib (**Fig. 2B**). However, unlike olaparib, AZD2461 was also effective in the high P-gp-expressing KB2P3.4R cell line. Consistent with this difference being based on P-gp, the olaparib sensitivity in KB2P3.4R cells was restored on co-treatment with tariquidar, a new generation P-gp inhibitor.

Increased expression of the *Abcb1a* and *Abcb1b* genes encoding the mouse drug efflux transporter P-gp contributes to olaparib resistance in *BRCA1*;p53-deficient mouse mammary tumors (28). To determine whether AZD2461 could overcome olaparib resistance *in vivo*, small tumor fragments of an olaparib-resistant tumor (T6-28) exhibiting an 80-fold increased expression of the *Abcb1b* gene were transplanted orthotopically into syngeneic WT female mice. When the tumor volume reached about 200 mm^3, the animals were treated with olaparib, combination of olaparib with the P-gp inhibitor tariquidar, or with AZD2461. As expected, olaparib resistance was successfully overcome by tariquidar pre-treatment (**Fig. 2C**). We also found that these tumors were sensitive to AZD2461 without the need for additional tariquidar treatment, consistent with the idea that spontaneous *BRCA1*;p53-defective mammary tumors, in which resistance is caused by increased P-gp-mediated drug efflux, remain sensitive to AZD2461. Consistent with these findings, in a separate study, long-term AZD2461 treatment in the *BRCA1*;p53-defective mouse tumor model suppressed the development of drug resistance (37).

Together, these data demonstrate that AZD2461 is a potent inhibitor of PARP1 and PARP2 that can provide effective inhibition of SSB repair and has significant single-agent activity in *BRCA*-deficient cancer cells, comparable to that of olaparib. Moreover, we have shown that AZD2461 is a poor substrate for P-gp and has activity in olaparib-resistant cancer cells that over-express P-gp *in vitro* and is capable of anti-tumor activity *in vivo* in olaparib-resistant tumors where resistance is based on the over-expression of P-gp. In this respect, the initial goal to develop a follow-on compound to olaparib that has comparable activity but without olaparib's P-gp liability has been successful. As a result, AZD2461 represents an excellent preclinical tool to study PARP inhibitor resistance, as it avoids this common mechanism of resistance and may help to identify additional mechanisms of PARP inhibitor resistance.

AZD2461 is as efficacious as, and better tolerated than, olaparib in combination with temozolomide in a mouse xenograft model

In addition to the utility of PARP inhibitors as single agents, there is also a strong rationale for combination with DNA-damaging chemotherapies such as temozolomide or camptothecins that induce SSBs (38, 39). As a single agent, the synthetic lethality of olaparib relies on the endogenously generated SSBs and the inability of *BRCA* mutation or other HRR deficiency to repair the ensuing DSBs, ultimately resulting in cancer cell death. In combination with DNA-damaging chemotherapies, the number of SSBs being generated by the chemotherapy agent is much larger than those that occur endogenously (40) and if repair is prevented (e.g. by treatment with a PARP inhibitor), cell death can be induced even when the HRR pathway is functional, purely because the DNA damage threshold that can be tolerated is exceeded. This has two important implications: first, a combination of a PARP inhibitor and SSB-inducing agent can result in the killing of HRR-proficient as well as HRR-

deficient cancer cells; second, there is also the potential for increased damage in normal tissue compartments, which is evidenced in the increased hematological toxicities observed in patients when PARP inhibitors and alkylating agents are combined (41).

To assess AZD2461 in combination with temozolomide *in vivo*, we used a mouse colorectal xenograft (SW620) model to compare the anti-tumor activity of 50 mg/kg temozolomide given once daily for 5 days either alone or in combination with 10 mg/kg AZD2461 or 10 mg/kg of olaparib where the PARP inhibitors were given for 7 days. This dose and schedule of olaparib had previously demonstrated potentiation of temozolomide anti-tumor activity **(Suppl Fig. 4A).** The data presented in **Fig. 3A** show that, as expected, temozolomide alone demonstrated anti-tumor activity and that this was improved considerably by combining with a PARP inhibitor. Statistical analysis of the results on day 55 (when the temozolomide treatment group was culled) showed that this difference was significant ($p<0.001$) and that the effect between the combination groups (i.e. temozolomide plus olaparib versus temozolomide plus AZD2461) was not statistically significant either at day 55 ($p=0.53$) or when the study was stopped at day 73 ($p=0.57$). Moreover, both combination treatments conferred considerable delay in tumor re-growth compared with temozolomide alone.

To examine the potential impact of AZD2461 in terms of tolerability, we looked at body weight loss as a gross indicator and at myelosuppression as a more clinically relevant indicator of combinatorial toxicity. Body weights of mice decreased relative to their weight at the start of the experiment but recovered quickly after the dosing was finished; weight loss was greater in the two combination groups (**Suppl Fig. 5A**).

To assess the impact of the combinations on bone marrow populations compared with single agent chemotherapy, we took cohorts of mice and gave the same dose and regimen as used in

the combination efficacy study. Mice were culled at time points across the dosing phase to create a time course in which the impact of the treatments on the bone marrow cells could be assessed (**Fig. 3B and 3C**). At the designated time points, femurs were excised and bone marrow cells flushed out and counted using flow cytometry. Using forward and side scatter parameters, three distinct white blood cell populations could be identified (lymphocytes, monocytes, and neutrophils) and these data showed that alone, temozolomide led to a reduction in the total number of white blood cells in bone marrow with the nadir after the last dose and a failure to return to the starting levels at day 8. Temozolomide in combination with olaparib led to a significantly greater impact on bone marrow (a nadir at day 6 and a worse state of recovery at day 8).

An unexpected finding, however, was that AZD2461 in combination with temozolomide did not result in the same severity of bone marrow effects as the olaparib combination. In fact, the nadir was not statistically different from temozolomide alone and there was a good recovery by day 8. Although lower doses of olaparib could result in less bone marrow toxicity, these are also likely to be less efficacious, as demonstrated by **Suppl Fig. 4**, which shows that doses of 3 mg/kg of olaparib and below in combination with temozolomide (50 mg/kg) do not confer a statistically significant benefit over temozolomide alone (maximum tolerated dose 68 mg/kg).

Taken together, these data suggest that AZD2461 might have two potential advantages over olaparib. First, while having a similar level of anti-tumor activity, AZD2461 did not have the same level of P-gp liability as olaparib; second, AZD2461 appeared better tolerated in combination with a DNA-damaging chemotherapy. The basis for this latter observation, though, could not obviously be attributed to PARP1/PARP2 inhibition, as AZD2461 and olaparib are similar in this respect, suggesting an as yet unidentified mechanism.

AZD2461 and olaparib have differential activity against PARP3

One possible explanation for the hematological toxicity differences observed between olaparib and AZD2461 could be differential PARP3 activity. The PARP3 protein plays an important role in non-homologous end joining (NHEJ), where it is stimulated by DSBs *in vitro* and it functions in the same pathway as the PAR-binding protein APLF to accelerate chromosomal DSB repair (32). Following IR treatment, phosphorylated histone H2AX (γH2AX) can be used as a marker of DSB damage (42, 43). The repair of DSBs and the decline in γH2AX over time following IR was abrogated by either the loss of PARP3, APLF, or treatment with the PARP inhibitor KU-58948, which is related chemically to olaparib (32).

The link between PARP3, NHEJ, and mouse bone marrow was highlighted in studies of murine hematopoietic stem and multipotent progenitor cells (HSPCs), which were shown to be more resistant to IR-induced damage than more differentiated progenitor cells (44). This difference was based on both an increased resistance to apoptosis and the ability to repair DNA by NHEJ. The NHEJ pathway is a less reliable alternative to HRR DSB repair, and mice bone marrow stem cells appear to utilize NHEJ while human HSPCs undergo apoptosis in response to a DNA damage insult instead (45). It has been suggested that the different ways the hematopoietic cells from mice and humans handle DNA damage may reflect the different challenges faced by mammals with diverse life spans and ages of reproductive maturity (46).

To investigate whether differential PARP3 activity could provide the basis for the differential hematological toxicity observed with olaparib and AZD2461 in mice, we looked at the effect of both of these PARP inhibitors on γH2AX following IR treatment (**Fig. 4A**). Unlike olaparib, which results in persistence of γH2AX following IR to the same degree as APLF knockdown, AZD2461 had no effect on γH2AX dynamics. To confirm if this observation

was due to PARP3 inhibition, we carried out PARP3 enzyme inhibition assays where PARP3 auto-ADP-ribosylation activity was assessed following the addition of increasing doses (2.5 nM–1.5 μM) of olaparib or AZD2461. **Fig. 4B** shows that AZD2461 did not inhibit PARP3 to the same extent as olaparib. There was a 50-fold difference in PARP3 activity, with the IC_{50} value for PARP3 being 4 nM for olaparib and 200 nM for AZD2461 (**Fig. 4B**). The data in **Fig. 4A, C, and D** suggest this difference at the enzyme level is sufficient to translate into a failure to inhibit NHEJ DSB repair, based on γH2AX kinetics.

Increased bone marrow tolerability of AZD2461 in combination with temozolomide is mouse specific and is not seen in rat models

The finding that AZD2461 does not inhibit PARP3, coupled with previous studies that suggest mouse bone marrow HSPCs preferentially use NHEJ when dealing with DNA damage, suggests the observation of better tolerability of chemotherapy combination reported here could be specific to mice and not translate into humans. Prior to initiating clinical trials to test whether AZD2461 was better tolerated with chemotherapy than olaparib, we looked to repeat the experiment presented in **Fig. 3** but in an athymic rat model. First we assessed PARP3 levels (and compared with PARP1 and PARP2 levels) in HSPCs from mice, rats, and humans (**Fig. 5 and Suppl Fig. 6 and Suppl Fig. 7**). We found that PARP3 levels were about 3.7 times higher in mice than in rats (**Fig. 5A**), consistent with their use of NHEJ as a primary repair mechanism. Because of a lack of the same sequence identity, it was not possible to directly compare levels of PARP3 expression across mice, rats, and human bone marrow cells using the TaqMan probe-based assay. However, using SYBR green dye detection RT-PCR, we designed specific primers for human and rat PARP3 and demonstrated

that the relative level of PARP3 expression is very similar between rat and human bone marrow cells (**Fig. 5B**).

In order to increase confidence around the finding of better hematological tolerability, we repeated the temozolomide combination study depicted in **Fig. 3C** in an athymic rat model (**Fig. 5C**). Contrary to the result we obtained in mice, we observed that AZD2461 was no better tolerated than olaparib in terms of bone marrow toxicity (**Fig. 5C**) or total body weight loss (**Suppl Fig. 5B and Suppl Fig. 5C**). A repeat of this experiment in male athymic rats (data not shown) confirmed these results. This observed difference could not obviously be explained by differences in PK between mice and rats, as they are comparable (10 mg/kg in both cases giving an area under the curve [AUC_t] of 1–2 μmol.h/L).

Here, we have provided an explanation for the better tolerability observed in mice by providing data showing the differential PARP3 activity for olaparib and AZD2461, and the different impact of the two compounds on NHEJ DSB repair as inferred by the effects on the recovery of radiation-induced γH2AX. To date, technical challenges around the isolation and analysis of rat HSPCs have prevented us from directly demonstrating that rats are different from mice in utilizing NHEJ and therefore we cannot formally rule out alternative reasons for combination toxicity differences observed between the two rodent species. However, the clinical experience of combining PARP inhibitors with temozolomide (41) does argue that rat and not mouse is the better predictive model.

Structural analysis of the PARP3 active site

Previous studies have made the observation that PARP3 differs from PARP1 and PARP2 in the D-loop structure within the catalytic domain (47). A sequence alignment of this region

(**Fig. 6, Suppl Materials and Methods**) shows that PARP3 contains a shorter loop within this region versus PARP1 and PARP2. A graphical model of the PARP3 crystal structure bound to PARP inhibitor KU58948 is shown in **Fig. 6**,see also (47), with the variant region highlighted. The model shows that PARP3 may be more restrictive for binding by PARP inhibitors with charged or extended groups beyond the carbonyl linker region of these molecules, whereas the PARP1 and PARP2 structures have a more extended D-loop and may be more permissive for inhibitor binding. **Fig. 6** also shows a comparison of the chemical structures of KU58948, olaparib and AZD2461, with the variable groups beyond the linker region highlighted. This illustrates that derivatives of olaparib could be generated for selectivity for or against PARP3 by targeting the variable region in the PARP3 D-loop structure.

The demonstration of differential PARP3 activity of AZD2461 compared with olaparib has two important ramifications. First, we are only just beginning to understand the biological roles of the different PARP family members and their interplay. For example, this study demonstrates that AZD2461 could represent a useful tool to distinguish the cellular functions of PARPs 1–3 in DNA repair. Specifically, previous *in vitro* studies have shown that PARP3 preparations can activate PARP1, and it has been suggested that PARP3 plays a role in regulating the DNA damage response through PARP1 activation (48). However, our study shows that in cells treated with AZD2461, PARP3 is active in the NHEJ pathway even in the absence of detectable PARP1 activity, suggesting that the roles of PARP1 and PARP3 in chromosomal DNA repair are, in fact, independent. Second, the structural similarity between olaparib and AZD2461 that results in this observed differential activity against PARP3 has allowed a rationalization of the mechanistic basis of this difference in specificity via the modeling of the PARP inhibitors in the PARP3 active site.

An increase in our understanding of inhibitor-PARP family member interactions, as demonstrated by an independent study (49), will also facilitate our understanding of PARP family biology. Together, advances in both an ability to generate inhibitors with a particular PARP specificity profile, as has been demonstrated very recently (50), along with an understanding of the biological roles of the different PARP family members, should provide the opportunity to generate inhibitors with the optimal PARP inhibitory profile to maximize anti-tumor activity and therapeutic index.

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Figure legends

Fig. 1. AZD2461 has comparable effects on DNA single-strand break repair and efficacy as olaparib *in vitro*. A, Chemical structure of the PARP inhibitors AZD2461 and olaparib and respective enzymatic IC50 against PARP1 and PARP2. **B,** Human A459 cells were pre-incubated in the presence of 500 nM olaparib or AZD2461 and then subjected to ionizing radiation treatment. SSB repair was quantified using the alkaline comet assay. Graph and statistical analyses were derived from three independent experiments. **C,** AZD2461 potentiates the activity of the alkylating agent MMS in the HeLa cell line when used in combination and assessed using an SRB 96-well assay. **D,** Clonogenic survival assays were used to assess the single-agent activity of AZD2461 and olaparib and then IC50 values determined. Both olaparib and AZD2461 demonstrate potent single-agent activity against *BRCA1*-deficient breast cancer cell lines MDA-MB-436, SUM1315MO2, and SUM149PT, but are significantly less active against the breast cancer lines T47D, BT549, and MDA-MB-231 that have wild-type *BRCA1*. IC_{50} values of greater than 10 μM have been recorded as equal 10 μM. Data were generated from three independent experiments.

Fig. 2. AZD2461 overcomes P-gp-associated resistance to olaparib. A, Activity of AZD2461 and olaparib in the matched cell lines KBA1 (a genetically modified version of HeLa that over-expresses high levels of P-gp) and KB31 that does not over-express P-gp. **B,** Immunofluorescence staining for P-gp protein expression in the parental KB2P3.4 $BRCA2^{-/-}$ mouse cell line and an acquired olaparib-resistant clone KB2P3.4R and relative growth inhibition in parental KB2P3.4 $BRCA2^{-/-}$ mouse cell line and an acquired olaparib-resistant clone. **C,** Response of the olaparib-resistant $Brca1^{\Delta5\text{-}13/\Delta5\text{-}13};p53^{\Delta2\text{-}10/\Delta2\text{-}10}$ tumor T6-28 to AZD2461. Animals carrying orthotopically transplanted tumors were treated daily with 10 mL 0.5% HPMC (vehicle) per kg p.o., 100 mg AZD2461 per kg p.o., 50 mg olaparib per kg

i.p., or 2 mg tariquidar per kg i.p. and 50 mg olaparib per kg i.p. when tumors reached a volume of 150 to 250 mm^3 (100%, day 0).

Fig. 3. AZD2461 is as effective as olaparib in potentiating the anti-tumor efficacy of temozolomide and shows lower impact on mouse bone marrow cells. A, Anti-tumor activity of AZD2461 and olaparib in combination with temozolomide in an SW620 tumor xenograft model. Mice were dosed orally once daily for 5 consecutive days with the combination therapies followed by once-daily dosing of the PARP inhibitor for an additional 2 days. Tumor volumes were plotted relative to the first day of dosing. Comparisons were made to the single agent temozolomide (one-sided) and between the AZD2461 combination and olaparib combination groups (two-sided) employing Student's t-test comparisons using pooled inter-animal variability. **B,** Kinetics of nucleated cells in mouse bone marrow following treatment with AZD2461 and olaparib in combination with temozolomide. Mice bone marrow samples were analyzed 24 hours after the third, fifth, and seventh dose by flow cytometry using forward (FSC-H) and side scatter (SSC-H) on linear scales. **C,** Mouse bone marrow analysis on day 5 of treatment demonstrates the impact of temozolomide with and without the two different PARP inhibitors on the total population of nucleated cells.

Fig. 4. AZD2461 inhibits PARP3 to a lesser extent than olaparib, which results in a lack of inhibition of non-homologous end-joining repair in cancer cells. A, AZD2461, unlike olaparib, does not impact on DSB repair kinetics in human A549 cells. Following pre-incubation with 500 nM olaparib or AZD2461, cells were irradiated with 2 Gy γIR and allowed to recover for the times indicated. DSBs were detected by γH2AX immunofluorescence. A stable APLF knockdown cell line is shown as a control for cells lacking PARP3/APLF-dependent NHEJ (APLF KD). Graph shows the mean +/- SEM from four independent experiments. **B,** AZD2461 is a much weaker inhibitor of PARP3 than

olaparib. PARP activity assay used 200 nM PARP3 in the presence of olaparib or AZD2461 at the concentrations shown. Assays were carried out in the presence of 25 μM Biotin-NAD and 200 ng Sau3Al-cut plasmid DNA. Products were separated by SDS-PAGE and detected using streptavidin-HRRP and ECL. PAR signal integrated density was quantified by ImageJ software and was used to assess AZD2461 and olaparib IC_{50} values for PARP3 inhibition. **C**, Repair of DSBs in primary PARP3 +/+ or **D,** PARP3 -/- mouse embryonic fibroblasts. Cells were pre-treated with 500 nM olaparib or AZD2461 before being subjected to 2 Gy IR and allowed to repair for the indicated times. The average number of γH2ax foci per cell is shown. Graph shows the mean +/- standard error of the mean from four independent experiments. ** = p<0.01, *** = p<0.0001 by paired t-test.

Fig. 5. PARP3 levels are significantly higher in mouse but not rat or human bone marrow cells and consistent with this is a lack of differential bone marrow toxicity between AZD2461 and olaparib in rats. A, PARP1, PARP2, and PARP3 gene expression were analyzed in mouse and rat bone marrow cells using customized TaqMan assays cross-reacting and performing with comparable efficiencies in both species. Comparative quantification of genes expression was based on six biological samples. Rat average ΔCt was used as the reference level to calculate fold change differentials for each sample. A two-tailed t-test with Welch's correlation was applied to analyze differences between gene expression in rat versus mouse (PARP1 means difference = 0.1517, p = 0.0632; PARP2 means difference -0.3883, p = 0.0318; PARP3 means difference = -3.717, p = 0.0169) **B,** PARP3 gene expression in rat and human bone marrow cells was measured by SybrGreen RT-PCR assay. Rat average ΔCt was used as the reference level to calculate fold change differentials for each sample (biological n = 6). A two-tailed t-test with Welch's correlation was applied to analyze differences between gene expression in rat versus human tissue (PARP3 means difference -0.01548, p=0.9232). Box-and-whiskers graphs show mean from three experimental repeats.

C, Female athymic rat bone marrow analysis (day 5 of treatment) using flow cytometry analysis as in **Fig. 3C**. The graph represents the mean ± SD of total nucleated cell population (% of parent) in individual treatment groups (n = 3).

Fig. 6. Comparison between the catalytic domains of PARP1 and PARP3. Sequence alignment of a portion of the catalytic domains of PARPs 1, 2, and 3 highlights residues forming the "HYE triad" within the catalytic core (green arrows) and a PARP3-specific deletion (grey box) are shown. The model shows the PARP3 crystal structure bound by the PARP inhibitor KU58948 (magenta) with the putative PARP3 selectivity pocket shown in yellow and "HYE triad" residues in green. The right panel shows a closer image of the PARP inhibitor pocket with the PARP3 variant region in yellow and the corresponding PARP1 structure aligned in cyan. Also shown are the chemical structures of the PARP inhibitors KU58948, olaparib, and AZD2461, with the variable side chains thought to be contributing to PARP3 selectivity highlighted.